The Amino-terminal Region of the Fusion Peptide of Influenza Virus Hemagglutinin HA2 Inserts into Sodium Dodecyl Sulfate Micelle with Residues 16–18 at the Aqueous Boundary at Acidic pH

OLIGOMERIZATION AND THE CONFORMATIONAL FLEXIBILITY*†§

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The conformation and interactions with membrane mimics of the NH₂-terminal fragment 1–25 of HA2, HA2 (1–25), of influenza virus were studied by spectroscopic methods. Secondary structure analysis of circular dichroism data revealed 45% helix for the peptide at pH 5.0. Tryptophan fluorescence quenching by acrylamide and NMR experiments established that the Trp¹⁴ is inside the vesicular interior and residues 16–18 are at the micellar aqueous boundary. NBD fluorescence enhancement of the NH₂-terminal labeled fluorophore on the vesicle-bound peptide indicated that the NH₂ terminus of the fusion peptide was located in the hydrophobic region of the lipid bilayer. No significant change in insertion depth was observed between pH 5.0 and 7.4. Collectively, these spectroscopic measurements pointed to an equilibrium between helix and non-helix conformations, with helix being the dominant form, for the segment in the micellar interior. The conformational transition may be facilitated by the high content of glycine, a conformationally flexible amino acid, within the fusion peptide sequence. Self-association of the 25-mer peptide was observed in the N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine SDS-gel electrophoresis experiments. Incorporating the NMR signal attenuation, fluorescence, and gel electrophoresis data, a working model for the organization of the fusion peptide in membrane bilayers was proposed.

The hemagglutinin (HA) glycoprotein of influenza virus is responsible for viral attachment to and fusion with the target cell (1, 2). HA molecules are expressed on the viral surface as homotrimers (3). Proteolysis of the proprotein HA (4) generates two polypeptide chains, HA1 and HA2, linked by a disulfide bond. HA1 acts as the receptor-binding subunit while HA2 anchors the HA molecule onto the viral membrane and mediates fusion with the target cell membrane. The fusion activity of proteolyzed HA is triggered by lowering the pH to about 5.0. HA has been shown to associate with and disrupt the membrane bilayer in order to exert its fusogenic function (5, 6).

Structures of the ectodomain of enzymatically cleaved HA2 have been determined by x-ray crystallography at both neutral and acidic pH (3, 7). In essence, the conformational change from neutral to low pH states consists in the transition of B-loop (55–76) to helix (8) and transition of helix (105–113) to a loose turn. The resultant trimeric helical rod spans more than 100 Å and is packed on its external face by three shorter COOH-terminal helices in an antiparallel orientation. However, the NH₂-terminal fusion peptide, which has been shown to be exposed at the fusion pH (9, 10) and to insert into and disrupt the membrane bilayer (11, 12), was not included in the structural determination at low pH (13), particularly in the membranous environment. Recently, the HA2-mediated fusion mechanism has been studied in considerable detail by several laboratories. Thus, Kemble et al. (14), Melikyan et al. (15), and Song et al. (16) reported intermediate steps which include hemifusion or stunted fusion in the mechanism leading to full membrane fusion. These intermediate structures involve reorientation of lipid molecules in the outer and inner leaflets of membranes in contact. It has also been deduced from Fourier transform infrared and electron paramagnetic resonance (EPR) spectroscopic experiments that the fusion peptide of influenza virus inserted into the membrane at a tilted angle (17–19). These results necessitate examination of the structure of HA2 fusion domain and its interactions with the membrane at high resolution.

The NH₂-terminal region of HA2 encompassing the first 15 amino acids is highly conserved among various strains of influenza viruses. It is characterized by high content of glycine and alanine residues, followed by a more polar region containing glycine renders HA fusion incompetent (6). The orientation and the depth of insertion of HA2 into the membrane bilayer may provide some insight into the HA2-mediated fusion mechanism.

Synthetic peptides corresponding to the NH₂-terminal sequence of HA2 have been shown to cause liposome fusion and red blood cell lysis (20–22). From EPR and infrared spectro-
scopic experiments (22), it was deduced that the HA2 fusion peptide inserted into the vesicle at an oblique angle and the NH₃ terminal of the fusion segment is located near the hydrocarbon-polar head group interface of the bilayer. The hydrophobic photolabeling technique can be used to probe the protein insertion into the membrane, but is unable to directly determine the insertion depth (23).

It was also found that substitution of glutamic acids at positions 11 and 15 affected little the capacity of hemolysis and CD data analysis implied a conformational equilibrium for the latter property was substantiated by SDS-gel electrophoresis. Propensity for the latter was pointed to the oligomerization of the peptide. Propensity for the insertion depth (23).

Fluorescence measurements have been employed in the previous investigations on the insertion depth of HA2 fusion peptide into the model membrane (25, 26). The data by Clague et al. (26) placed Trp 14 approximately 8 Å from the center of the vesicular bilayer and suggested no appreciable positional change between neutral and acidic pH. For these experiments, only the location of the tryptophan probe can be deduced.

In order to understand the structural basis of the fusion peptide of HA2, we carried out investigation on the peptides corresponding to HA2(1–25) and HA2(1–20) from strain X31 of the influenza virus, using NMR spectroscopy which affords information on the dynamics and structure at the atomic resolution as well as insertion depth on the amino acid level. Data from circular dichroism (CD), fluorescence, and NMR experiments enabled us to locate residues 16–18 of the fusion peptide at the interface of aqueous phase and hydrocarbon interior of micelle or vesicle. The result strikingly indicated the localization of Glu11 in the hydrophobic core of SDS micelle and pointed to the oligomerization of the peptide. Propensity for the latter property was substantiated by SDS-gel electrophoresis. Identification of the region residing in the membranous core and CD data analysis implied a conformational equilibrium for the segment, thus providing a rationalization of highly conserved glycine residues in the fusion peptide sequence.

**EXPERIMENTAL PROCEDURES**

**Materials**

Two peptides corresponding to residues 1–25, HA2(1–25) (NH₂-GLFPGAAGFIENGWGMIDGWYGR) and residues 1–20, HA2(1–20), of HA2 (strain X31) of influenza virus were synthesized in an automated mode by a solid phase synthesizer from Applied Biosystems (Foster City, CA) model 431A using 9-fluorenylmethoxy carbonyl (Fmoc) chemistry. The peptides were cleaved from the resins by trifluoroacetic acids and purified by high performance liquid chromatography on a Vydac C18 reverse-phase column. The primary sequence of the peptide was ascertained by electrospray mass spectrometry.

SDS was acquired from Roche Molecular Biochemicals (Mannheim, Germany) and d₂₀-SDS from Cambridge Isotope (Andover, MA). Lipids, 1,2-ditetracetylcyano-5,6-dihexadecanoyl-sn-glycero-3-phosphocholine (DMPC), and 1,2-dihexadecanoyl-sn-glycero-3-phospho-L-serine (DPPS) were acquired from Calbiochem (San Diego, CA). Acrylamide, 5-doxyl-stearic acid (5-DXS), proteinase K, and 7-nitrobenz-2-oxa-1,3-diazole (NBD) were purchased from Sigma. Osteodeyl rhodamine B chloride (R18) was acquired from Molecular Probes (Eugene, OR). Reagents for electrophoresis and molecular weight markers were products of Amersham Pharmacia Biotech. All reagents were used in the experiments without further purification. Solutions containing vesicles were prepared by solubilizing the lipids in a chloroform/methanol (4:1, v/v) mixture and drying the sample under nitrogen stream before dissolving in buffer solution. Mixtures of peptides and SDS or phospholipids were sonicated for 30–60 min before measurements.

The amino-terminal labeling of the peptide with NBD was prepared by the standard procedure (27). Briefly, 1 mg of pure peptide was reacted with 2 equivalents of the NBD probe in dry dimethyl formamide at room temperature for 20 h. The conjugated peptide was further purified by high performance liquid chromatography as described above for the unlabeled peptide.

**NMR Experiments**

Micellar solutions containing HA2(1–25) and SDS 1.2:120 mM were used for NMR measurements. One-dimensional and two-dimensional 1H NOESY and TOCSY NMR experiments were performed on a Bruker AMX-500 spectrometer as described previously (28). In deuterium-hydrogen (D/H) exchange experiments, the peptide-incorporated SDS sample in the NMR tube was lyophilized three times with pure H₂O. D₂O·H₂O (9:1) (v/v) was added immediately before acquiring NMR data (28). The 1H signal attenuation with varying pH is due to an increase in amide proton exchange rate at pH 8.0 compared with pH 5.0 (29, 30), resulting in higher relaxation rate and weaker signal (30, 31) at higher pH. Relaxation enhancement by 5-DXS is due to dipolar interaction between its unpaired electron on the doxyl moiety and the proton, and hence is highly sensitive to the distance between them.

**Structure Calculations**

A total of 365, including 118 non-sequential, NOE interactions and 24 intra-residue dihedral angles were utilized in the structural computations using distance geometry/simulated annealing protocols of Biosym programs Insight II, Discover, and NMRlitect (version 97.0) from Molecular Simulations, Inc. (San Diego, CA). NOE data were converted into interproton distance using H2/H3 cross-peak of the aromatic ring of phenylalanine as reference. A range of 0.6 to 1.0 Å was allowed to vary in the distance constraints. In the simulated annealing protocol, the temperature was raised to 1000 K in four steps followed by a molecular dynamics run for 34 ps to allow for more conformational space to be explored. The system was subsequently annealed to 300 K in 10 steps for a total of 66 ps and minimized by the steepest-descent and conjugated-gradients methods before final refined structures were obtained.

**Circular Dichroism Experiments**

CD experiments were carried on a Jasco 720 spectropolarimeter at ambient temperature. Cells with path lengths of 0.1 and 1.0 cm were employed for sample solutions containing final concentrations of 75 μM, 15 mM HA2(1–25):SDS and 12 μM, 1.2 mM HA2(1–25):DMPC, respectively. Peptide concentration was determined by UV absorbance denatured in 6 M guanidinium chloride, using ε = 12,660 liter mol⁻¹·cm⁻¹ at 280 nm for HA2(1–25) containing one tyrosine and two tryptophan residues (32). Spectra were recorded from 184 or 190–260 nm at scanning rate of 20 nm min⁻¹ with a time constant of 4 s, step resolution of 0.1 nm, and bandwidth of 1 nm. For each of the peptide preparations, final CD profile was obtained by averaging four scans.

CD data were represented in units of extinction coefficient, ε (liter mol⁻¹·cm⁻¹), which is converted by 2ε = [θ]θ/3300, where [θ] is the molar residue ellipticity (degree cm²·dmol⁻¹). In turn, [θ] is obtained from the obliquity of dihedral (θ) according to [θ] = ε*π·l·c·n, where l is the cell length in mm, c is the molar concentration, and n is the number of amino acid residues in the peptide. Quantitative prediction of the secondary structure (helix, in particular) was accomplished by fitting CD data with Hennessey-Johnson (H.-J.) algorithm (33), by the program Varselec, using 33 proteins of known secondary structure as the basis set.

**Fluorescence Experiments**

**Steady State Fluorescence Studies—** Fluorescence measurements of HA2(1–25) in aqueous buffer, SDS micellar solution, and DMPC or DPPS vesicular solution were performed on a JASCO spectrofluorometer, model FP-777, using a cell of 1 cm in length at 25 °C. Fluorescence emission spectra in 380–450 nm range were recorded by using 280 nm excitation wavelength with a scan rate of 100 nm min⁻¹, response time of 1 s and data interval of 0.1 or 0.2 nm. The band widths for excitation and emission were 5 and 1.5 nm, respectively. The average from two independent scans was taken for all spectral measurements. Appropriate blanks were subtracted to obtain the corrected spectra. The solutions used in this experiment contained 10 μM of the peptide, 50 mM sodium chloride as well as 25–40 mM SDS or 1.2 mM phospholipid.

**Acrylamide Quenching Studies—** Fluorescence quenching study monitors accessibility of the fluorophore to the acrylamide quencher. An incremental amount of acrylamide stock solution (1 M) was added to the peptide (10 μM) solutions to make final concentrations of acrylamide up to 50 μM. Corrections due to dilution were made to the observed fluorescence intensities. The data were analyzed by the Stern-Volmer equation (34).
Interactions of Influenza HA2 with Model Membranes

\[ F_0/F = 1 + K_{SV}[Q] \]  
(Eq. 1)

where \( F_0 \) is the fluorescence intensity at the zero quencher concentration, \( F \) is the fluorescence intensity at any given quencher concentration \([Q]\), whereas \( K_{SV} \) represents the apparent Stern-Volmer quenching constant, obtained from the slope of \( F_0/F \) versus \([Q]\) plot. The reported \( K_{SV} \) values were the average of two independent measurements.

Binding of Peptide to Membrane and Accessibility of Peptide to Proteolytic Cleavage—The ability of the peptide to bind to lipid membrane was studied by using fluorescent-labeled peptide. The peptide and the lipid concentrations were 1 and 500 \( \mu \)M, respectively. NBD fluorescence is sensitive to its environment (27, 35). A blue shift (from 550 to 528 nm) is observed upon elevating the pH. The protease (\(-30 \mu\)g/ml) was added to the NBD-conjugated peptide bound to the lipid bilayer and loss in fluorescence was measured with time (35). In the control experiment, the peptide was preincubated with the same amount of protease followed by the addition of phospholipid. The net retention of fluorescence is attributed to the protection of peptide from protease cleavage. The percent protection was defined as fluorescence intensity after addition of the protease relative to that before the protease treatment.

Octadecylrhodamine Chloride B (R18) Human Erythrocyte Content Mixing Assay—Self-quenching of R18 was used to study the fusion efficiency of the 25-mer peptide. Dequenching is observed as a result of association of the labeled peptide with the membrane. Spectra were collected by employing 467 nm excitation wavelength. The protease cleavage experiment was performed under similar experimental conditions at neutral or acidic pH. The protease relative to that before the protease treatment.

The concentration of R18 was 10 \( \mu \)g/ml and that of HA2-(1–25):DMPC is 12 \( \mu \)M. Excitation and emission wavelengths were 556 and 590 nm, respectively, for fluorescence measurements. The suspension was acidified to pH 5.0 by sodium citrate prior to the addition of peptide for acidic condition. The measured dequenching was normalized by the dequenching obtained with adding 5 \( \mu \)l of 10% (v/v) Triton X-100 according to,

\[ \% \text{ Dequenching} = \frac{(F_t - F_0)}{(F - F_0)} \]  
(Eq. 2)

where \( F_t \) and \( F_0 \) are fluorescence intensities at a given time \( t \) and at time 0 (before addition of the peptide), respectively, while \( F \) is the fluorescence after introduction of Triton X-100 and is taken as fluorescence at infinite dilution of probe. The time course was followed for 10 min with excitation and emission band widths of 5 and 1.5 nm,

\[
\begin{array}{ccc}
\text{pH 5.0} & \text{pH 7.0} \\
\text{helix} & 45 & 38 \\
\beta\text{-sheet} & 28 & 28 \\
\text{turn} & 24 & 17 \\
\text{others} & 3 & 17 \\
\end{array}
\]

FIG. 1. Kinetics of R18 mixing due to HA2-(1–25)-induced fusion of RBC at pH 5.0 (top trace) and 7.4 (lower trace). Final peptide concentration in reaction medium was kept at 50 \( \mu \)M. Time scans were recorded at the emission wavelength of 590 nm using 556 nm excitation wavelength at room temperature. Addition of 0.1% (v/v) Triton X-100 to R18-entrapped RBC suspension was taken as infinite dilution of R18 as described under "Experimental Procedures."

FIG. 2. Far-UV CD spectra of HA2-(1–25) at pH 5.0 and 7.0 in SDS (top) and DMPC (bottom) solutions at 298 K. The concentration of HA2-(1–25):SDS is 75 \( \mu \)M, 15 mM and that of HA2-(1–25):DMPC is 12 \( \mu \)M, 1.2 mM. The analyzed data of SDS micellar solution of secondary structure using Hennessy-Johnson protocol is represented in the inset of the top panel. As judged from the ellipticity at 222 nm, helicity is essentially the same in SDS and DMPC dispersions. Moderate decrease in helicity can be observed for the peptide in both media upon elevating the pH.

TABLE I

Effect of pH on influenza HA2 fusion sequences, HA2-(1–25) and HA2-(1–20), studied in different media at 25 °C by fluorescence spectroscopy

| Media | pH 5.0 | pH 7.4 |
|-------|--------|--------|
|       | \( \lambda_{\max} \) | \( K_{SV} \) | \( \lambda_{\max} \) | \( K_{SV} \) |
| Buffer | 349 | 448 | NA | 16.2 | 350 | 353 | NA | 17.2 |
| SDS | 342 | 339 | 10.9 | 8.1 | 343 | 343 | 11.1 | 8.9 |
| DPPS | 336 | NA | 9.8 | NA | 338 | 337 | 10.0 | 6.6 |
| DMPC | 332 | 330 | 4.8 | 3.3 | 333 | 331 | 6.4 | 5.2 |
It gives rise to a spectrum very close to trace e.  

traces d–e indicate a dramatic increase in the NBD fluorescence in e, which signifies that the NH₂ terminus of HA2-(1–25) is in the apolar environment when the peptide binds to the phospholipid bilayer. Higher fluorescence intensity at acidic pH than at neutral pH (compare a to b and c to d) suggests a slightly deeper penetration into the bilayer at acidic pH for the peptide. Binding of the NBD-labeled peptide to the DMPC vesicle at acidic pH as probed by proteasine K cleavage. In curve a, the lipid suspension was added to the peptide solution at time point a, causing an enhanced fluorescence which maintains a steady value. Curve 2 displays drop in fluorescence intensity with proteasine K treatment at time point b to probe the accessibility of peptide in the lipid bilayer to the enzyme. Curve 3 represents the control experiment with the peptide precleaved by the proteasine. A marginal increase in fluorescence was due to scattering of the lipid suspension. Curve 4 represents the data obtained with the control solution by adding 0.1% Triton X-100 to the R18-encapsulated RBC dispersion.}

**RESULTS**

**R18 Content Mixing Assay Shows That HA2-(1–25) Induces Fusion of Vesicles at Low pH Analogous to HA2**—Fluorescence dequenching measurements were conducted on the HA2-(1–25) and R18-incorporated RBC dispersion at acidic and neutral pH to examine the fusion efficiency of the peptide. Fig. 1 displays time courses of the R18 spread in the presence of the fusion peptide at pH 5.0 and 7.4. The rate and extent of lipid fusion are greatly diminished at neutral pH as compared with those at acidic pH. The fusion peptide is thus active at low pH characteristic of HA2 fusion activity.

**CD Data of HA2-(1–25) Indicate That Substantial Non-helix Structure Exists in SDS Micellar Solution in Addition to 45% of α-Helix**—The CD profile of fusion peptide in SDS solution and secondary structure analyzed by the Hennessey-Johnson prediction procedure are shown in Fig. 2. The stacking, spacer, and running gels with 4, 10, and 16.5% of acrylamide, respectively, were used as described by Schägger and Jagow (39). Peptide samples were incubated with buffer solutions containing 1% of SDS, 0.06 M Tris-HCl (pH 6.8), and 20% of sucrose or glycerol. Fixing, staining, and destaining times were adjusted to 1 min, 1 h, and overnight, respectively, to reduce the extent of diffusion (38).

**CD Data of HA2-(1–25)**

**Self-assembly of HA2-(1–25) revealed by the Tricine SDS-PAGE experiment.** The molecular mass of the HA2 fusion peptide was 2.76 kDa. Lanes a and b represent the peptide and standard molecular weight markers, respectively. The two bands in lane a correspond to monomeric and dimeric species. The arrows indicate the standard molecular weights, respectively.

The percentage of fluorescence dequenching was measured relative to the increased value obtained with the control solution by adding 0.1% Triton X-100 to the R18-encapsulated RBC dispersion. Tricine SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) Tricine SDS-PAGE was performed according to the procedures used by Pristka et al. (38).

**FIG. 4.** Self-assembly of HA2-(1–25) revealed by the Tricine SDS-PAGE experiment. The molecular mass of the HA2 fusion peptide was 2.76 kDa. Lanes a and b represent the peptide and standard molecular weight markers, respectively. The two bands in lane a correspond to monomeric and dimeric species. The arrows indicate the standard molecular weights.
Insertion of HA2-(1–25) into Micelles and Phospholipid Bilayers Is Deduced from Fluorescence of Tryptophan Residues within the Peptide and of the NBD-labeled Peptide—Fluorescence experiments were conducted making use of the tryptophan residues at positions 14 and 21 for HA2-(1–25) and position 14 for HA2-(1–20). Table I summarizes emission peak and quenching results at acidic and neutral pH. For HA2-(1–25) at acidic pH, the emission maximum shifts from 349.0 nm in buffer solution to 342.0 nm in SDS micelle solution and to 332 nm in DMPC solution, indicating that tryptophan residues are in the hydrophobic environment when bound to the micelle or vesicle.

Acrylamide $K_{SV}$ quenching data reveal that Trp$^{14}$ is localized within about 5 Å from the lipid bilayer surface (40) at pH 5.0. For both 20-mer and 25-mer peptides, $K_{SV}$ in DMPC dispersion is somewhat smaller than that in SDS solution at the same pH. These results suggest that the position of Trp$^{14}$ in the hydrophobic region of the two membrane mimics is similar, since the difference in $K_{SV}$ between the two media reflects in part the larger head group of the phospholipid, leading to a larger separation between the acrylamide probe and the fluorophore. Hence SDS micelle is a reasonable model for study on the insertion of fusion domain into membrane bilayer.

$K_{SV}$ is slightly smaller for the two peptides at pH 5.0 than at pH 7.4 in the same micellar or vesicular suspension (Table I), suggesting a slightly deeper penetration of both peptides at acidic pH (41). A large blue shift in tryptophan fluorescence and much deeper penetration into large unilamellar vesicles at acidic pH relative to neutral pH was reported by Rafalski et al. (42) on HA2-(1–20). However, a lack of insertion of HA2-(1–20) into vesicles has been noted as the pH was changed to 7.0 from 5.0 at which the peptide was found inserted (24).

Further evidence of penetration of the peptide into the membrane is provided by NBD-labeled peptide. Fig. 3A illustrates an increase of NBD fluorescence, along with a blue shift, upon incubating the labeled peptide in the DMPC and DPPS suspensions. In support of the result deduced from data in Table I, Fig. 3A indicates a slightly deeper insertion at pH 5.0 than at pH 7.4.

Accessibility of the fusion peptide in the lipid bilayer was probed by proteinase K cleavage. Fig. 3B shows a drop in NBD fluorescence of the labeled peptide in the DMPC bilayer upon addition of proteinase K at point b of curve 2. This suggests that the peptide undergoes fluctuation when inserting into the membrane such that the NH$_2$ terminus of HA2-(1–25), to which NBD is attached, is accessible to the enzyme external to the membrane. The result also implies that the NH$_2$ terminus is near the apolar-aqueous interface of the vesicle. Curve 3 of the figure indicates that NBD is externally bound to the vesicle when it is excised from the fusion peptide prior to incubation with the vesicle solution.

Tricine SDS-PAGE Experiment Demonstrates the Propensity of Self-assembly of HA2-(1–25)—Since the fusion peptide has been shown to insert into the membrane bilayer, we examined the self-assembly state of the fusion peptide in the SDS detergent solution. The result shown in Fig. 4 indicates that both monomeric and dimeric species are present in the SDS environment, demonstrating the tendency of HA2-(1–25) to oligomerize.

NMR Experiments Show the NH$_2$-terminal Portion of HA2-(1–25) Penetrates into the SDS Micelle with Significant Helix Form in Equilibrium with Non-helix Form—Fig. 5 presents the fingerprint region of the NOESY spectrum of HA2-(1–25) in the presence of SDS micelles. Nonsequential NOE cross-peaks are summarized in Fig. 6. ω-Helix structure, characterized by d(i, i+3) and d(i, i+4) relations, are seen in the regions 2–13 and 17–24, with the segment 14–16 exhibiting weaker helical character. The NOE results are consistent with the data in Fig. 7 which displays deviation of the chemical shifts of $\alpha$H and $\beta$H of a given amino acid from that of the random structure, as well as coupling constants $\hbar_{ij}$ evaluated from one-dimensional spectrum and XEASY program.

The results of backbone amide proton peak attenuation by varying pH or by 5-DXSA are presented in Fig. 8A, along with data on the D-H exchange rate. NOESY spectra for HA2-(1–25) before and 7 h after introduction of 90% D$_2$O into the fusion peptide/SDS suspension are also shown. The spectrum in Fig. 8B highlights the effect of amide D-H exchange on some of the resonance peaks. At higher pH, the protons exposed to the aqueous phase exhibit higher exchange rate, resulting in broadened and weaker peaks in NOESY or TOCSY spectrum. For all three methods, it is clear that the residues 3–11 region resides in the apolar milieu of SDS micelle. The pH variation result indicates that the Met$_{17}$-Ile$_{18}$ stretch is more exposed to the solvent at the micellar-aqueous boundary at neutral pH.
On the other hand, the D-H exchange experiment (Fig. 8, A and B) suggests that this stretch is less accessible to solvent molecules at pH 3.5. The 5-DXSA experiment indicated that Trp 14 and Ile 18 are in the apolar environment of micelle near the head group interface. Fig. 8A shows that at low pH Ile 18 is located in the apolar region of micelle but near the head groups. Asp19 is clearly outside of the SDS micelle. Note also that the polar Glu11 and Asn12 are in the interior of the micelle. Our result is consistent with photolabeling data (43), which suggested that the NH2-terminal residues 1–22 of HA2 insert into the membrane bilayer. Of the two glutamic acids, the one at position 15 is clearly more exposed to solvent molecules than that at position 11, especially based on D-H exchange data displayed in Fig. 8B.

Calculated Structures Based on NOE Data Reveal a Larger Fluctuation in the Polar, COOH-terminal Region of HA2-(1–25) in Association with SDS Micelle—To further explore the structural heterogeneity of the peptide, molecular simulation using distance constraints derived from NOE data in the presence of SDS micelle was performed. Fig. 9 shows the root mean square deviation of each of the residues in the peptide, with the superposition of 16 calculated structures shown in the inset. The fluctuation is larger at both ends owing, in part, to the fraying effect. However, larger structural fluctuation is observed in the region 19–24 as compared with the corresponding region 2–7 (with the same sequential distance from the two respective termini). Hence, greater conformational heterogeneity at the COOH-terminal segment is not totally due to the fraying end effect. This result corroborates with the idea that residues 16–18 are located at the micelle-water boundary, and consequently larger root mean square deviation values are obtained for the residues 19 and beyond which lie outside the micelle.

A model of trimeric assembly of HA2-(1–25) using one of the structures shown in Fig. 9 is depicted in Fig. 10. Side chains of residues 6, 9, and 11–19 are explicitly displayed to show the accessibility of water molecules in the interhelical space. It is
noteworthy in this arrangement that the side chains of Glu\textsuperscript{11}, Glu\textsuperscript{15}, and Asp\textsuperscript{19} are shielded from contact with the apolar region of the membrane external to the trimer as the fusion peptide molecules assemble in the membrane. On the other hand, side chains of Trp\textsuperscript{14}, Met\textsuperscript{17}, and Ile\textsuperscript{18} are on the external face of the trimer, rendering them exposed to the hydrophobic environment in our insertion mode.

**DISCUSSION**

The acrylamide fluorescence quenching (Table I) and CD (Fig. 2) experiments in vesicular and micellar solutions suggest the suitability of using the SDS micelle to probe the insertion of HA2-(1–25) into lipid bilayers. NMR attenuation technique detects the \(^1\)H signal change by pH variation, D-H exchange and spin label on the contiguous amino acids along a fusion peptide sequence (31). Supported by fluorescence experiments using acrylamide quenching and NBD-labeled peptide, the NMR results shown in Fig. 8 allow a precise determination of the penetration position of the fusion peptide in the membrane environment. Compared with another method of probing the burial depth, fluorescence quenching of fluorophore attached to fusion peptide by spin-labeled lipids (40), the present approach offers two advantages. First, the attenuation profile is along the entire peptide chain, allowing a clear identification of boundary of the two regions between which there is a large distinction in water accessibility. In contrast, the fluorescence method is based on the distance dependent quenching between the fluorophore and the quencher moieties on the two different molecules and therefore subject to uncertainties such as the orientation of the interacting groups and flip-flop of the labeled phospholipid. Second, there is no additional perturbation of membrane structure since no spin-label and fluorophore are necessary. Using the EPR technique, Macosko et al. (19) determined that the NH\textsubscript{2}-terminal fusion peptide of the HA2-(1–127) fragment inserted into the membrane bilayer with a maximum depth of 15 Å from the phosphate group, suggesting that the fusion peptide did not traverse both leaflets of the bilayer. Still another method, hydrophobic photolabeling, is not capable of directly pinpointing the depth of penetration (44).

Among the three NMR methods adopted in the present work, D-H exchange affords a more sensitive \(^1\)H signal attenuation for residues near the apolar-polar boundary. Thus resonance intensity of Gly\textsuperscript{13}-Gly\textsuperscript{16} is largely reduced in D-H exchange experiments whereas these signals are gradually affected by pH variation (Fig. 8A). This is in part due to the fact that D-H exchange measurements were made at minutes or longer after the onset of introducing deuterium oxide into SDS solution. For HA2-(1–25) in SDS dispersion, the helix content is found to be 45\% (Fig. 2), which amounts to 11 helical residues. However, data from NMR, and fluorescence measurements on the Trp quenching by acrylamide (Table I) as well as NBD-labeled peptide (Fig. 3A) at pH 5.0 enable us to locate the residues (Gly\textsuperscript{13}, Ile\textsuperscript{18}) near the micelle-water or vesicle-water interface but inside the apolar interior of micelle and the NH\textsubscript{2} terminus of the peptide in the hydrophobic region. Hence it is impossible for the micelle-inserted portion of the peptide to adopt a purely helical structure. On the other hand, NMR data (Fig. 7) indicate that segment 2–14 is located inside the micelle primarily as helix. Taken together, our data lead to the proposition that there is an equilibrium between helix and non-helix conformations for the region, with helix being the predominant form. This may be rationalized by the presence of a high content of glycine, known for its conformational plasticity, within the segment. A mutational study by Steinhauser et al. (21) on the effect of substituting glycine within HA2 by bulky non-polar amino acids indicated that the fusogenicity was greatly impaired by changes at positions 1 and 8, and only the alanine substitution was tolerated.

Identification of residues near the membrane-water interface and the proposition of transition between helix and non-helix forms for the portion of HA2 immersed in the membrane bilayer may be germane to the HA2-mediated fusion. First, it is impossible to span both leaflets of the membrane bilayer for a helical peptide sequence of about 16 amino acid residues, especially if an oblique insertion mode is adopted by the fusion peptide as deduced from IR and EPR measurements (17–19). This means that, at some stage of fusion process, only the outer leaflet of the membrane is perturbed. Moreover, it is speculated that, in order to destabilize the membrane, e.g. for fusion pore formation and dilation, a transformation between helix and other forms such as \( \beta \) strand may occur for the membrane-immersed segment. However, based on our data alone, it is not certain that these non-helix forms are essential in the fusion process. Helix-to-\( \beta \) strand transition has been found for HA2-(1–20) in phospholipid vesicle solution containing cholesterol and lipopolysaccharide (45), an inhibitor for pore formation. It is of interest to note that the 20-mer peptide undergoes the transformation only when the lipopolysaccharide is inserted into the outer, but not the inner, leaflet of lipid bilayer, a phenomenon consistent with the idea that insertion of HA2-(1–25) with enhanced helical structure does not lead to its spanning both leaflets. Helix was implied as the fusion active form of the peptide in the discussion.

Decrease in the conjugated NBD fluorescence (Fig. 3A) and in the \( \mathcal{K}_{ov} \) value (Table I) for SDS- and PC-bound HA2-(1–25) when pH was changed from 7.4 to 5.0 suggests that a slightly deeper burial of the peptide at acidic pH. This is also consistent with the result inferred from Fig. 3A and the protease K protection assay shown in Fig. 3C.

Propensity of self-assembly of the fusion peptide in the SDS environment is presented in Fig. 4 under shearing flow in the electric field. Trimeric form has been found for the HA2 ectodomain excluding the fusion peptide sequence in crystal diffraction studies. Our result suggests that tendency for the fusion peptide to oligomerize in the membrane-mimic medium may play a role in the virus-mediated fusion, as proposed previously for the NH\textsubscript{2}-terminal region of gp41 of HIV-1 (29). The paradox of the polar (and ionic) amino acid residues Glu\textsuperscript{11} and Asn\textsuperscript{12}...
embedding in the apolar region of SDS micelle can be resolved by the oligomerization of fusion peptide molecules.

In the present study, helix is found as the primary form for the inserted segment of the 25-mer peptide, hence it is likely to constitute a fusion active conformation. Additionally, the self-assembly propensity is observed for the peptide. Incorporating the spectroscopic results from the present work, we propose a model illustrated in Fig. 10 for the organization of HA2-(1–25) in the membrane bilayer at an intermediate step of fusion. The peptide helical monomers are oriented with the polar face (Glu11, Asn12, and Glu15) pointing to each other in the inner lumen of the oligomer, to reduce the unfavorable free energy caused by immersing these residues in the hydrophobic milieu. Interactions between the polar residues may be mediated by water molecules; thus the oligomeric assembly in the membrane interior may be a loose association between monomers of the fusion peptide domain. In support of the importance of polar amino acids at these positions in self-association of the fusion peptide, only amino acids such as Gln and Thr are found to replace Glu15 among influenza virus strains. A lack of close interaction between the spin labels in the HA2-(1–127) polypeptide was observed from an EPR study by Macosko et al. (19).

Results of Fig. 8 can be explained by our model. Thus Trp14 and Ile18 are on the face exposed to apolar region near the micellar head groups. This leads to a larger attenuation of Trp14 and Ile18 peaks by 5-DXSA but a smaller reduction in proton signal intensity of Ile18 by deuteron exchange, as exhibited in Fig. 8A. The arrangement is in agreement with acrylamide fluorescence quenching experiments, which suggest that Trp14 is inside the hydrocarbon core of the vesicle near the aqueous boundary. The arrangement renders these residues more accessible to the nitroxide moiety attached to the acyl chain of 5-DXSA, but impedes its access to residues 12, 13 and 16, which experience less signal reduction by the spin label.

It is of interest to note that the BHA2 hydrophobic photolabeling data in the liposome solution can be rationalized by the model illustrated in Fig. 10. Thus the side chains of Ile6, Phe8, Trp14, and Met17, which are at the labeling maxima (44), are more exposed to the hydrophobic environment. The photolabeling data in liposome dispersion are, insofar as the model shown in Fig. 10 is concerned, consistent with those from various biophysical techniques undertaken in micellar and vesicular solutions.

We have studied the fusion peptide of human immunodeficiency virus type 1 (HIV-1) which also possesses high Gly/Ala content (28). It was found that the highly conserved Ala15-Gly16 is localized at the micellar-aqueous boundary and Gly16 is conformationally flexible. A role similar to Gly16 of HIV-1 may be played by the highly conserved Gly20 (or Gly16) of HA2. The fusion peptides of both HIV-1 and influenza virus are at the NH2 terminus of their transmembrane subunit. Hence glycines may confer the flexibility in conformational transition of the segment in the membrane apolar core during the fusion mediated by viruses with similar structural features of fusion peptides (37).

REFERENCES
1. Stegmann, T., Doms, R. W., and Helenius, A. (1989) Annu. Rev. Biophys. Biophys. Chem. 18, 187–211
2. White, J. M. (1992) Science 258, 917–924
3. Wilson, I. A., Skehel, J. J., and Wiley, D. C. (1981) Nature 289, 366–373
4. Klenk, H.-D., Rott, R., Ortlich, M., and Blidorn, J. (1975) Virology 68, 426–439
5. Schoch, C., and Blumenthal, R. (1993) J. Biol. Chem. 268, 9267–9274
6. Gething, M. J., Doms, R. W., York, D., and White, J. (1986) J. Cell Biol. 102, 11–23
7. Bullough, P. A., Hughson, F. M., Skehel, J. J., and Wiley, D. C. (1994) Nature 371, 37–43
8. Carr, C. M., and Rums, P. S. (1993) Cell 73, 823–832
9. Skehel, J. J., Bayley, P. M., Brown, E. B., Martin, S. R., Waterfield, M. D., White, J. M., Wilson, I. A., and Wiley, D. C. (1992) Proc. Natl. Acad. Sci. U. S. A. 79, 968–972
10. Daniels, R. S., Douglas, A. R., Skehel, J. J., and Wiley, D. C. (1983) J. Gen. Virol. 64, 1657–1662
11. Tsurudome, M., Glück, R., Graf, R., Falchetto, R., Schaller, U., and Brunner, J. (1992) J. Biol. Chem. 267, 20225–20232
12. Stegmann, T., Delfino, J. M., Richards, F. M., and Helenius, A. (1991) J. Biol. Chem. 266, 18404–18410
13. Wharton, S. A., Calder, L. J., Ruigrok, R. W. H., Skehel, J. J., Steinhauser, D. A., and Wiley, D. C. (1988) J. Gen. Virol. 69, 1847–1857
14. Kemble, G. W., Danieli, T., and White, J. M. (1994) Cell 76, 383–391
15. Melikyan, G. B., Brener, S. A., Ok, D. C., and Cohen, F. S. (1997) J. Cell Biol. 136, 995–1005
16. Song, L., Ahkong, Q. F., Gevgescauld, G., and Lucy, J. A. (1991) Biochim. Biophys. Acta 1065, 54–62
17. Ishiguro, R., Kimura, N., and Takahashi, S. (1993) Biochemistry 32, 9792–9797
18. Gray, C., Tatulian, S. A., Wharton, S. A., and Tamm, L. K. (1996) Biophys. J. 70, 2275–2286
19. Maceko, J. E., Kim, C.-H., and Shin, Y. K. (1997) J. Mol. Biol. 267, 1139–1148
20. Wharton, S. A., Martin, S. R., Ruigrok, R. W., Skehel, J. J., and Wiley, D. C. (1988) J. Gen. Virol. 69, 1847–1857
21. Steinhauser, D. A., Wharton, S. A., Skehel, J. J., and Wiley, D. C. (1995) J. Virol. 69, 6643–6651
22. Lüneberg, J., Martin, I., Nubler, F., Ruyschaert, J.-M., and Herrmann A. (1995) J. Biol. Chem. 270, 27606–27614
23. Harter, C., Bachi, T., Semenza, G., and Brunner, J. (1988) Biochemistry 27, 1856–1864
24. Long, M. L., Waring, A. J., and Hammer, D. A. (1997) Biophys. J. 73, 1430–1439
25. Lear, J. D., and DeGrado, W. F. (1987) J. Biol. Chem. 262, 6500–6505
26. Clague, M. J., Knutson, J. R., Blumenthal, R., and Herman, A. (1991) Biochemistry 30, 5491–5497
27. Rapaport, D., and Shai, Y. (1991) J. Biol. Chem. 266, 23769–23775
28. Chang, D. K., Cheng, S. F., and Chien, W. J. (1997) J. Virol. 71, 6593–6602
29. Chang, D. K., Cheng, S. F., and Trivedi, V. D. (1999) J. Biol. Chem. 274, 5299–5309
30. Wüthrich, K. (1986) NMR of Proteins and Nucleic Acids, John Wiley & Sons, Inc., New York
31. Chang, D. K., and Cheng, S. F. (1998) J. Biomol. NMR 12, 549–552
32. Johnson, W. C., Jr. (1990) Proteins Struct. Funct. Genet. 7, 205–214
33. Hennessey, J. P., Jr., and Johnson, W. C., Jr. (1981) Biochemistry 20, 1085–1094
34. Lakowicz, J. R. (1983) Principles of Fluorescence Spectroscopy, Plenum Press, New York
35. Rajarathnam, K., Hochman, J., Schindler, M., and Ferguson, M. S. (1989) Biochemistry 28, 3168–3176
36. Morris, S. J., Sarkar, D. P., White, J. M., and Blumenthal, R. (1989) J. Biol. Chem. 264, 3972–3978
37. Pécheur, E. I., Sainte-Marie, J., Bienvenüe, A., and Hoeckstra, D. (1999) J. Membr. Biol. 167, 1–17
38. Pritscher, M., Rucker, J., Hoffman, T. L., Doms, R. W., and Shai, Y. (1999) Biochemistry 38, 11359–11371
39. Schagger, H., and Jagow, G. V. (1987) Anal. Biochem. 166, 368–379
40. Breukink, E., van Kraaij, C., van Dalen, A., Demel, R. A., Siezen, R. J., de Kruijff, B., and Kuipers, O. P. (1988) Biochemistry 27, 8153–8162
41. Barratt, M. D., and Lagger, P. (1974) Biochem. Biophys. Acta 363, 127–133
42. Rafalski, M., Ortiz, A., Rockwell, A., van Ginkel, L. C., Lear, J. D., DeGrado, W. F., and Wilschut, J. (1991) Biochemistry 30, 10211–10220
43. Durrer, P., Galli, C., Hoenke, S., Corti, C., Glück, R., Vorherr, T., and Brunner, J. (1996) J. Biol. Chem. 271, 13417–13421
44. Harter, C., James, D., Bachi, T., Semenza, G., and Brunner, J. (1989) J. Biol. Chem. 264, 6459–6464
45. Razinkov, V., Martin, I., Turco, S. J., Cohen, F. S., Ruyschaert, J.-M., and Epand, R. M. (1999) Eur. J. Biochem. 262, 890–899
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