

Putative role of membranes in the HIV fusion inhibitor enfuvirtide mode of action at the molecular level

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Partition of the intrinsically fluorescent HIV fusion inhibitor enfuvirtide into lipidic membranes is relatively high ($\Delta G = 6.6 \text{ kcal} \cdot \text{mol}^{-1}$) and modulated by cholesterol. A shallow position in the lipidic matrix makes it readily available for interaction with gp41. No conformational energetic barrier

prevents enfuvirtide from being active in both aqueous solution and lipidic membranes. Lipidic membranes may play a key role in the enfuvirtide biochemical mode of action.

Key words: AIDS, enfuvirtide, fusion inhibitor, fuzeon, HIV, T20.

INTRODUCTION

Despite the promising clinical studies and trials, which led to a fast and recent approval by the United States Food and Drug Administration [1] for clinical use, action of the HIV fusion inhibitor enfuvirtide (Fuzeon; Figure 1) at the molecular level is largely unknown. Unlike other peptides based on the C-region of gp41 (C-peptides), enfuvirtide lacks the eight-amino-acid sequence (628–635) thought to be essential for association with the N-region [2].

The sequence of enfuvirtide and its eventual arrangement in an α -helix (Figure 1), with amphipathic segments, clearly suggests that it may interact with biological membranes. A theoretical analysis (Membrane Protein Explorer, MPEx version 2.04; <http://blanco.biomol.uci.edu/mpex/>) on the hydrophobicity and interface affinity reinforces this idea (results not shown). Thus we were prompted to study (i) the extent of the partition of enfuvirtide into biological membrane models, (ii) its location in the lipidic matrix and (iii) the interplay between partition and conformation. The presence of tryptophan residues in enfuvirtide makes fluorescence techniques suitable tools to probe this molecule with no need for chemical derivatization.

EXPERIMENTAL

Materials

Enfuvirtide was a kind gift from Roche (Palo Alto, CA, U.S.A.). POPC (1-palmitoyl-2-oleyl-*sn*-glycero-3-phosphocholine), DPPC (1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine) and POPG {1-palmitoyl-2-oleyl-*sn*-glycero-3-[phospho-rac-(1-glycerol)]} were purchased from Avanti Polar-Lipids (Alabaster, AL, U.S.A.), while cholesterol was from Sigma (St. Louis, MO, U.S.A.). 5NS (5-doxy-stearic acid) and 16NS (16-doxy-stearic acid) were from Aldrich Chem. Co. (Milwaukee, WI, U.S.A.). L-Tryptophan, acrylamide, Hepes and NaCl were from Merck (Darmstadt, Germany). The spectrofluorimeter used was an SLM Aminco 8100 (double monochromators; 450 W Xe lamp) and the CD spectrometer was a Jasco J720 (450 W lamp).

Methods

We used 10 mM Hepes, pH 7.4/150 mM NaCl buffer, and prepared lipidic LUVs (large unilamellar vesicles) by extrusion techniques [3]. Quenching studies were carried out by solubilization of the quenchers in ethanol followed by direct injection in the lipidic vesicles suspension (ethanol in the final sample was kept < 2%). Effective lipophilic quencher concentrations inside the membrane were calculated for data-analysis purposes. Excitation and emission wavelengths were 280 and 350 nm, respectively, in all fluorescence measurements. For details of transient-state fluorescence experiments, see [4]. We prepared enfuvirtide stock solutions in buffer (2.2×10^{-4} M), diluted to a final concentration of 7×10^{-6} M (fluorescence quenching), 1×10^{-5} M (partition coefficient calculation) or 7×10^{-5} M (CD experiments). Partition coefficient determination was carried out by successive addition of small aliquots of LUV (15 mM), with 10 min incubations in between. The dilution effect on fluorescence intensity was corrected.

RESULTS AND DISCUSSION

UV-visible absorption and fluorescence spectra in buffer of enfuvirtide are identical to those of Trp in aqueous solution (results not shown). No red-edge excitation effect (i.e. red shift of the emission spectrum) was detected, which indicates that all Trp residues sense similar local environments. Accordingly, the fluorescence quenching of enfuvirtide by acrylamide (a hydrophilic molecule) revealed that no hydrophobic pockets are present (linear Stern–Volmer plots; results not shown). Although the existence of hydrophobic pockets does not seem likely in a small peptide, aggregation or clustering could eventually take place. Aggregation is probably prevented by the intercalation of charged residues among hydrophobic ones (Figure 1a).

In the presence of LUV of POPC, the fluorescence intensity of enfuvirtide increases (Figure 2). Concomitantly, a fluorescence emission blue shift occurs (11 nm when [POPC] = 5 mM), which shows that the Trp residues in enfuvirtide are being incorporated progressively into the lipid and sensing a more hydrophobic

Abbreviations used: LUV, large unilamellar vesicles; POPC, 1-palmitoyl-2-oleyl-*sn*-glycero-3-phosphocholine; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; POPG, 1-palmitoyl-2-oleyl-*sn*-glycero-3-[phospho-rac-(1-glycerol)]; 5NS, 5-doxy-stearic acid; 16NS, 16-doxy-stearic acid.

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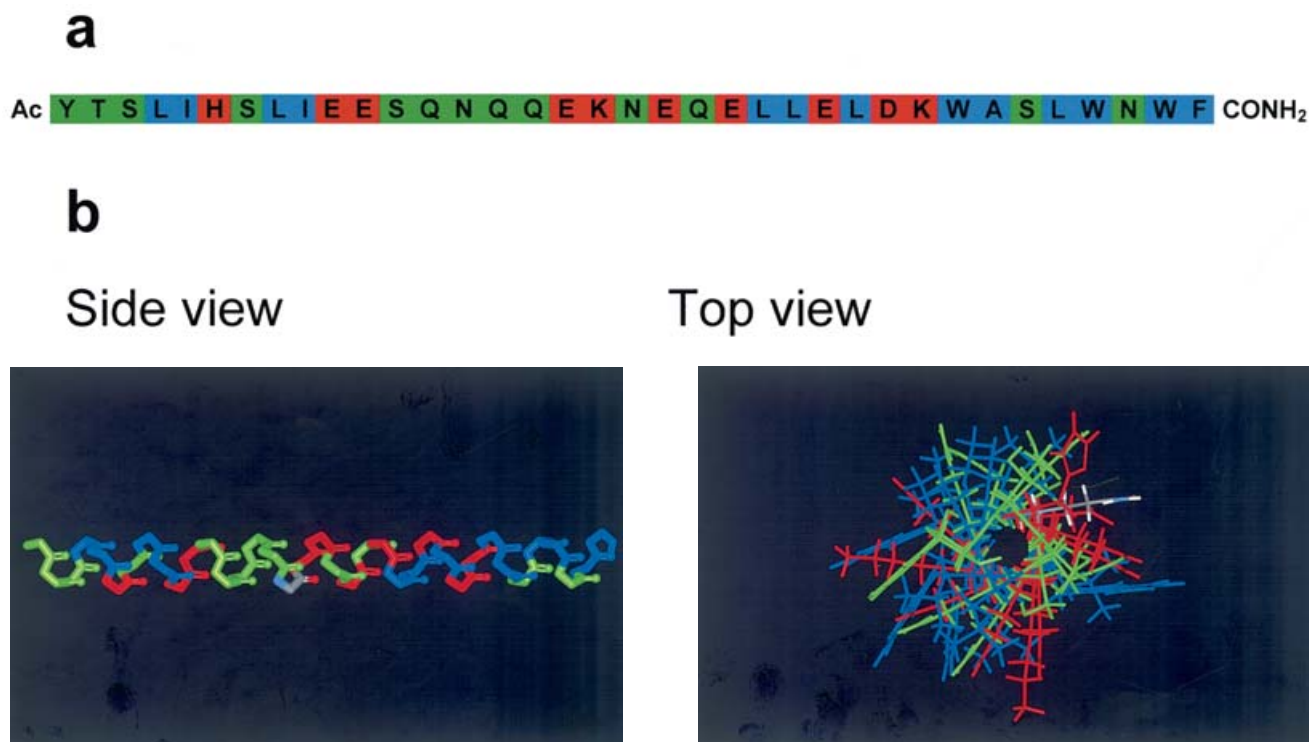


Figure 1 Amino acid sequence of enfuvirtide (a) and its arrangement in an α -helix (b), as it appears in the crystallographic structure of gp41

Hydrophobic (blue), non-charged polar (green) and charged polar (red) residues form amphipathic segments in the helix. The top view in (b) shows that the hydrophobic side chains point towards the same side of the chain. This is also apparent from the side view, when only the backbone is represented. Uncapped enfuvirtide at both ends is usually referred to as T20.

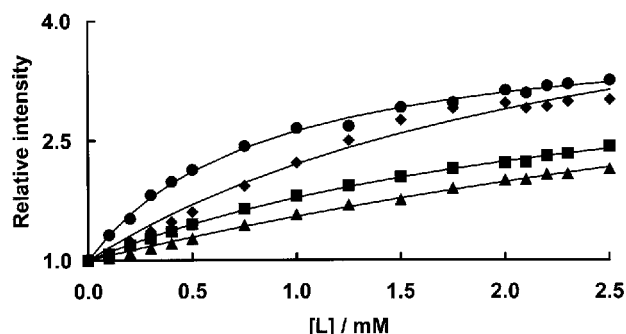


Figure 2 Partition coefficient determination

The fluorescence intensity of enfuvirtide increases upon addition of lipidic LUV as a result of a higher quantum yield of the molecules inserted into the lipidic matrix. POPC without added cholesterol (\bullet) is the most efficient in the uptake of the peptides, followed by DPPC (\blacklozenge). The differences are probably due to fluidity: POPC forms fluid bilayers (similar to biological membranes) while DPPC is in the gel state. POPC + cholesterol [33% cholesterol (in molarity); \blacktriangle] is the least efficient. The solid lines are fittings of eqn (1) to the experimental data [POPC, $K_p = (1.6 \pm 0.1) \times 10^3$; $\Delta G = 6.6 \text{ kcal} \cdot \text{mol}^{-1}$]. The presence of the negatively charged lipid POPG (20%; \blacksquare) mimics the inner leaflet of mammals' cell membranes and results in very weak incorporation. Outer leaflets are electrically neutral and mimicked by POPC. [L] is the outer leaflet lipidic (or lipid + cholesterol) concentration.

environment. The partition coefficient describing the distribution of enfuvirtide between aqueous and lipidic media is $K_p = [\text{enfuvirtide}]_L / [\text{enfuvirtide}]_w$, where $[\text{enfuvirtide}]_L$ and $[\text{enfuvirtide}]_w$ are the peptide concentrations in the lipidic and aqueous

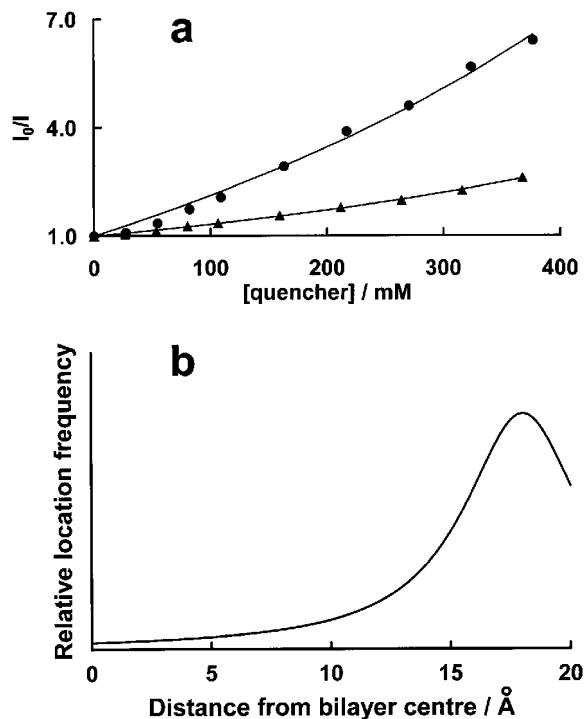


Figure 3 Location of enfuvirtide Trp residues inside the membrane

(a) Stern–Volmer plot for the quenching of enfuvirtide fluorescence by 5NS (\bullet) and 16NS (\blacktriangle) in POPC (5 mM) vesicles. 5NS is a better quencher; therefore Trp residues are located in a shallower position. (b) Quenching data (Table 1) were used to calculate the enfuvirtide Trp residues in-depth frequency distribution along the lipidic bilayer using the SIMEXDA method [9]. The shallow location is clear.

Table 1 Average fluorescence lifetimes (τ) of enfuvirtide

The mean fluorescence lifetime (τ) of enfuvirtide increases upon incorporation in lipidic membranes. Differential diffusional fluorescence quenching by 5NS and 16NS inside the lipidic matrix enables in-depth location of the Trp residues.

System	τ (ns)
Buffer	2.4
5 mM POPC	4.9
5 mM POPC/0.4 mM 5NS	3.0
5 mM POPC/0.4 mM 16NS	4.0

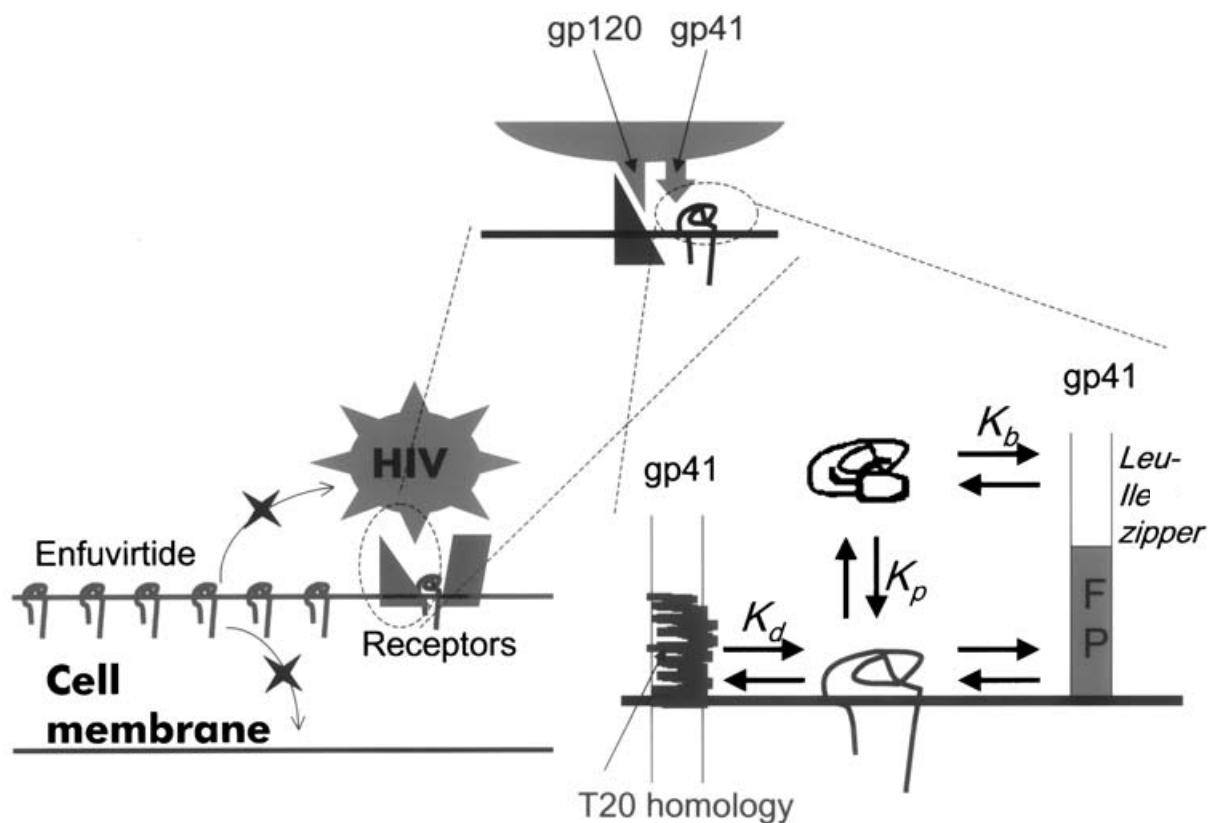
media, respectively. This can be calculated from the data (Figure 2) by fitting the following equation [5,6]:

$$\frac{I}{I_w} = \frac{1 + K_p \gamma_L [L] I_L / I_w}{1 + K_p \gamma_L [L]} \quad (1)$$

where I is the measured fluorescence intensity, I_w and I_L are the fluorescence intensities when all the molecules are in the aqueous or lipidic environment, respectively, γ_L is the lipidic molar volume [7] and $[L]$ is the molar concentration of the accessible lipid, i.e. that of the outer leaflet of the bilayer. For POPC, a lipid with packing density and fluidity properties similar to biological membranes, a K_p value of $(1.6 \pm 0.1) \times 10^3$ ($\Delta G =$

$6.6 \text{ kcal} \cdot \text{mol}^{-1}$) is obtained and this implies that, depending on the lipid concentration, the local concentration in the membrane can increase by several hundred- to thousand-fold relative to the bulk (≈ 700 -fold at $[\text{POPC}] = 4 \text{ mM}$; ≈ 1600 -fold at $[\text{POPC}] < 0.1 \text{ mM}$). However, this is severely reversed by the presence of cholesterol or physiological concentrations [8] of negatively charged lipids (Figure 2). The cholesterol effect does not seem totally caused by the condensation effect imposed by the sterol on the lipid because peptide partition into gel-phase membranes is more pronounced (Figure 2). The effect of negatively charged lipids is probably due to simple electrostatic repulsion (enfuvirtide has a overall negative net charge of -5).

The in-depth location of fluorescent molecules in lipidic membranes can be evaluated from quenching experiments with lipophilic molecules derivatized with quencher groups at selected sites [9]. Stearic acid, for instance, can be derivatized at carbon-5 or -16 with doxyl groups (5NS and 16NS, respectively). When placed in the membrane, the quencher group in 5NS locates at a shallow position, near the interface, whereas in 16NS it locates close to the hydrophobic core. 16NS is a better quencher for fluorescent molecules buried deeply in the membrane while 5NS is a better quencher for molecules near or at the interface. Enfuvirtide is better quenched by 5NS (Figure 3a), meaning that the Trp residues are located in a shallow position in the membrane. Fluorescence-lifetime quenching data (Table 1) enabled the application of the SIMEXDA method [9] to recover the Trp residues'

**Figure 4 Schematic representation of enfuvirtide action at the molecular level**

Enfuvirtide attaches to membranes in an interfacial position reaching local high concentrations. Translocation is prevented due to charge effects (among others). When the virus approaches the cell surface, its outer membrane will not compete for enfuvirtide uptake due to its high content of cholesterol. Therefore, enfuvirtide molecules remain as 'guardians' of the cell surface. The cell membrane is a reservoir of enfuvirtide; the gp41 fusiogenic approach exposes the Leu-Ile zipper-like region to the peptides, which binds them (K_b) [12]. However, local concentration of aqueous enfuvirtide is kept high due to partition equilibrium (K_p). Moreover, a high local concentration at the cell-membrane surface enables direct contact of the peptide with the gp41 C-region (K_d) when the enfuvirtide homologous sequence comes into contact with the lipidic matrix to form the fusion pore. Fusion peptide (FP) binding [2] also benefits from a high concentration of enfuvirtide at the cell surface.

in-depth distribution in the membrane (Figure 3B). A mean shallow location and a fairly narrow distribution were observed.

Like other HIV gp41 C-peptides [10], enfuvirtide is not an α -helix in solution (CD data not shown). It is known that several peptides go through a random-coil-to-helix transition upon binding to membranes [11]. However, this is not the case with enfuvirtide, which remains in a random-coil conformation when inserted in membranes (no alterations are detected in the CD spectra; results not shown).

Based on the data described above, one can depict in general terms what might be the action of enfuvirtide at the molecular level. As shown in Figure 4, enfuvirtide inserts into the external layer of the cell plasmalemma (non-charged lipids) and is prevented from translocation due to the repulsion caused by the negatively charged lipids of the inner layer, among other reasons. When the HIV approaches the cell, the virus lipidic membrane does not remove the enfuvirtide from the cell outer surface because of its high cholesterol content; i.e. enfuvirtide concentrates at cell surfaces and tends to stay there. Therefore, cell membranes are an enfuvirtide reservoir. The partition equilibrium tends to stabilize the enfuvirtide concentration in the aqueous environment when binding to gp41 occurs (i.e. improving fusion inhibition efficiency). Moreover, the high levels of enfuvirtide accumulated at the cell surface render the interaction with other segments of gp41 possible if they reach the cell surface (i.e. if enfuvirtide binding to the Leu-Ile-rich region fails and the fusion process proceeds). Binding of enfuvirtide and related molecules to the fusion peptide and other gp41-derived peptides has been reported [2,12].

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