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Determination of the equilibrium micelle-inserting position of the fusion peptide of gp41 of human immunodeficiency virus type 1 at amino acid resolution by exchange broadening of amide proton resonances

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Abstract

The exchange broadening of backbone amide proton resonances of a 23-mer fusion peptide of the transmembrane subunit of HIV-1 envelope glycoprotein gp41, gp41-FP, was investigated at pH 5 and 7 at room temperature in perdeuterated sodium dodecyl sulfate (SDS) micellar solution. Comparison of resonance peaks for these pHs revealed an insignificant change in exchange rate between pH 5 and 7 for amide protons of residues 4 through 14, while the exchange rate increase at neutral pH was more prominent for amide protons of the remaining residues, with peaks from some protons becoming undetectable. The relative insensitivity to pH of the exchange for the amide protons of residues 4 through 14 is attributable to the drastic reduction in $[OH^-]$ in the micellar interior, leading to a decreased exchange rate. The A15-G16 segment represents a transition between these two regimes. The data are thus consistent with the notion that the peptide inserts into the hydrophobic core of a membrane-like structure and the A15-G16 dipeptide is located at the micellar-aqueous boundary.

The proton exchange technique has been used to study the structure and dynamics of biomacromolecules through exchange between labile protons and protons (or deuterons) of solvent molecules (Wagner and Wüthrich, 1979; Englander et al., 1996). For proteins, the method provides a straightforward way to differentiate amino acid residues on the periphery from those buried in the interior. Thus the method is useful in elucidating the protein folding mechanism (Baldwin, 1993; Bai et al., 1995). It was also employed to map the combining site on the protein in the complex formation by the decrease in exchange rate at the complex interface (Paterson et al., 1990; Werner and Wemmer, 1992). The principle has been used to probe the interaction of a glucagon-like peptide with a micelle (Thornton and Gorenstein, 1994). Proton-deuterium or proton-tritium exchange was utilized in the majority of investigations which therefore monitor the kinetic aspect of structural study. The method is also limited to the exchange life time between minutes and weeks. Several proposals were made for the exchange mechanism: structure unfolding (global or subglobal), native (folding) and solvent penetrating mechanisms (Wagner and Wüthrich, 1979; Kim et al., 1993). In these mechanisms, steric hindrance, for example by aromatic or hydrophobic side chain, and hydrogen bond have been considered as factors for a slowing in amide hydrogen exchange.

Insertion of the viral fusion protein into the target cell membrane is considered an important step in virus-mediated fusion which is essential for viral infection (White, 1990). Hence it is crucial to understand the mode of insertion, in particular, the sequence in the fusion protein that penetrates into, and thus destabilizes, the membrane. For example, White and co-workers reported that a lipid-anchored influenza hemagglutinin (HA) promoted hemifusion, but not complete fusion reaction (Kemble et al., 1994). This may be due to insertion of the fusion domain of HA into the outer leaflet of the membrane bilayer, a problem that can be answered by identifying the sequence

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located in the hydrophobic core of the membrane. We have previously employed proton-deuterium exchange, spin label reagents, fluorescence and molecular modeling techniques using data derived from NMR experiments to probe the amino terminal 23-mer fusion peptide of the transmembrane subunit of HIV-1 gp41 (Chang et al., 1997a).

For exposed labile protons, the exchange rate is highly sensitive to the pH of the solution, with a minimum occurring near pH 3 for the backbone (bb) amide proton. In the range of pH 2 to pH 8, the bb amide proton is in the slow exchange regime with water protons in comparison with the chemical shift difference between the bb amide protons and water protons. The observed transverse relaxation rate, $(T_{2,\mathrm{obs}})^{-1}$, which is related to linewidth is then described by

$$(T_{2,\mathrm{obs}})^{-1} = (T_{2,\mathrm{intr}})^{-1} + k_{\mathrm{ex}} \qquad (1)$$

where $(T_{2,\mathrm{intr}})^{-1}$ and k_{ex} are the intrinsic relaxation rate and exchange rate of the amide protons, respectively. k_{ex} is given by

$$k_{\mathrm{ex}} = k_A[\mathrm{H}^+] + k_B[\mathrm{OH}^-] + k_W[\mathrm{H_2O}] \qquad (2)$$

where k_A, k_B and k_W are exchange rate constants for catalysis by acid, base and water, respectively. For pH>3, the exchange reaction of the bb amide proton is base-catalyzed, k_{ex} is therefore larger for higher pH in the pH>3 range. Thus under physiological conditions near pH 7 at room temperature, amide protons accessible to bulk water are undetectable due to exchange broadening. However, protection of the proton from attack of solvent molecules by the hydrophobic core of membranous environment may render those protons observable by slowing the exchange with solvent protons. In other words, the concentrations of protons, hydroxyl ions and water molecules are much lower in the micellar interior than in the bulk water phase. Because in the micellar core k_B is not likely to vary to the same extent as the hydroxyl ion concentration, k_{ex} is greatly diminished, and likely remains small even as the solution pH is raised to 7 or 8. On the other hand, the $(T_{2,\mathrm{intr}})^{-1}$ is increased when the peptide is associated with the SDS micelle, because of an increase in the rotational correlation time. In addition, the hydroxyl ions are readily accessible at the micellar exterior. Consequently, amide protons located outside the micelle are broadened beyond detection at neutral pH, due to a large $(T_{2,\mathrm{obs}})^{-1}$, the sum of increased k_{ex} and $(T_{2,\mathrm{intr}})^{-1}$.

Because of the large difference in the contribution of k_{ex} to the linewidth of amide proton peaks, particularly at neutral pH, between protons inside and outside the micelle, arising mainly from the drastic decrease of hydroxyl ions in the micellar apolar core, it is possible to use variation of the lineshape of amide proton resonances with pH to determine the position of amide groups with respect to the micelle.

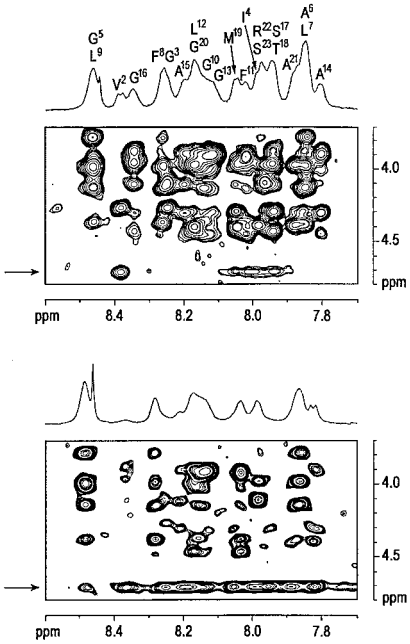

Figure 1. Amide proton resonance region of 1D and 2D PFG-NOESY spectra of the 23-mer fusion peptide of HIV-1 gp41 (0.85 mM, 305 K) at pH 5 (top) and at pH 7 (bottom) in 150 mM SDS aqueous (1:9 v/v D_2O:H_2O) solution. Comparison of 1D spectra obtained at the two pHs indicates that the amide proton of V2 is undetectable at pH 7. 2D spectra further show that amide protons of G16 through S23 are greatly attenuated or undetected at neutral pH. The mixing time used in NOESY experiments was 600 ms. The protons from the solvent resonate at 4.71 ppm. Arrows in both panels indicate the peaks arising from exchange between protons of solvent molecules and various amide protons. The enhanced intensity of crosspeaks from solvent protons with the amide protons of G5, L9, G16, G3, A15, M19, R22, S23, S17, T18, A21 and A14 at pH 7 relative to pH 5 is consistent with the notion that the exchange rate of these amide protons with solvent protons is higher at pH 7.

In the following, a 23-mer peptide (AVGIGALFLGFLGAAGSTMGARS; gp41-FP) encompassing the N-terminal portion of HIV-1 gp41 was used to study the location of penetration into the SDS micelle. The peptide was found to possess β-form in equilibrium with disordered structure in water, but is induced into helix both in 50% TFE and in SDS micellar solutions (Chang et al., 1997b). The F8-G10 segment, the FLG motif, forms a type I β-turn which is thought to be the initiation site for helix formation of the peptide.

Figure 1 shows the expanded region of 1D and 2D PFG-NOESY spectra (Piotto et al., 1992) of the fusion peptide of the transmembrane subunit of HIV-1 in the SDS micellar suspension at pH 5 and pH 7. The experiments were performed on a Bruker Avance-500 Spectrometer. Both 1D and 2D data indicate that, at pH 7, the signals of bb amide protons from residues at the amino and carboxyl termini are markedly attenuated or undetectable. Analogous result was obtained for pH 8. The results of linewidth analysis for the bb amide proton resonances of micelle by XEASY (Bartels et al., 1995) and inspection of resolved peaks in the 1D spectrum are shown in Table 1. At pH 5 and 305 K, the linewidth ranges between 6.4 and 11.2 Hz. Linewidth increases between 0.5 and 6.1 Hz are observed for the protons located in the micellar core as the solution pH is raised from 5 to 7. This is due to the small increase in $[OH^-]$ at higher pH in the membrane apolar interior. In contrast, resonance peaks external to the SDS micelle are broadened to the baseline (i.e. linewidth is greater than 100 Hz) at neutral pH, indicating a large increase of $[OH^-]$ accessible to these protons as pH changes from 5 to 7. It is noted in Figure 1 that by raising the pH, intensity is increased for the crosspeaks from the water resonance along F_1 axis and some of the amide protons along F_2 axis (marked by arrows in Figure 1); the result indicates that these peaks are due to chemical exchange rather than nuclear Overhauser effect. This is because the intensity of an exchange crosspeak is proportional to the rate of exchange between the water protons and the protons to which the crosspeak is attributed (Ernst et al., 1987). In support of the contention, in the ROESY spectrum, positive sign (relative to the diagonal peaks) is observed for these crosspeaks, the intensity of which increases with higher temperature (Chang, unpublished data).

Another quantitative analysis of the 2D data, taken as deviation from unity of the ratio of the corresponding crosspeak intensity at pH 7 and pH 5 for each of the residues in the peptide, is displayed in Figure 2. The attenuation in the amide proton signal is attributed to the spin relaxation during the time delays in NOESY experiments (cf. Equation 1). The signal loss is larger for larger relaxation rate. The result reveals that the middle section is most solvent-inaccessible due to its location in the micellar interior, and the A14-G16 comprises the transition region to the solvent-exposed carboxyl terminal portion. Similar result is obtained for the mutant of the peptide in which G10 is substituted by a valyl residue. The present

Table 1. Linewidth [in Hz] of the backbone amide protons of gp41-FP in SDS micellar solution at pH 5 and pH 7 at 305 K analyzed from XEASY protocol and 1D spectrum inspection

Residue	pH 5		pH 7	
	XEASY	1D	XEASY	1D
A^1	–	–	–	–
V^2	6.8 ± 0.2	6.3	–	–
G^3	–	–	–	–
I^4	8.3 ± 0.3	8.5	12.8 ± 0.6	12
G^5	–	–	–	–
A^6	–	–	16.7 ± 1.1	–
L^7	9.8 ± 0.4	–	15.2 ± 0.9	–
F^8	7.6 ± 0.3	–	10.5 ± 0.5	–
L^9	9.9 ± 0.4	–	12.6 ± 0.7	–
G^{10}	–	–	–	–
F^{11}	6.2 ± 0.2	6.0	13.3 ± 0.8	14
L^{12}	9.9 ± 0.4	–	14.9 ± 1.1	–
G^{13}	–	–	–	–
A^{14}	7.6 ± 0.3	–	8.1 ± 0.3	8
A^{15}	10.3 ± 0.4	–	16.4 ± 0.9	–
G^{16}	–	–	–	–
S^{17}	7.4 ± 0.3	–	–	–
T^{18}	8.1 ± 0.3	–	–	–
M^{19}	6.4 ± 0.2	6.3	–	–
G^{20}	–	–	–	–
A^{21}	9.4 ± 0.4	–	–	–
R^{22}	–	–	–	–
S^{23}	11.2 ± 0.5	–	–	–

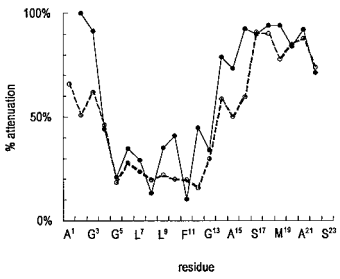

Figure 2. Attenuation, due to pH change, of intensity of the intraresidue crosspeak from the amide proton to C_α proton for each of the amino acid residues in the fusion peptide of HIV-1 gp41 (solid trace) and its G10V variant (dashed trace) in SDS micellar solution. pH of 5 and 7 were used in the wild-type peptide, while pH 5 and pH 8 were used for the G10V analog. For both peptides, exchange with the solvent protons for amide protons in the region between I4 and G13 is enhanced significantly less than that for those protons in the remainder of the peptide. The data suggest the region I4-G13 is less solvent-accessible, consistent with the idea that the region is embedded in the hydrophobic interior of the micelle.

data extend previous proton-deuterium exchange experiments in which amide protons of F8, L9 and L12 were found to be slowly exchanging (Chang et al., 1997a). The data lend support to the conclusion made from previous experiments that the A15-G16 stretch is at the interface of micelle and aqueous phase.

Helical structure can be identified for the region spanning residues 4–15 and 16–22 of the fusion peptide, with a loose structure between the two segments (Chang et al., 1997a). Although the helical form for the former segment is more stable than the latter, it is not likely to account for the difference in the pH sensitivity of the crosspeak intensity arising from the residues of these two regions. This is because the A15-G16 stretch constitutes the loose linker between the two helical sections and therefore should have weaker hydrogen bonds with the residues in the remainder of the peptide, resulting in faster exchange of the amide protons of A15 and G16 with solvent molecules. However, the exchange rates for these two bb amide protons are found to be intermediate between those from its N-terminal and C-terminal regions. Hence the intramolecular hydrogen bonding is inadequate in accounting for the observed exchange rate variation among the amide protons of the residues in the peptide.

The intrinsic exchange rate of bb amide protons under the influence of amino acid side chains has been quantitatively investigated by Bai et al. (1993). The steric and inductive factors arising from neighboring side chains were found to be additive. Thus the rate was found to be smaller for the protons adjacent to apolar side chains while the exchange was generally enhanced by the presence of neighboring polar side chains for base-catalyzed exchange reaction. The difference of a few Hz is, however, too small to account for the broadening to the baseline of those resonance peaks attributed to protons external to the SDS micelle at neutral pH observed in the present study.

Li and Montelione (1993) have shown that, in a trimeric peptide, the attenuation of the amide proton signal in a PFG-HSQC experiment at neutral pH was due to partial non-equilibrium distribution of water proton spin state and exchange broadening. Both effects depend on the chemical exchange between the amide protons and the water protons.

The uniform and small attenuation of the NH/αH crosspeaks from residues of a large segment of a short peptide over the range of pH 5 to 8 at room temperature is a strong indication that these residues are buried in the interior of some hydrophobic matrix. The method thus provides an effective means to determine the penetration of fusion peptides or other membrane-inserting proteins into the membranous environment at the resolution of amino acid residue. It is simple and straightforward in that the uniform broadening out of the amide proton resonances at neutral pH is taken to be an indicator of the amide groups external to the micelle. Because the method does not involve proton-deuterium exchange, it reports the equilibrium, but not transient, property of the insertion. Accordingly, there is no upper or lower limit to the exchange rate to be measured, in contrast to the H-D exchange approach. Application to a bicelle suspension is in progress in our laboratory.

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References

Bai, Y., Milne, J.S., Mayne, L. and Englander, S.W. (1993) *Proteins Struct. Funct. Genet.*, **17**, 75–86.

Bai, Y., Sosnick, T.R., Mayne, L. and Englander, S.W. (1995) *Science*, **269**, 192–197.

Baldwin, R.L. (1993) *Curr. Opin. Struct. Biol.*, **3**, 84–91.

Bartels, C., Xia, T., Billeter, M., Güntert, P. and Wüthrich, K (1995) *J. Biomol. NMR*, **6**, 1–10.

Chang, D.K., Cheng, S.F. and Chien, W.J. (1997a) *J. Virol.*, **71**, 6593–6602.

Chang, D.K., Chien, W.J. and Cheng, S.F. (1997b) *Eur. J. Biochem.*, **247**, 896–905.

Englander, S.W., Sosnick, T.R., Englander, J.J. and Mayne, L. (1996) *Curr. Opin. Struct. Biol.*, **6**, 18–23.

Ernst, R.R., Bodenhausen, G. and Wokaun, A. (1987) *Principles of Nuclear Magnetic Resonance in One and Two Dimensions*, Oxford University Press.

Kemble, G.W., Danieli, T. and White, J.M. (1994) *Cell*, **76**, 383–391.

Kim, K.S., Fuchs, J.A. and Woodward, C.K. (1993) *Biochemistry*, **32**, 9600–9608.

Li, Y.C. and Montelione, G.T. (1993) *J. Magn. Res.*, **B101**, 315–319.

Paterson, Y., Englander, S.W. and Roder, H. (1990) *Science*, **249**, 755–759.

Piotto, M., Saudek, V. and Sklenar, V. (1992) *J. Biomol. NMR*, **2**, 661–665.

Thornton, K. and Gorenstein, D.G. (1994) *Biochemistry*, **33**, 3532–3539.

Wagner, G. and Wüthrich, K. (1979) *J. Mol. Biol.*, **134**, 75–94.

Werner, M.H. and Wemmer, D.E. (1992) *J. Mol. Biol.*, **225**, 873–889.

White, J.M. (1990) *Annu. Rev. Physiol.*, **52**, 675–697.