NMR structure of a viral peptide inserted in artificial membranes: a view on the early steps of the birnavirus entry process

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Running title: Entry of a non-enveloped virus

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Non-enveloped virus must penetrate the cellular membrane to access the cytoplasm without the benefit of membrane fusion. For birnavirus, one of the peptides present in the virus capsid, pep46 for infectious bursal disease virus, is able to induce pores into membranes as an intermediate step of the birnavirus-penetration pathway. Using osmotic-protection experiments, we demonstrate here that pep46 and its pore-forming N-terminal moiety (pep22) form pores of different diameters, 5-8nm and 2-4nm, respectively, showing that both pep46 moieties participate to pore formation. The solution structures of pep46, pep22 and pep24 (the pep46 C-terminal moiety) in different hydrophobic environments and micelles determined by 1H-NMR studies provide structural insights of the pep46 domain interaction. In CDCl3/CD3OH mixture and in dodecylphosphocholine (DPC) micelles, the N-terminal domain of pep46 is structured in a long kinked helix, while the C-terminal is structured in one or two helices depending upon the solvents used. We also show that the folding and the proline isomerization status of pep46 depend of the type of hydrophobic environment. NMR spectroscopy with labelled-phospholipid micelles, differential scanning calorimetry and plasmon waveguide resonance studies show the peptides parallel to the lipid-water interface, perturbing the fatty acid chain packing. All these data lead to a model in which the two domains of pep46 interact with the membrane to form pores.

Viruses developed different strategies to deliver their genome into the host cells. Enveloped viruses possess viral proteins that promote fusion of the viral and cellular membranes (1). In contrast, non-enveloped viruses destabilize cellular membrane by mechanisms that are not yet well understood. The role of membrane lytic peptides in the entry process has been well established for different virus families. Peptides are generated by autocatalytic cleavage of a precursor such as in the case of the nodavirus γ peptide (2,3), the poliovirus VP4 (4), and µ1C from reovirus (2,3,5,6), by trypsin cleavage such in the case of rotavirus VP4 (7,8), and by cellular proteolysis such as in the case of adenovirus protein VI (9) or by viral proteolysis such as in
the case of birnavirus pVP2 generating pep46 and additional peptides (10).

Birnaviruses belong to the non-enveloped double-stranded RNA (dsRNA) viruses. Some of them being economically important, such as the infectious bursal disease virus (IBDV) which is the causative agent of a highly contagious disease of young chickens, and the aquatic birnaviruses, the most widespread pathogens of aquatic animals. As for other dsRNA viruses, their genome must be hidden throughout the viral cycle to avoid degradation by cell defence mechanisms. Consequently, during cell entry, the birnavirus particle or at least a large molecular transcription complex has to cross the target membrane to reach the cytoplasm.

To carry out virus penetration into the cell, most dsRNA viruses, such as those belonging to the *Reoviridae* family, exhibit a capsid harbouring several concentric protein shells. The most external shell is generally lost during the entry process and is assumed to insure a local destabilization of the target membrane (11,12). The remaining inner shell constitutes the viral transcription machinery (13,14). In contrast to the viruses belonging to the *Reoviridae* family, birnaviruses possess a single-layered capsid competent for membrane translocation (15,16).

The major components of the birnavirus particle derive from the autoproteolytic processing of the polyprotein pVP2-VP4-VP3 encoded by one of the two genomic segments (segment A). VP4 is a protease that cleaves its own N and C termini in the polyprotein, thus releasing pVP2 and VP3 (17). The sequential maturation of pVP2 takes place only upon virus particle assembly and generates VP2 and the peptides pep46, pep7a, pep7b and pep11 (46, 7, 7, and 11 residues long, respectively) (18). The structures of IBDV and of its capsid protein VP2 were determined by X-ray crystallography (19). Fitting the atomic model of VP2 into the virion electron density map revealed that VP2 is the only component of the virus to follow the icosahedral symmetry. Although the four peptides (pep46, pep7a, pep7b and pep11) were not visualised in the IBDV particle by X-ray crystallography (19), they are accessible to mild trypsin digestion or to biotinylation, demonstrating their close proximity to the virus surface (20).

While pep7a, pep7b and pep11 were shown to be inactive on membranes, pep46 has a strong activity. It interacts with liposomes and destabilizes the target cell membrane. Consequently, it has been proposed that pep46 plays a role in the cell entry process of the virus particle (18). The first 15 residues constituting the N-terminal domain of pep46 show an amphipathic character with positively charged and hydrophobic residues, similarly to some other membrane active peptides found in non-enveloped viruses such as the γ peptide of nodavirus (21), the N-terminus of VP1 of poliovirus (22), the N-terminus of protein VI of adenovirus (23) and antimicrobial peptides such as melittin (24) and magainin (25). Pep22, the N-terminal moiety of pep46 (22 first residues), was shown to conserve the membrane destabilization function of pep46, and the residue Pro16 was demonstrated to play a critical role in this function (18). Pep46 and its N-terminal domain pep22 are able to form pores in membranes with a diameter always found to be less than 10 nm (18).

In the present study, we measure the membrane pore size formed by pep22 and pep46 using osmotic protectants such as polyethylene glycols (PEG). To understand how these peptides act on the membrane, we determined by nuclear magnetic resonance (NMR) experiments the structures of pep46 and its two N- and C-terminal derived domains, pep22 and pep24 respectively, in a hydrophobic solvent mixture, as well as in association with a micelle detergent which mimics the membrane environment. The orientation of pep46 and pep22 relative to the dodecylphosphocholin (DPC) micellar surface was probed by the effects of paramagnetic agents, such as the spin labeled 5- and 16-doxyl-stearic acids and Mn$^{2+}$, on the NMR resonances of the peptides. Additionally, differential scanning calorimetry (DSC) and plasmon waveguide resonance (PWR) were employed to monitor the interaction of pep46 with lipids, its affinity and its orientation in respect to the lipid surface. We show that pep46 is almost totally buried inside the micelle with a parallel orientation relative to the micelle surface. Our results allow us to propose a model for the membrane destabilization by pep46.
**Experimental Procedures**

**Peptide synthesis** - Pep46 and pep22 were synthesized by automated solid-phase synthesis using the Fmoc strategy, and purified by reverse-phase HPLC using procedures already reported for the production of other proteins (26). During pep22 synthesis, 22 labeled amino acids (95% 15N, 15% 13C) were incorporated. Pep24 and pep22 were also purchased from Epytop (Nîmes, France), and were biotinylated at their N-terminus. The sequences of the peptides derived from pep46 are described Figure 1.

**Inhibition of hemolysis by osmotic protection** - Immediately before use, 50µl of sheep erythrocytes were washed repeatedly in phosphate buffered saline (PBS) and centrifuged at 3000 rpm for 5 min. Erythrocytes were resuspended in 200µl of PBS and incubated with or without 10µM pep22 or pep46 in the presence of polyethylene glycols (PEG) of different sizes for 1 hour at 37°C. The PEG concentrations were either 30mM (27) or equivalent to about 100 milliOsmol (mOsm). The osmotic pressure of PEGs were first estimated from the data of Reid and Rand (28), taking into account the peculiar particularity of high molecular weight PEGs and then measured with a cryoscopic osmometer (Roebling Messtechnik). Experiments at constant PEG osmotic pressure were carried out at 100mOsm, the mean value of the osmotic pressures corresponding to 30mM PEG concentrations. All PEGs were dissolved in PBS. Similar results were obtained when the PEG osmolarity was lowered to 40mOsm, the osmolarity of the haemoglobin in the erythrocyte (29) and the all total osmolarity adjusted to 300mOsm with Tris-buffered-saline. Preparations were then centrifuged at 3000rpm for 3 min to pellet cells and the absorbance of supernatant at 405 nm \((A_{405})\) was measured in a microplate reader (EL800, Bio-Tek). Percentage of peptide induced hemolysis was calculated as \([(A_{405} \text{ sample}) - A_{405} \text{ (blank)}] / (A_{405} \text{ (peptide without PEG)} - A_{405} \text{ (blank)})\) x100%, where the blank contained all components except pep22 or pep46 and PEG, and the peptide reaction contained only peptide.

**Sample preparation and NMR spectroscopy** - The pep46, pep22 and pep24 peptides were dissolved at pH 3.5, and at pH 6.5 only for pep22 and pep24, in pure H₂O. As these peptides were shown to interact with membrane, DPC (10 to 100mM) was added to the aqueous solutions (S.D.S., Peypin, France). In presence of DPC, pep22 and pep24 were dissolved at pH 6.0 and pep46 at pH 3.5 and 6.2. The pH was checked and adjusted after each addition of DPC, using small amount of NaOH and HCl. The peptides were also studied in a mixture of CD₃OH/CDCl₃ (1:1). In all cases the final concentrations were about 1mM. Two-dimensional phase-sensitive ¹H Clean-TOCSY (30) with a 70ms spin lock, and 100ms and 200ms mixing times NOESY experiments (31), were recorded at 293K, 303K, 313K and 333K on a AVANCE Bruker spectrometer operating at 600.14 MHz without sample spinning with 2K real points in t2, with a spectral width of 6000 Hz and 512 t1-increments. Pulsed-field gradients (32) were used for water suppression by a WATERGATE pulse sequence. The data were processed using XWINNMR software (Bruker). A π/6 phase-shifted sine bell window function was applied prior to Fourier transformation in both dimensions (t1 and t2). ¹H-¹⁵N HSQC experiments were performed on the pep22 sample at 1.0mM, pH 6.5 in DPC at 293 K. The GARP sequence was used for decoupling during acquisition. Experiments were recorded on the phase sensitive mode using echo/antiecho gradient selection and trim pulses in inept transfer. A total of 256 FIDs of eight scans were collected for each experiment. A π/2 phase-shifted sine square bell window function was applied prior to Fourier transformation in both dimensions (t1 and t2). The temperature was externally controlled using special temperature control system (BCU 05 Bruker). Solvent accessibility of the amide backbone signals was determined by proton/deuterium exchange studies. Samples were prepared by dissolving the lyophilised protein sample in DPC into D₂O. We monitored the disappearance of the NH peaks as a function of time, by 2D NOESY spectra.

**NMR structure of pep46, pep22 and pep24** - NOE cross-peak volumes measured on NOESY spectrum recorded for pep46 in solution in CD₃OH/CDCl₃ (1:1), and in aqueous solution in presence of 100 mM DPC for pep46, pep22 and pep24, were converted into distances, semi quantitatively, by counting contour levels. Using the Tyr-2,6H geminal, and Asp-Hβ protons as calibration peak, NOEs
signals were classified into 5 categories with upper distance limits ranging 2.5 to 5 Å. Pseudoatom corrections were added when necessary. Standard protocols using distance geometry and simulated annealing were performed in X-PLOR 3.84 (33,34) on an SGI O2 R12000 computer.

**Peptide location in micelles** - For locating pep46 and pep22 relative to the micelle surface, we used three different paramagnetic agents: MnCl₂ (0.1/1 to 1.5/1 Mn²⁺/pep22 and 0.1/1 to 5/1 Mn²⁺/pep46), 5-doxyl stearic acid (0.3/1 to 6/1 5-doxyl/pep22 and 0.3/1 to 6/1 5-doxyl stearic acid /pep46), and 16-doxyl stearic acid (0.3/1 to 3/1 16-doxyl/pep22 and 0.3/1 to 1.5/1 16-doxyl stearic acid/pep46) (35). The effects of the spin labels were observed by comparing the peak volume intensities in 1H-15N HSQC for pep22 and in NOESY spectra (35,36) for pep46 of labelled with those of unlabeled samples. The amplitude of the spectra in the presence of the three paramagnetic labels was normalized to the least affected cross-peaks.

**Study of the interaction of pep46 with DMPC and DMPG micelles by DSC** - Lipid film preparation was made by dissolving the appropriate amount of lipid (dimyristoylphosphatidylcholine, DMPC or dimyristoylphosphatidylglycerol, DMPG; Genzyme) in a mixture of CHCl₃ and CH₃OH, 2/1 (v/v), followed by solvent evaporation under nitrogen to deposit the lipid as a film on the wall of a test tube. Final traces of solvent were removed in a vacuum chamber attached to a liquid nitrogen trap for 3-4 h. Films were hydrated with 10 mM Tris, 0.1 M NaCl, 2 mM EDTA, pH 7.6 (Tris buffer) and vortexed extensively at a temperature above the phase transition temperature of the lipid to obtain MLVs with a lipid concentration of 1mg/mL. The peptide was added after the formation of the MLVs. The calorimetry was performed on a high-sensitivity Differential Scanning Calorimeter (Calorimetry Sciences Corporation). A scan rate of 1°C/min was used and a delay of 10 min between sequential scans in a series was applied to allow thermal equilibration. Data analysis was performed with the fitting program CPCALC provided by CSC and plotted with Igor. As for the peptide, P/L molar ratios of 1/100, 1/50, 1/25 and 1/10 were used in those studies. Samples containing the peptide alone, dissolved in buffer at peptide concentrations corresponding to those at the higher peptide/lipid molar ratios studied (P/L 1:10) exhibited no thermal events over the temperature range of 0-100°C. This indicates that the peptides do not contribute to the endothermic events observed in this study, which arise solely from lipid phase transitions and their modulation upon lipid/peptide interactions. A minimum of at least three to four heating and cooling scans were performed for each analysis.

**Study of the interaction of pep46 and orientation relative to a lipid bilayer by PWR** - PWR spectra are produced by resonance excitation of conduction electron oscillations (plasmons) by light from a polarized CW laser (He-Ne; wavelength of 632.8 and 543.5 nm) incident on the back surface of a thin metal film (Ag) deposited on a glass prism and coated with a layer of SiO₂ (see (37) for additional information). Experiments were performed on a beta PWR instrument from Proterion Corp. (Piscataway, NJ) that had a spectral resolution of 1 mdeg. The sample to be analyzed (a lipid bilayer membrane) was immobilized on the resonator surface and placed in contact with an aqueous medium, into which peptides can be introduced. The self-assembled lipid bilayers were formed using a solution (in butanol/squalene 0.93/0.07 v/v) of 8 mg/mL of egg phosphatidylcholine (Avanti). The method used to make the lipid bilayers is based on the procedure by Mueller and Rudin to make black lipid membranes across a small hole in a teflon block (38). To accomplish this, a small amount of lipid solution was injected into the orifice in a teflon block separating the silica surface of the PWR resonator from the aqueous phase. Spontaneous bilayer formation was initiated when the sample compartment was filled with aqueous buffer solution (37). The molecules (such as lipids and peptides) deposited onto the surface plasmon resonator change the resonance characteristics of the plasmon formation and can thereby be detected and characterized. PWR spectra, corresponding to plots of reflected light intensity versus incident angle, can be excited with light whose electric vector is either parallel (s-polarization) or perpendicular (p-polarization) to the plane of the resonator surface. Spectral simulation (37) and/or graphical analysis (39) allow one to obtain information about changes in the mass density, structural asymmetry, and molecular orientation induced by bimolecular interactions.
occurring at the resonator surface. Here, the graphical analysis method was employed. Briefly this method consists in deconvoluting the components of the PWR spectra that are due to changes in mass in the lipid film from those that are due to changes in structural anisotropy. Such distinction can be done based on the magnitude and direction of the PWR spectra shifts observed in the $p$- and $s$-polarized light. Thus, alterations in mass density (due to addition or subtraction of mass from the membrane) result in shifts in $p$- and $s$-polarization with the same magnitude and direction (isotropic changes), whereas structure alterations lead to anisotropic changes (shifts for $p$- and $s$-pol that are distinct in magnitude and direction). By plotting the spectral changes observed in a $(s, p)$ coordinate system where mass ($\Delta m$) and anisotropy ($\Delta str$) axes are represented based on the PWR sensitivity factor, the contribution of mass and structural changes can be obtained (39). Each point in the mass and anisotropy axis can be expressed by changes in the original coordinates ($\Delta p$ and $\Delta s$) by the following equations:

$$\Delta m = [(\Delta s)^2 m + (\Delta p)^2 m]^{1/2}$$

$$\Delta str = [(\Delta s)^2 str + (\Delta p)^2 str]^{1/2}$$

The sensitivity factor ($S_i$), a measure of the sensitivity of the instrument for the $s$-pol relative to $p$-pol ($S_i = \Delta s/\Delta p$), necessary to determine the mass and anisotropy axes has been determined, for the prism used in those experiments, to be 0.74 [46].

Affinities between the peptide and the lipids were obtained by plotting the PWR spectral changes that occur upon incremental additions of ligand to the cell. Since the PWR is mainly sensitive to the optical properties of material that is deposited on the resonator surface [when a similar emergent medium (buffer) is employed, which is the case here], there is little interference from the material that is in the bulk solution. Moreover, the amount of bound material is much smaller than the total amount of ligand present in the bulk solution, and it is assumed that the bulk material is able to freely diffuse and equilibrate with the membrane. Data fitting (GraphPad Prism) through a hyperbolic saturation curve provides the dissociation constants. It should be noted that since concomitantly with the binding process other processes such as membrane reorganization and solvation occur, the dissociation constants correspond to apparent dissociation constants.

**RESULTS**

**Hemolysis induced by pep46 and its membrane-active domain pep22 is inhibited by osmotic protection.** Pep46 and pep22 (Fig. 1) perforate synthetic and biological membranes, allowing liposomes and cells to release their content in the medium. Leakage occurs either through large pores directly formed by the peptides, or by osmolysis after the peptides formed small pores that semi-permeabilize the membrane. To discern between these two possible mechanisms and to estimate the size of the pores induced by pep46 and pep22, we assessed the hemolysis protection by PEGs of different molecular size (37-39) in the presence of pep46 and pep22 (Fig. 2). Hemolysis protection by PEGs was carried out either at constant concentration (30mM) (Fig. 2A) or at constant osmotic pressure (100mOsm) (Fig. 2B). Similar results were obtained with the two procedures. High molecular weight PEGs do inhibit the release of hemoglobin from erythrocytes in the presence of pep46 and pep22. Pep46 and pep22 form small pores, having characteristic diameters. For pep46, only the 6K-PEG and the 8K-PEG partially inhibited the hemoglobin release (Fig. 2A and 2B). 6K- and 8K-PEG have estimated hydrodynamic diameters of 5.0 and 7.6 nm respectively (41,42), suggesting a diameter in the range 5-8nm for the pores formed by pep46. For pep22, whereas the largest PEGs tested (4K, 6K and 8K) strongly inhibited hemoglobin release, the smallest PEGs (1K and 2K) had little effect. In these experiments, most of the protection selectivity occurred for PEGs of molecular weight in the range 2-4K (Fig. 2A and 2B). The hydrodynamic diameter of these molecules has been estimated to about 2.5 and 3.8 nm respectively (41,42), suggesting a diameter in the 2-4 nm range for pores formed by pep22. Although pore diameter measurements using hemolysis protection by poly ethylene glycols are not accurate (40), these results suggest that pep46 forms larger pores than pep22 and that both N- and C-ter domains of pep46 participate to the pore formation.

**NMR studies of pep46 and derived**
peptides (pep22 and pep24) in micelles. Pep46 was found soluble in pure water at pH 3.5 but not structured (Fig. S1A), and insoluble at a more biological pH, such as 6.0. DPC was chosen to study pep46 and derivates since it contains a zwitterionic headgroup, which is a proper cell membrane lipid mimetic. It is also a relatively small molecule, which facilitates NMR studies (41,42), by solubilizing efficiently hydrophobic or amphipathic α-helices (36). Pep46 was dissolved in presence of 10 mM DPC, at pH 3.5, and increasing amounts of DPC were added until no modification was observed on the 1D spectra, which indicates the stabilization of the pep46 structure in micelles. Until 50mM, DPC increments induced chemical shift variations and surprisingly, led to a decrease in the line width of 1H resonances (for comparison, Fig. S1B and S1C). Between 50mM and 100mM of DPC, the spectra were almost identical (Fig. S1C and S1D). Similar folding variations have been observed by CD for pep46, in function of DPC concentration. Indeed, until 50 equivalent of DPC, changes in CD spectra are observed, and between 50 and 100 equivalent of DPC the spectra are almost identical (Fig. S2). In presence of DPC, pep46 is also soluble at a more biological pH value, such as 6.2. Unfortunately we observed a broadening of the signals with increasing pH (Fig. S1D, S1E, S3) or in phosphate buffer pH 6.8 (Fig. S1F). Nevertheless, most of the resonances in the NOESY spectra recorded at pH 6.2 were assigned by comparison with the NOESY spectra recorded at pH 3.5. Although some amide protons underwent significant chemical shift variations (Fig. S3), we confirmed that pep46 very likely adopts the same three-dimensional conformation at pH 3.5 and 6.2 since all the secondary structure medium NOEs observed at low pH have also been assigned in the NOESY spectra at pH 6.2, in spite of the significant increase of the line widths (Fig. S3). Moreover we observed that the Hα chemical shift index (CSI) analysis which represent the difference between the observed chemical shift and the random coil values (43), are very similar whatever the pH values, 3.5 or 6.2 (Fig. S4). An upfield trend (i.e., negative secondary shift) for the Hα resonances of residues suggests that this peptide consists predominantly of helical structural elements at these two pH values. These results are confirmed by the circular dichroism (CD) spectra (Fig.S2). The increase of DPC concentration induces a structural transition of pep46 from a random coil to an α-helical structure characterized by the presence of two minima at 209 and 220nm in the CD spectra. Similar spectra are obtained at pH 3.5 and 6 for pep46 in the presence of 100 equivalent of DPC, suggesting the peptide presents similar folding in these conditions.

Contrary to pep46, pep22 and pep24 are soluble in pure H2O at pH 6.0 but no characteristic NOEs of any secondary structure were found in these conditions. DPC increments did not induce a decrease in the line width of these two peptides, but as for pep46, we observed chemical shift variations, characteristic of the secondary structure formations. Only trans-proline conformers were observed in presence of DPC. The structures of pep22 and pep24 were calculated using the NMR restraints collected for the peptides in 100 mM DPC at pH 6.0. The pH has no effect on the line widths of these two shorter peptides. Pep22 adopts a similar conformation to its corresponding counterpart in pep46, an α-helix spanning residues Phe3 to Phe22. On the contrary, pep24 adopts only one α-helix instead of the two observed in the corresponding domain in pep46 (Fig. S5), arising from additional secondary structure NOE contacts involving the three glycines, Gly33, Gly35 and Gly41, a residue known to often provide flexibility in the protein structure (Table S1). The cis/trans isomerism of Pro16 and Pro23 noted in pep46 (18) was not observed in pep22 and pep24 respectively. Indeed, the duplication of Val15, Val17 and Val18 resonances on one hand, and of Ala25, Ala26 and His30 on the other hand, observed for pep46, were not detected in the NMR spectra of pep22 and pep24.

NMR studies of pep46, pep22 and pep24 in hydrophobic solvent. To better characterize the potential function of the N- and C-ter domains of pep46 in membrane destabilization, pep46 and its two derivates were studied in a mixture of solvents (CD3OH/CDCl3), shown to mimic the hydrophobicity of membranes (44-46). The complete chemical shift assignment of the backbone and side chain protons was achieved for all the amino acids of pep46 (Fig. S6), Only the sequential NOE cross-peaks,
characteristic of a trans conformation of prolyl bonds were observed in NMR spectra for pep46 solubilized in CD$_3$OH/CDCl$_3$ (1:1). Analysis of the NOESY experiments based on the observed medium range NOEs, reveals that pep46 is organized into two domains. Indeed, typical NOEs corresponding to a classical $\alpha$-helix were found for the (3-22) and the (27-46) stretches (Fig. S7 and S8). In CD$_3$OH/CDCl$_3$, pep46 shows almost the same general characteristics as those obtained in DPC (Fig. 3). The structure is characterized by a N-terminal curved $\alpha$-helix showing approximately a 115° angle (120° in DPC) between the two helical domains, spanning residues (3-22), followed by a loop constituted by the proline-rich region (23-27), and ended by one $\alpha$-helix (28-41). The first helix (3-22) in pep46 is perfectly well defined with an average rmsd for the ten best structures calculated on the backbone atoms of 0.70 (± 0.29) Å (Table 1). This helix has the characteristics of an amphipathic helix in the region (3-15). The amino acid side chains of Lys4, Asp5, Arg8, Arg11, and Arg12 form its hydrophilic face, while the side chains of Phe3, Ile6, Ile7, Ala9, Ile10, Ile13, and Ala14 provide an uninterrupted hydrophobic surface. The Pro16 appears to induce a break in the helix (3-22). The segment comprising residues 17-22 is exclusively composed of hydrophobic residues. The C-terminal domain (27-41) is better defined in CD$_3$OH/CDCl$_3$ than in presence of DPC, and is characterized by one well defined long helix with an average rmsd for the backbone atoms of 0.33 (± 0.16) Å (Table 1). The region (32-41) presents the characteristics of a short amphipathic helix, with the hydrophilic face constituted by the amino acid side chains of Glu34, Asp37, and Tyr38, and the hydrophobic face composed of the side chains of Ile32, Val36, Leu39 and Leu40. Altogether these data show that the C-terminal domain of pep46 and pep24 exhibits a structure that strongly depends on the environment.

**Positioning of pep22 and pep46 in detergent micelles by spin-labeled probes and Mn$^{2+}$.** To investigate how pep22 and pep46 form pores in membranes and to understand the contribution of the C-terminal domain of pep46 in the pore formation, we determined their orientation in DPC micelles through the analysis of the effects of 5-, 16-doxyl stearic acids and Mn$^{2+}$ ions on the proton signals of the peptides in micelles. While manganese ions were used to probe the residues exposed to the solvent close to the micelle surface, both 5-doxyl stearic acid and 16-doxyl stearic acid were used for identification of the membrane-embedded residues (47). The 16-doxyl spin label is located in the center of DPC micelles, whereas the 5-doxyl stearic spin label is positioned near the phosphate moiety, below the head group (47).

**Positioning of pep22.** The effect of paramagnetic probes was estimated from $^1$H-$^1$N HSQC experiments by comparing the spectra with and without 5- or 16-doxyl stearic acid and Mn$^{2+}$ (Fig. 4). With 16-doxyl stearic acid/pep22 ratio of 3:1, the resonances of Ile10, Ala14, Val15 disappeared and those corresponding to residues located on both sides of these amino acids (Phe3, Ile6, Ile7, Ala9, Ile10, Ile13, and Ala14) provide an uninterrupted hydrophobic surface. The Pro16 appears to induce a break in the helix (3-22). The segment comprising residues 17-22 is exclusively composed of hydrophobic residues. The C-terminal domain (27-41) is better defined in CD$_3$OH/CDCl$_3$ than in presence of DPC, and is characterized by one well defined long helix with an average rmsd for the backbone atoms of 0.33 (± 0.16) Å (Table 1). The region (32-41) presents the characteristics of a short amphipathic helix, with the hydrophilic face constituted by the amino acid side chains of Glu34, Asp37, and Tyr38, and the hydrophobic face composed of the side chains of Ile32, Val36, Leu39 and Leu40. Altogether these data show that the C-terminal domain of pep46 and pep24 exhibits a structure that strongly depends on the environment.
the hydrophobic residues (Phe3, Ile6, Ile7, Ala9, Ile10, Ile13, Ala14, Val15), and the 5-doxyl stearic acid on the five first N-terminal residues and on the positively charged arginine, 8, 11 and 12 (Table 2). The results showed that the 3-15 helical domain probably, lies parallel to the micelle surface, with the hydrophobic face toward the interior of the micelle, and the positively charged residues, (Arg8, Arg11 and Arg12) interacting with the negatively charged phosphate head groups, anchoring the peptide just below the micelle surface (Fig. 7B).

In contrast, strong differences were observed on the (17-22) domain within pep22 and pep46. While the resonance intensities of Val17, Leu21 and Phe22 are clearly influenced by Mn$^{2+}$ in pep22 (Fig. 4D), in pep46 these same residues did not show significant reduction in their resonance intensities upon addition of this paramagnetic probe (Table 2). Residues 17-22 of pep46 are more buried into the lipid micelle than the corresponding ones of pep22. A significant periodic reduction in the cross-peak intensities of Ala14, Val15, Val17, Val18, Leu21 and Phe22 was observed in presence of both the 16-doxyl and the 5-doxyl stearic acid (Table 2), while a significant reduction in the intensity of the cross-peaks was observed only in presence of 5-doxyl for the following residues: Pro16, Ser19, Thr20, Pro23, Ala25, Ala26 and Pro27. These residues are located on the same side of the structure (Fig. S9).

A periodicity in the remaining amplitude versus the residue number was also apparent in the C-terminal segment (28-40) (Fig. 7A and Table 2). The most affected residues under the 16-doxyl stearic acid effect are Ile32 and Val36 whose side chains interact with the lipid acyl chains and penetrate deeper into the micelle. The 5-doxyl stearic acid probe caused the largest changes in Leu28, Ala29, Gly33 and Gly35, Leu39, Leu40 and to a lesser degree, in His30, Ala31, Glu34, Asp37 and Tyr38. Therefore, these residues are buried into the lipid acyl chain region nearby the head group region. Leu28, Ala29, Ile32, Val36, Leu39 and Leu40 on one hand and His30, Glu34, Asp37 and Tyr38, on the other hand are located respectively on the hydrophobic and hydrophilic faces of the C-terminal helix (Fig. 3A). This part of the protein appears also inserted in the micelle, although to a lesser extent when compared to the first N-terminal helix, with a parallel orientation relative to the lipid surface. Only the residues of the C-terminal part of pep46 underwent attenuation, although low, of their cross-peaks in the presence of Mn$^{2+}$ (Table 2). These results are corroborated by the amide exchange experiments. We solubilized the protein/micelle complex in D$_2$O. Slowly exchanging protons were detected by NOESY spectra acquired every 8h for a period of 64h. The most solvent-protected amino acids were observed in the regions comprising residues 6-20 on one hand, and residues 30-40 on the other hand (Table 2).

The effect of pep46 on DMPC and DMPG lipid phase transition. The degree of interaction of pep46 with lipids was monitored by following the changes in the lipid phase pre-transition arising from the conversion of lipid phase structure L$_{β'}$ to P$_{β'}$, and the main phase transition corresponding to the conversion from P$_{β'}$ to L$_α$ (T$_m$) upon peptide/lipid interaction. In the present study the peptide was added to the lipid after multilamellar vesicles (MLVs) formation to better mimic the biological system. The lipids employed in this study are the dimyristoylphosphatidylcholine (DMPC) and dimyristoylphosphatidylglycerol (DMPG) (48-50). These lipids have been often used in DSC studies as they possess a highly energetic phase transition that occurs at room temperature. DMPC constitutes a good model of eukaryotic cell membranes. Moreover, this zwitterionic lipid resembles DPC used here in the NMR studies. Since electrostatic interactions between peptides and lipids have been shown to play an important role and because eukaryotic cells possess some charged lipids, the anionic lipid DMPG was also used in these studies. In terms of the lipid model, MLVs were chosen because they produce sharp and energetic phase transitions and have been routinely used in DSC studies. The interaction of pep46 with the lipids clearly affects the thermotropic lipid behaviour with either a great decrease (in the case of DMPC) or abolishment of the pre-transition (DMPG), at P/L of 1/10) (Fig. 8). The pre-transition, which is observed in some saturated lipids is due to the tilting of the hydrocarbon side chains and is sensitive to the presence of...
interacting molecules. The untilting of the hydrocarbon chains by pep46 may be explained by a simple neutralization of the head group charge by the cationic peptide, which will result in reduced electrostatic head group repulsion and concomitantly in a smaller head group area observed in the case of DMPG. Moreover, the cooperativity and the enthalpy of the main phase transition were also affected by the peptide. The enthalpy of the main phase transition is mainly due to the disruption of van der Waals interactions between the fatty acid chains, and perturbations on this transition are indicative of intercalation of the peptide between the fatty acid chains. This indicates a strong interaction of pep46 with the lipids not only at the level of the head group but with an intercalation in between the fatty acid chains.

**The interaction of pep46 with egg PC lipid bilayers studied by plamon-waveguide resonance (PWR).** This technique was used to directly monitor the interaction of pep46 with the lipid bilayer and the changes induced by the peptide lipid bilayer organization and total mass (37). PWR spectral changes occurring after addition of pep46 to the PWR cell compartment containing an egg PC bilayer are shown in Fig. 9A and 9B. The binding of pep46 to the bilayer leads to a decrease in the resonance angle position for both p- and s-polarizations and to changes in the spectral depth. In order to characterize the mass and the structural changes that accompany the interaction of the peptide with the lipid bilayer, (see (39) for details) we performed a graphical analysis, which, by plotting the data points on a (s, p) coordinate system containing both mass and structural axis placed according to the sensitivity factor of the PWR sensor, allows the determination of the mass and structural anisotropy contributions to the process. Thus, the origin of the plot corresponds to the lipid bilayer in absence of ligand and the data points shown correspond to the shifts induced by peptide binding to the bilayer. The binding of pep46 to the lipid bilayer produced very large spectral changes (~ -30 mdeg for p-pol and ~ -53 mdeg for s-pol), which corresponds in magnitude to about 1/3 to 1/4 of that obtained upon the formation of the lipid bilayer. Such large spectral changes indicate that the peptide induces large structural and mass changes in the lipid bilayer. Indeed, the graphical analysis indicates a ~ 70% change is mass (decrease) vs ~ 30% change in structure. A decrease in mass density results mainly from a decrease in the mass of the lipid bilayer due to removal of lipid from the bilayer accompanied by lipid structural rearrangements. Additionally, it should be noted that the magnitude of the resonance angle shift was much larger for s-than p-polarization, meaning that larger structural and mass changes are occurring parallel rather than perpendicular to the bilayer. This suggests an orientation of the peptide with its long axis parallel to the lipid bilayer. Regarding the affinity of pep46 to the bilayer, a Kd of about 1nM was calculated (Fig. 9C). Moreover, the changes in spectral depth induced by the peptide indicate alterations in bilayer thickness.

**DISCUSSION**

Pep46 was shown to be the central actor of the entry mechanism of birnaviruses (18). In this study, we enlighten the pore-forming activity of pep46 in membranes. The diameter of the pore was estimated to be near 5-8 nm by following osmotic protection of erythrocytes from hemolysis. This value is in agreement with data obtained previously by cryo-microscopy that showed the pore diameters always less than 10 nm. It is worthwhile to note that pep22, the perforating domain of pep46, gives smaller pore diameter (2-4 nm). These data prove that pep24 that has no permeabilization activity, cooperates with pep22 to form larger pores. The different diameters supposedly arise from different sizes or self-aggregation properties of the perforating peptides pep22 or pep46.

We combined DSC, PWR and NMR spectroscopy studies to gain more information on pep46 structure-function relationships; in particular its position in the membrane. We show that the structures of pep46 and the two peptides derived from its N- or C- extremities, pep22, and pep24, respectively, significantly depend on the solvent employed. When aqueous solution and physiological pH are used, pep22 and pep24 are soluble but are not folded, in contrast to pep46 that is only soluble at acidic pH. In more hydrophobic solution (CD3OH/CDCl3 (1/1)), or in presence of DPC, pep46 shows similar solubility properties than pep22 and pep24. Surprisingly, we observed that increasing DPC concentration in pep46
solution induced a narrowing in the line-widths of pep46 in the $^1$H NMR spectra. This suggests that pep46 has a high tendency to aggregate. The hydrophobic environments reduce drastically intermolecular hydrophobic interactions between the accessible hydrophobic domains of pep46, allowing its complete dissolution whatever the pH. Aggregation takes place most probably through the highly hydrophobic domain located between amino acids 20 and 29 of pep46 (Fig. 3), since each N- and C-terminal domain taken separately does not aggregate in the same conditions. Moreover, the fact that pep24 acquires a secondary structure in hydrophobic environment shows that although it has no permeabilization activity, it interacts with membranes.

The major difference between the structures of pep46 obtained in CD$_3$OH/CDCl$_3$ (1/1) and in presence of DPC concerns its C-terminal domain, which was shown to be folded in either one $\alpha$-helix (28-41) or in two successive short helices, (28-33) and (35-41), respectively (Fig. 3). The structural heterogeneity observed in DPC micelles between the two C-terminal helices results from the high degree of chain flexibility at the position of two glycines (Gly33 and Gly35). This structural heterogeneity is not found for pep24. Indeed, a perfectly well defined $\alpha$-helix involving the (27-41) region is observed for pep24 in DPC micelles (Fig. S5). On the contrary, the same $\alpha$-helical structure is observed in pep22 and in its corresponding N-terminal domain in pep46. Pep24 and its corresponding domain in pep46 do not interact in the same way with DPC micelles. This difference is probably governed by the interaction of the N-terminal domain of pep46 with DPC micelles which constrains the conformation of its C-terminal domain. Gly33 and Gly35 provide the necessary flexibility in the C-terminus of pep46 to undergo the conformational adaptability that favours a better orientation of the peptide relative to the membrane.

As the DPC micelles are prolate ellipsoids with a thickness comparable to that of a plasmic membrane (51), the topology of the pep22 and pep46 peptides relative to the surface and the interior of the DPC micelle was investigated using paramagnetic probes, such as Mn$^{2+}$, 5-doxyl stearic acid and 16-doxyl stearic acid. Important information on the location of the peptides in the membrane can be obtained at low protein/lipid ratio. The first fifteen residues of pep22 and pep46 are buried in the micelle. Indeed, they form an helix that lies more or less parallel to the micelle surface with the hydrophobic face toward the center, and Arg8, Arg11 and Arg12 anchoring this domain to the head group of the DPC by interactions between the arginine positively charged guanidinium groups and the negatively charged DPC phosphates (Fig. 7B). In the very hydrophobic domain (14-22) of pep46, some residues are strongly affected by both the 5- and the 16-doxyl stearic acid such as Ala14, Val15, Val17, Val18 and Phe22. These hydrophobic residues are located on the same side of pep46(14-22) helix, and constitute an hydrophilic platform (Fig. S9). This N-terminal domain is followed by a relatively more flexible region, pep46(23-30) containing three prolines (Pro23, Pro24, Pro27). These three successive residues impose nevertheless rigid constraints on rotation about the N-C$\alpha$ bond of the backbone, and are responsible for the formation of a turn (Fig. 7A), which probably imposes the relative orientation of the N- and C-terminal domains of pep46. Although less buried inside the micelle than the first fifteen residues of pep46, its C-terminal domain is also parallel to the surface of the DPC micelle, but it does not insert so deeply in the lipid aliphatic chains.

The interaction of pep46 with DMPC and DMPG was monitored with DSC by following the effect of the peptide on lipid phase transitions. The results obtained with DMPC confirmed that the peptide interacts strongly with the lipid phosphate head groups and intercalates within the fatty acid chain region as indicated by the perturbation of both the pre- and main lipid phase transitions. PWR studies indicate that the peptide has a strong affinity (Kd ~ 1nM) for egg PC lipid bilayer and produces important changes both in the structure and mass of the membrane. Therefore, the decrease in mass of the lipid bilayer could be explained by either a peptide-induced efflux of lipid into the plateau Gibbs border caused by peptide intercalation between the fatty acid chains or by bilayer solubilization (with or without pore formation). In agreement with NMR data, PWR studies demonstrate that pep46 is oriented parallel to the bilayer, at least in the first stage of peptide-
lipid interaction at a time the decrease in mass is not observed. Critical residues of pep46 for virus rescue have been identified using a reverse genetic system (18). Indeed, among the 46 substitutions generated for each residue of pep46, 17 did not allow virus recovery (Phe1, Gly2, Lys4, Ile7, Ile10, Arg11, Ala14, Pro16, Ala25, Leu28, Ala29, Ile32, Gly35, Leu39, Leu40, Gln45, Ala46). As represented in Figure 7A, the hydrophobic residues Ile7 and Ile10 and Leu28, Ile32, and Leu39, are respectively located on one side of each of the N- and C-terminal helices of the peptide. These residues are located toward the hydrophobic center of the micelles. The basic residues, Lys4 and Arg11, are located on the same side of the N-terminal helix, and are responsible for the anchoring of this domain at the polar heads (Fig. 7B). The results imply that the helical kink introduced by the Pro16 residue is probably crucial for the permeabilization activity of pep46. The appearance of such kinked helix is also present in the flock house virus (FHV) γ peptide (nodavirus) (21), and as well in some antimicrobial peptides such as, gaegurin (52), maculatin (53), buforin II (54) and melittin (55).

Cryomicroscopy experiments in a previous publication (18) showed that liposomes incubated with pep46 undergo structural modifications of the thickness of the membrane leading to the formation of pores. PWR, DSC studies and NMR data are in good agreement with these results, and suggest that electrostatic attractions provided by DPC head groups are probably essential for driving pep46 to the membrane surface to achieve primary binding with a parallel orientation. The aggregation properties of pep46 could induce its deep insertion within the fatty acid chain region, leading to important lipid rearrangements, changes in bilayer thickness and a decrease in the mass of the lipid bilayer due to removal of lipids. According to these results, we propose a model where pep46 adsorbs on the bilayer surface, pushes the lipid head groups aside leading to a thinning of the membrane. Pep46 could replace one layer of the lipid bilayer and could represent the first step of a membrane deformation model (18) (Fig. 7B).

The structure of another viral peptide with a membrane binding activity, γ peptide from Flock House virus (FHV), an insect nodavirus has been determined (21,56-58). The amphipathic helix corresponding to the N-terminal residues (21-residue-long) can disrupt membranes in vitro and was shown to be the host membrane-interacting region of the virus during entry (5,57,59). γ peptide and pep46 display similar conformations and activities. Pep46, or at least its pep22 domain, seems to behave like other surface-oriented peptides, including pro-apoptotic peptides such as Bax (60) hunter-killer peptides (61) as well the antimicrobial peptides, such as gramicidin (62), magainin (63,64), PGLa (65), piscidin (66) and LL-37 (67). In aqueous solutions these peptides adopt a random coil conformation, and an amphipathic helical structure in both organic solvents and detergent micelles (25,66,68-71). NMR experiments, fluorescence quenching, and calorimetry experiments on these antimicrobial peptides at low concentration, indicate that the helix is oriented parallel to the bilayer surface (64,66,67,72). It has been shown by molecular dynamics simulations that a single peptide binding to the interface is not enough for pore formation (73). The amphipathic peptides bind to membranes and form pores when the required concentration is reached inducing membrane thinning which consequently led to pore formation (74-79). For example, melittin has been found to bind to lipid membranes in monomeric form (80) but it may self-associate at higher concentration. Multimerisation could also play a role in membrane defect formation under some conditions (81,82). However, at higher concentrations, oriented CD spectroscopy indicated that this peptide maybe oriented perpendicular to the bilayer surface (76,83). The release of the 780 copies of pep46 present in the IBDV particle (19) within an endosome is probably sufficient to induce aggregation and pore formation.

The toroidal pore mechanism has been proposed for a series of antimicrobial peptides, e.g, magainin (84), melittin (85), pleurocidin (86), syringomycin (87), LL-37 (88) and cathelicidin (89), but accumulation of peptide may, as for melittin, leads to a detergent-like disintegration of the bilayer structure via a carpet mechanism (90). We can foresee that the N-terminal domain of pep46 (pep22) depending on its concentration behaves as the antimicrobial peptides described above, with formation of toroidal pores. As already described in the case of reovirus (6), the pores
are of insufficient size (around 10 nm) for the passage of a virus particle. Indeed, the pores formed by pep46 are ten times smaller than the IBDV particle (700 Å diameter) (19). We showed like others for reoviruses (27), that osmotic protection of the cells depends on PEG size. PEGs large enough do not pass through the pores, retaining water on the outside and preventing cell lysis. As already discussed (27), the pore formation is a necessary step resulting in the endosome degradation by influx of water.

Our data allow us to propose a model for the entry of IBDV into cells. Firstly, the release of pep46 from the capsid allows pep46 to interact with the endosomal membrane. The interaction of pep46 with the lipid polar heads induces a conformational change of pep46 that allows the helical structure adopted by the N-terminal amphipathic region to penetrate the lipid bilayer. The high hydrophobic domain pep46(20-32) leads to the aggregation of pep46, which subsequently induces the formation of pores in the membrane. Although the C-terminal domain was shown to modulate pep46 activity, no specific activity has been assigned so far to this domain. The flexibility of the pep46(23-27) loop could explain the interaction of pep46 with membranes through its N-terminus, its C-terminus could be responsible of the pep46 oligomerization leading to larger pores.

FOOTNOTES

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Figure legends

**Fig. 1.** Primary sequence of the peptide pep46 and of the two peptides deriving from its N- or C-terminal domains (pep22, and pep24).

**Fig. 2.** Inhibition of peptide induced hemolysis by osmotic protection. Hemolysis reactions were performed with or without 10µM pep46 or pep22 in the presence of polyethylen glycols (PEGs) of different sizes. For both cases, PEGs were added at a concentration of 30mM (A) or at a concentration equivalent to 100mOsm (B). In absence of peptide, no hemolysis is observed in constant PEG concentration (30mM (-) pep (A)) or constant PEG osmolarity (100mOsm (-) pep (B)) conditions. Each experimental points and error bar are the mean and standard deviation of four independent experiments.

**Fig. 3.** Comparison of the pep46 structures obtained in presence of DPC (A) and CD$_3$OH/CDCl$_3$ (1:1) (B). The helices are represented as green cylinders, and the residues are colored according to their hydrophobicity (red: hydrophobic residues, blue: hydrophilic residues).

**Fig. 4.** Effect of paramagnetic agent on $^{15}$N labelled pep22 at 293K. $^1$H-$^{15}$N HSQC of 1mM pep22 with (A) 100mM DPC (100:1 DPC/pep22 mole ratio); (B) 100mM DPC and 3mM 16-doxyl stearic acid; (C) 100mM DPC and 6mM 5-doxyl stearic acid; (D) 100mM DPC and 3mM MnCl$_2$. Cross peaks are labeled using amino acid residue one-letter code.

**Fig. 5.** Paramagnetic quenching obtained for pep22 in DPC micelles using 5- and 16-doxyl stearic acid. Intensity retention plot for pep22 in the presence of (A) 3mM 16-doxyl stearic acid; (B) 6mM 5-doxyl stearic acid.

**Fig. 6.** Effects of the spin-labeled molecules 16- and 5-doxyl stearic acid, and Mn$^{2+}$, on the NOESY spectra of pep46, in 100mM DPC micelles. (A) NH-aliphatic region of a NOESY spectrum of pep46, recorded with a mixing time of 200ms, at 323K and pH 3.5. Corresponding region with (B) 16 doxyl stearic acid, (C) 5 doxyl stearic acid and (D) MnCl$_2$ are represented.

**Fig. 7.** Stereoview of one of the pep46 structures obtained in presence of DPC (A). The signal reductions greater than 50% are mapped onto the pep46 structure. Residues that are the most affected upon addition of 16-doxyl stearic acid are colored dark purple. Residues that are quenched by the addition of 5-doxyl stearic acid are colored light blue. Residues that at the same time strongly quenched by the 5-doxyl stearic acid and 16-doxyl stearic acid are colored dark blue. Residues that are affected strongly by 5-doxyl stearic acid and moderately by 16-doxyl stearic acid are colored light purple. (B) Schematic representation of the interaction of pep46 DPC micelle. One representative structure of pep46 is positioned with the basic residues interacting with the negatively charged phosphate head group of DPC, and the hydrophobic residues plunging into the fatty acid chains of the lipid.

**Fig. 8.** High-sensitivity DSC heating scans illustrating the effect of the addition of pep46 (bold) on the thermotropic phase behavior of DMPC and DMPG at a P/L 1/25. Thermodynamic parameters are given in Table 3.

**Fig. 9:** Interaction of pep46 with an egg PC lipid bilayer monitored by PWR. PWR spectra (A and B) obtained for the lipid bilayer (solide line) and after addition of pep46 to the bilayer (solid line with +) obtained for p- and s-polarized light, respectively. The resonance position shifts obtained for p- (•) and s- (_) polarizations for the incremental addition of pep46 are represented and the hyperbolic binding curve from which a Kd value of 1 nM was obtained (C).
Table 1. NMR restraints and structural statistics

| NMR derived distance restraints | Total | 483  |
|--------------------------------|-------|------|
|                                | Intraresidue | 211  |
|                                | Sequential (|i-j| =1) | 152  |
|                                | Medium-range (1< |i-j| ≤4) | 120  |
|                                | Long-range (|i-j| >5) | 0    |

Distance violations
- > 2 0

r.m.s.d. (Å) from experimental distance restraints
- (514*) 0.027 ± 0.004

r.m.s.d. from idealized geometry
- bonds (Å) (715) 0.0048 ± 0.0002
- angles (deg) (1299) 0.66 ± 0.01
- improper angles (deg) (45) 0.45 ± 0.02

Xplor potential energies ([Kcal/mol])
- $E_{\text{NOE}}$ 20.8 ±6.0
- $E_{\text{VDW}}$ -189.9 ± 6.6
- $E_{\text{BOND}}$ 13.1 ±1.4
- $E_{\text{ANGLES}}$ 86.8 ±2.0
- $E_{\text{IMPROPER}}$ 11.5 ± 0.8
- $E_{\text{TOTAL}}$ -1767.8 ± 45.2

Ramachandran analysis of residues
- favored region (%) 90.7
- additional allowed regions (%) 7.6
- generously allowed regions (%) 1.4
- disallowed regions (%) 0.2

Atomic rms deviation to mean structure (Å)

| Range | Backbone atoms | Heavy atoms |
|-------|----------------|-------------|
| (3-15)| 0.26 ± 0.12    | 1.75 ± 0.63 |
| (17-22)| 0.66 ± 0.38  | 1.29 ± 0.53 |
| (3-22)| 0.70 ± 0.29 | 1.80 ± 0.52 |
| (18-27)| 0.91 ± 0.43 | 1.05 ± 0.51 |
| (27-41)| 0.33 ± 0.16 | 1.12 ± 0.41 |
Semiquantitative classification according to L, low effect; M, moderate effect; S, strong effect. The exchange of labile protons in pep46 when bound to DPC micelles was studied by adding D$_2$O to a lyophilized sample. The exchangeable protons of residues Ile6, Arg8, Ala9, Arg11, Arg12, Ala14, Val15, Val17, Val18, Ser19, Thr20, His30, Ile32, Gly33, Gly35, Val36, Tyr38 and Leu40 are still present in the NOESY spectrum recorded after 8h (○), the Ile39 amide proton is still present after 24h (●), Ile13 after 32h (●), and Ile7 and Ile10 after 64h (●).

Table 2. Effects of the spin-labels on the residues of pep46.

|        | Hbond | 16-DOXYL stearic acid | 5-DOXYL stearic acid | MnCl$_2$ | Hbond | 16-DOXYL stearic acid | 5-DOXYL stearic acid | MnCl$_2$ |
|--------|-------|-----------------------|----------------------|---------|-------|-----------------------|----------------------|---------|
| Phe 1  | L     | M                     | S                    | L       | M     | S                     | M                    | S       |
| Phe 2  | L     | M                     | S                    | L       | M     | S                     | M                    | S       |
| Phe 3  | M     | S                     | Ala 26               | L       | M     | S                     | M                    | S       |
| Lys 4  | L     | M                     | Ala 25               | L       | M     | S                     | M                    | S       |
| Asp 5  | L     | M                     | Leu 28               | M       | S     | S                     | M                    | S       |
| Ile 6  | ●     | S                     | Ala 29               | M       | S     | S                     | M                    | S       |
| Ile 7  | ●     | S                     | His 30               | L       | M     | S                     | M                    | S       |
| Arg 8  | ●     | S                     | Ala 31               | L       | M     | S                     | M                    | S       |
| Ala 9  | ●     | M                     | Ile 32               | S       | M     | S                     | M                    | S       |
| Ile 10 | ●     | M                     | Gly 33               | O       | S     | S                     | S                    | S       |
| Arg 11 | ●     | M                     | Gly 34               | O       | S     | S                     | S                    | S       |
| Arg 12 | ●     | M                     | Gly 35               | O       | S     | S                     | S                    | S       |
| Ile 13 | ●     | S                     | Val 36               | O       | S     | S                     | M                    | S       |
| Ala 14 | ●     | S                     | Asp 37               | M       | L     | S                     | M                    | L       |
| Val 15 | ●     | S                     | Tyr 38               | O       | M     | S                     | M                    | L       |
| Pro 16 | ●     | S                     | Leu 39               | O       | M     | S                     | M                    | S       |
| Val 17 | ●     | S                     | Leu 40               | O       | M     | S                     | M                    | S       |
| Val 18 | ●     | S                     | Gly 41               | M       | L     | S                     | M                    | L       |
| Ser 19 | ●     | S                     | Asp 42               | M       | L     | S                     | M                    | L       |
| Thr 20 | ●     | L                     | Glu 43               | M       | L     | S                     | S                    | L       |
| Leu 21 | ●     | M                     | Ala 44               | S       | L     | S                     | M                    | L       |
| Phe 22 | ●     | S                     | Gln 45               | S       | L     | S                     | L                    | S       |
| Pro 23 | ●     | L                     | Ala 46               | S       | L     | S                     | L                    | S       |
Table 3. Thermodynamic parameters for the DMPC and DMPG phase transition in the absence and presence of peptide pep46 at different P/L molar ratios.

| P/L    | T pre-transition | ΔH pre-transition (Kcal/mol) | ΔS pre-transition (Kcal/mol) | Tm | ΔH main transition (Kcal/mol) | ΔS main transition (Kcal/mol) |
|--------|------------------|-----------------------------|-----------------------------|----|-----------------------------|-----------------------------|
| DMPC alone | 14.4             | 1.1                         | 0.004                       | 23.8 | 6.7                        | 0.022                        |
| 1/100   | 14               | 1.1                         | 0.004                       | 23.7 | 7.2                        | 0.024                        |
| 1/50    | 14               | 0.9                         | 0.003                       | 23.7 | 6.5                        | 0.022                        |
| 1/25    | 13.8             | 0.6                         | 0.002                       | 23.7 | 5.6                        | 0.019                        |
| 1/10    | 13.8             | 0.3                         | 0.001                       | 23.7 | 4.0                        | 0.014                        |
| DMPG alone | 9.1              | 0.4                         | 0.001                       | 22   | 6.1                        | 0.021                        |
| 1/100   | 9.3              | 0.4                         | 0.002                       | 22.1 | 6.6                        | 0.022                        |
| 1/50    | 9.2              | 0.4                         | 0.001                       | 22.1 | 7.5                        | 0.025                        |
| 1/25    | 9.2              | 0.1                         | 0.001                       | 22   | 5.4                        | 0.018                        |
| 1/10    | disappears       | disappears                  | disappears                  | 23.5 | 6.5                        | 0.022                        |

Pre-transition: L'β to the P'β
Main transition: P'β to Lα
| Peptides | Sequence                                      |
|----------|----------------------------------------------|
| Pep46    | 1 FGFKDIIRAIRRIAVPVVSTLFPPAAPLAHAIGEGVDYLLGDEAQA |
| Pep22    | 15 FGFKDIIRAIRRIAVPVVSTLFPPAAPLAHAIGEGVDYLLGDEAQA |
| Pep24    | 20 PPAAPLAHAIGEGVDYLLGDEAQA                  |

Figure 1
Figure 2

Graph A and B show the relationship between PEG molecular weight (x1000) and peptide induced hemolysis (%). The graphs compare the hemolytic activity of pep46, pep22, and (-) pep across different PEG MWs.

- **Graph A:**
  - X-axis: PEG MW (x1000)
  - Y-axis: Peptide induced hemolysis (%)
  - Lines represent pep46, pep22, and (-) pep

- **Graph B:**
  - X-axis: PEG MW (x1000)
  - Y-axis: Peptide induced hemolysis (%)
  - Lines represent pep46, pep22, and (-) pep
Figure 3
Figure 4
Figure 5
Figure 6
Figure 8

Figure 9
NMR structure of a viral peptide inserted in artificial membranes: a view on the early steps of the birnavirus entry process

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