HIV-1 Tat membrane interaction probed using X-ray and neutron scattering, CD spectroscopy and MD simulations

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Running Title: HIV Tat/membrane structure and properties

Keywords: Low-angle X-ray scattering (LAXS), wide-angle X-ray scattering (WAXS), circular dichroism spectroscopy, molecular dynamics (MD) simulations, lipid bilayer structure

4/9/2014

Abstract

We report the effect on lipid bilayers of the Tat peptide G_{48}RKKRRQRRRPPQ$_{60}$ from the HIV-1 virus transactivator of translation (Tat) protein. Synergistic use of low angle X-ray scattering (LAXS) and atomistic simulations (MD) indicate Tat peptide binding to neutral dioleoylphosphatidylcholine (DOPC) lipid headgroups. This binding induced the local lipid phosphate groups to move 3 Å closer to the center of the bilayer. Many of the positively charged guanidinium components of the arginines were as close to the center of the bilayer as the locally thinned lipid phosphate groups. LAXS data for DOPC, DOPC/dioleoylphosphatidyl-ethanolamine (DOPE), DOPC/dioleoylphosphatidylserine (DOPS), and a mimic of the nuclear membrane gave similar results. Generally, the Tat peptide decreased the bending modulus K_C. Further indications that Tat softens a membrane, thereby facilitating translocation, were provided by wide-angle X-ray scattering (WAXS) and neutron scattering. CD spectroscopy was also applied to further characterize Tat/membrane interactions. Although a mechanism for translation remains obscure, this study suggests that the peptide/lipid interaction makes the Tat peptide poised to translocate.

Abbreviations: dioleoylphosphatidylcholine (DOPC), dioleoylphosphatidylethanolamine (DOPE), dioleoylphosphatidylserine (DOPS), palmitoyloleoylphosphatidylcholine (POPC), palmitoyloleoylphosphatidylethanolamine (POPE), palmitoyloleoylphosphatidylserine (POPS), Soy phosphatidylinositol (Soy PI), sphingomyelin (SM), cholesterol (Chol), bis(monoacylglycerol)-phosphate) (BMP), phosphatidylglycerol (PG), cell-penetrating peptide (CPP), giant unilamellar vesicles (GUVs), large unilamellar vesicles (LUVs), low-angle X-ray scattering (LAXS), wide-angle X-ray scattering (WAXS), hexafluoroisopropanol (HFP), trifluoroethanol (TFE)

1. Introduction

The name cell-penetrating peptide (CPP) connotes a peptide that easily penetrates cell membranes (for Reviews see [1-3]). The present work focuses on the transactivator of translation, Tat, from the HIV-1 virus, which plays a role in AIDS progression. Early work showed that the HIV-Tat transactivator protein (86 amino acids) was efficiently taken up by cells, and concentrations as low as 1 nM were sufficient to transactivate a reporter gene expressed from the HIV-1 promoter [4, 5]. It has been reported that Tat protein uptake does not require ATP [6]. Studies using inhibitors of different types of endocytosis, including clathrin- and caveolae-mediated, or receptor-independent macropinocytosis reached the same conclusion that ATP mediated endocytosis is not involved in Tat protein permeation [7-10]. However, this issue is controversial, as other studies found evidence for endocytosis in Tat protein import [11-19]. Still other studies have concluded that an ATP requirement for Tat protein entry depends on the size of the cargo attached to Tat protein, or on the specific cell type [20-22].

The part of the Tat protein responsible for cellular uptake was assigned to a short region Tat (48-60), G_{48}RKKRRQRRRPPQ$_{60}$, which is particularly rich in basic amino acids [6]. Deletion of three out of eight positive charges in this region caused loss of its ability to translocate [6]. In this manuscript short basic regions will be called Tat, while the entire 86-amino acid protein will be called Tat protein. Tat was shown to be responsible for the Tat protein's permeation into the cell nucleus and the nucleoli [6], and this was confirmed using live cell fluorescence in SVGA cells [23]. Tat (48-60) was shown to have little toxicity on HeLa cells at 100 μM concentration [6], but the longer Tat protein (2-86) was toxic to rat brain glioma cells at 1-10 μM [24]. Interestingly, no hemolytic activity was found when human erythrocytes were incubated with a highly neurotoxic concentration (40 μM) of Tat (2-86) [24]. These results prompt the question, what is the mechanism of Tat's translocation through membranes?

To address this question, many biophysical studies have used simple models of biological membranes composed of a small number of lipid types. These studies are valuable because there is no possibility for ATP-dependent translocation, thus ruling out endocytosis if translocation occurs. For example, Mishra et al. reported that the rate of entry into giant unilamellar vesicles (GUVs) composed of PS/PC (1:4 mole ratio) lipids of rhodamine-tagged Tat is immeasurably slow, but it crosses a GUV composed of PS/PC/PE (1:2:1) lipids within 30 seconds [25]. This study suggests that negative curvature induced by the inclusion of PE facilitates translocation. In a subsequent study using much smaller unilamellar vesicles (LUVs), Tat did not release an encapsulated fluorescent probe in LUVs composed of lipids modeling the outer plasma membrane, PC/PE/SM/Chol (1:1:1:1.5), but did release the probe in LUVs composed of BMP/PC/PE (77:19:4) [26]; BMP (bis(monoacylglycero)-phosphate) is an anionic lipid specific to late endosomes. In that study [26], the inclusion of PE did not suffice to cause leaky fusion in LUVs in the absence of a negatively charged lipid. The contrasting results in these two experiments may also be due to the use of LUVs instead of GUVs since it was reported that Tat does not translocate across LUVS of PC/PG (3:2) but does translocate across GUVs of the same lipid composition [27]. In a similar experiment, Tat did not translocate into egg PC LUVs [28]. In another experiment confirming these results, Tat did not translocate into GUVS containing only PC with 20 mol% cholesterol, but when PS or PE was included with PC, then rapid translocation of Tat was observed [29]. These experiments demonstrate that the choice of lipids and model systems influences Tat translocation.

Is a pore formed during Tat translocation? Although direct conductance measurements of Tat and lipid membranes have not been carried out, two studies measured conductance with the somewhat similar CPP oligoarginine R9C peptide. Using single-channel conductance of gramicidin A in planar lipid membranes consisting of anionic, neutral or positively charged lipids, R9C did not increase conductance, even in anionic lipid membranes [30]. By contrast, in a similar experiment using planar lipid membranes, a current was induced by R9C in PC/PG (3:1) membranes, with increasing destabilization over time [31]. Thus questions remain about pore formation of Tat in membranes. In the GUV experiment with Tat mentioned above [29], Ciobanasu et al., using size exclusion methods, suggested a pore in the nanometer range, which could only be passed by small dye tracer molecules. Thus, if a true pore forms, it is likely to be small and transitory.

What is the secondary structure of Tat in membranes? Circular dichroism (CD) spectroscopy was carried out on, where the penultimate proline on Tat (48-60) was replaced by a tryptophan [27]. That study found a random coil secondary structure in aqueous solution as well as when mixed with PC/PG/PE (65:35:5) LUVs. The same result was obtained using CD in PC/PG (3:1) vesicles by Ziegler et al.[10], indicating that an alpha helix is not required for Tat's translocation ability. In addition, solid state NMR has identified a random coil structure of Tat in DMPC/DMPG (8:7 mole ratio) multibilayers [32]. In the larger Tat-(1-72)-protein NMR measurements at pH 4 have determined there is no secondary structure, with a dynamical basic region [33]. Similarly, NMR was used to study the full Tat protein and found a highly flexible basic region [34].

Regarding the mechanism of translocation of this randomly structured, short basic peptide, many models have been proposed based on the conflicting results listed above. Molecular dynamics simulations offer some insight into the molecular details of translocation. Herce and Garcia simulated the translocation of Tat (Y_{47}GRKKRRQRRR$_{57}$) across DOPC at various lipid:peptide molar ratios [35]. Their simulations indicated that Tat binds to the phosphate headgroups, with 1 Tat binding with 14 lipids, each positive charge on Tat-associated with nearly 2 phosphate groups [35]. Translocation involved a localized thinning, and snorkeling of arginine side chains through the hydrophobic layer to interact with phosphates on the other side of the membrane. This allowed some water molecules to penetrate the membrane along with Tat, forming a pore [35]. In this simulation, performed without inclusion of counterions, pore formation was only observed at high ratios of peptide:lipid (1:18) or at elevated temperature. However, a subsequent Gromacs simulation with counterions found no thinning and no pore formation when Tat was added to DOPC membranes [36]. Instead it found a membrane invagination associated with a cluster of Tat peptides, suggesting that micropinocytosis could be the model for Tat translocation across membranes [36].

In this work we primarily combine experimental low-angle X-ray scattering (LAXS) data with MD simulations to obtain the structure of fully hydrated, oriented lipid bilayers with Tat (47-57) added at several mole ratios. The lipid systems were DOPC, DOPC/DOPE (3:1 mole ratio), DOPC/DOPS (3:1), DOPC/DOPE (1:1) and a mimic of the nuclear membrane (POPC/POPE/POPS/SoyPI/Chol, 69:15:2:4:11). Accessory techniques, densitometry, wide-angle X-ray scattering (WAXS), neutron scattering, CD spectroscopy were also applied to further characterize Tat/membrane interactions.

2. Materials and methods

2.1 Lipids and peptides

Synthesized lipids were purchased from Avanti Polar Lipids (Alabaster, AL) and used without further purification. Membrane mimics were prepared by first dissolving lyophilized lipids in chloroform and then mixing these stock solutions to create the lipid compositions DOPC, DOPC/DOPE (3:1), DOPC/DOPE (1:1), DOPC/DOPS (3:1) and nuclear membrane mimic (POPC/POPE/POPS/SoyPI/Cholesterol, 69:15:2:4:11) (based on Ref. [37]). Peptide (Y_{47}GRKKRRQRRR$_{57}$) was purchased in two separate lots from the Peptide Synthesis Facility (University of Pittsburgh, Pittsburgh, PA); mass spectroscopy revealed >95% purity. This Tat peptide corresponds to residues (47-57) of the 86 residues in the Tat protein [6]. Tat was dissolved in HPLC trifluoroethanol (TFE) and then mixed with lipid stock solutions in chloroform to form mole fractions between 0.0044 and 0.108. Weight of Tat in these mole fractions was corrected for protein content (the remainder being 8 trifluoroacetate counter-ions from the peptide synthesis). Solvents were removed by evaporation in the fume hood followed by 2 hours in a vacuum chamber at room temperature.

2.2 Samples for X-ray and neutron scattering

Four mg dried lipid/peptide mixture was re-dissolved in HPLC chloroform/TFE (2:1 v:v) for most of the lipid compositions. However, DOPC/DOPS (3:1) mixtures required chloroform/HFP (1:1 v:v) in order to solubilize the negatively charged DOPS. 200 μl of 4 mg mixtures in solvents were plated onto silicon wafers (15x30x1 mm) via the rock and roll method [38] to produce stacks of ~1800 well-aligned bilayers; solvents were removed by evaporation in the fume hood, followed by two hours under vacuum. Samples were prehydrated through the vapor in polypropylene hydration chambers at 37^{o}C for 2-6 h directly before hydrating in the thick-walled X-ray hydration chamber [39] for 0.5–1 h. Pre-equilibration allowed sufficient time for equilibrium binding of peptides with membrane mimics.

2.3 Samples for densimetry

Multilamellar vesicles (MLVs) were prepared by mixing dried lipid mixtures with MilliQ water to a final concentration of 2-5 wt% in nalgene vials and cycling three times between -20^{o}C and 60^{o}C for ten minutes at each temperature with vortexing. Pure Tat was dissolved in water at 0.4 wt%.

2.4 Samples for circular dichroism (CD)

Thin films were prepared by spreading ~1 mg, x=0.11 (Peptide/(Lipid+Peptide)), in chloroform/TFE (1:1) onto one inside face of a quartz cuvette (Fisher Scientific, Pittsburgh, PA) and solvents were removed under vacuum. Our samples were purposely misoriented during spreading onto the cuvette side to minimize orientation effects on CD spectra [40, 41]. Hydration occurred through the vapor in sealed cuvettes at room temperature for 24 h. In addition, lyophilized Tat was also dissolved in 3 ml water (0.067 mg/ml) with no lipid.

2.5 X-ray scattering methods

LAXS. Low-angle X-ray scattering data from oriented fluid phase lipid mixtures at 37 ^{o}C were obtained at the Cornell High Energy Synchrotron Source (CHESS) using previously described methods [42, 43]. The analysis of diffuse LAXS from oriented stacks of fluctuating

fluid bilayers has been previously described [39]. Absolute form factors $|F(q_z)|$ were obtained as previously described [42]. Modeling to estimate the locations of Tat and the lipid components was performed using the SDP program [44].

WAXS. As described previously [45, 46], wide-angle X-ray scattering (WAXS) was obtained at a fixed angle of 0.5^o, background collected at -0.5^o was subtracted, and these data were analysed to obtain the S_{xray} order parameter. Further details can be found in Supplementary data near **Fig. S4.**

2.6 Densimetry

Volumes of lipid mixtures with and without peptides in fully hydrated multilamellar vesicles (MLV) were determined at 37±0.01 ^oC using an Anton-Paar USA DMA5000M (Ashland, VA) vibrating tube densimeter [47].

2.7 CD spectroscopy

CD spectroscopy was carried out as described in Ref. [48]. Additional details and results can be found near **Fig. S5.**

2.8 Molecular dynamics simulations

Three systems with different DOPC/Tat mole ratios (128:0 , 128:2 and 128:4, corresponding to 0, 0.015 and 0.030 mole fractions) were simulated atomistically using the Gromacs 4.6.1 package [49]. DOPC was modeled by the Slipid force field [50, 51] and HIV Tat was modeled by Amber 99SB [52]. The systems were simulated at 310 K with a constant area in the x-y plane for 100 nanoseconds. The Z direction was coupled to 1 atm with constant pressure. The center of mass (COM) distance between each peptide and the bilayer was constrained by an umbrella potential with a force constant of 3000 kJ/mol/nm^2. The DOPC/Tat system was explored using 45 independent simulations as a combination of 3 different constant areas and 7 different peptide insertion depths for two concentrations and the pure DOPC system. The depths of Tat insertion were 18, 16, 14, 12, 10, 8 and 5 Å from the bilayer center. Comparison to the X-ray form factors was performed using the SIMtoEXP software [53].

2.9 Neutron scattering methods

Grazing angle of incidence neutron scattering data were obtained at the MAGIK beamline at the NIST Center for Neutron Research in Gaithersburg, Maryland using a hydration chamber designed by Drs. Tristram-Nagle and Frank Heinrich. The chamber is able to fully hydrate the horizontally-held oriented lipid bilayers, by heating a small well containing D_2O or H_2O, and by cooling the samples relative to the humid vapor using two Peltier coolers. More details concerning the sample chamber can be found at http://www.humidity.frank-heinrich.net/. Although the chamber can hold up to 10 silicon wafers, each containing ~2000 bilayers, most scans were collected with a 3 mm vertical slit on the samples, so that only three wafers contributed to the scattering. The data were collected both out-of-plane (q_z) to observe the first order lamellar D-spacing, and in-plane (q_r) to observe aggregation using a Denex 2D detector.

3. Results

3.1 Low angle X-ray scattering (LAXS)

Fig. 1 shows the scattering intensity pattern from DOPC/DOPE (1:1) with mole fraction x=0.034 Tat. The diffuse lobes are due to equilibrium fluctuations that occur in these fully hydrated, oriented lipid/peptide samples. The intensity I(**q**) in the diffuse patterns provide the absolute values of the form factors $F(q_z)$, which are the Fourier transforms of the electron density profile, through the relation $I(\mathbf{q})=S(\mathbf{q})|F(q_z)|^2/q_z$, where $\mathbf{q}=(q_r,q_z)$, S(**q**) is the structure interference factor, and q_z^{-1} is the usual LAXS approximation to the Lorentz factor [39, 54, 55]. The first step in the analysis takes advantage of the q_r dependence of the scattering to obtain the bending modulus K_C with results shown in **Fig. 2**. As positively charged Tat concentration was increased, the lamellar repeat spacing D generally increased in neutral lipid bilayers and decreased in negatively charged bilayers, consistent with changes in electrostatic repulsive interactions. With few exceptions, the water space between bilayers exceeded 20 Å.

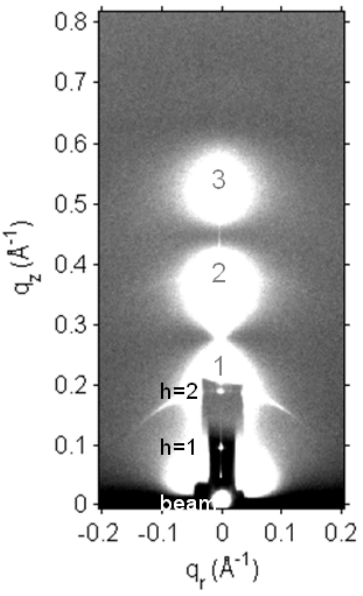

Figure 1. LAXS of DOPC/DOPE (1:1), x=0.034 Tat mole fraction (peptide/(lipid+peptide)) at 37 ^{o}C. White lobes of diffuse scattering intensity have large grey numbers, while lamellar orders and beam are shown to the left of the Molybdenum beam attenuator (short, dark rectangle). q_z and q_r are the projections of **q** along the direction normal and parallel to the membranes, respectively. The lamellar repeat spacing was D=66.2 Å.

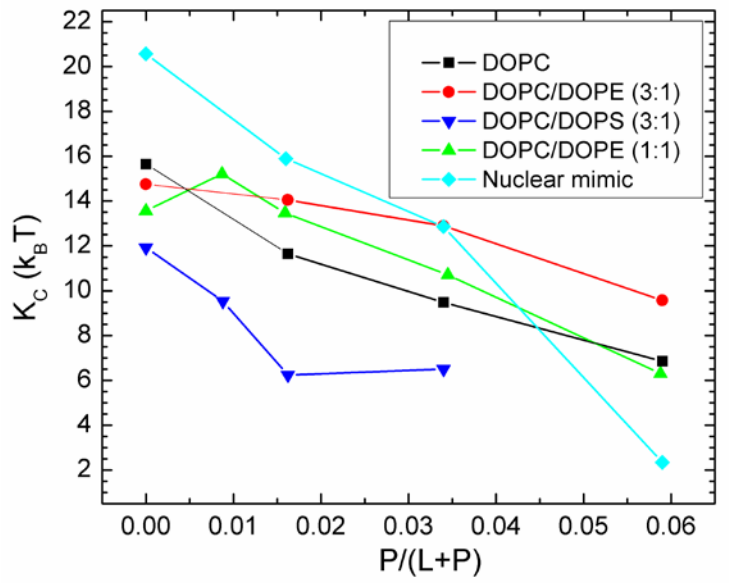

Figure 2. Bending modulus, K_C, vs. P/(L+P) mole fraction. D-spacings for DOPC/Tat mixtures varied from 64 to 68 Å, for DOPC/DOPE/Tat mixtures from 64 to 69 Å, for DOPC/DOPS/Tat (3:1) mixtures from 57 Å to >100 Å (pure DOPS was unbound), and for nuclear mimic/Tat mixtures from unbound (nuclear mimic) to 64 Å. Estimated uncertainty in all values is ± 2.

The analysis that obtains K_C also obtains the structure factor S(**q**) and then the unsigned form factors $|F(q_z)|$ are obtained from the intensity I(**q**) by division. Results for five different membrane mimics are shown in **Fig. 3.** Vertical lines indicate the "zero" position between the lobes of diffuse data where $F(q_z)$ change sign. In every sample, the zero positions shift to larger q_z, indicating a thinning of the membranes.

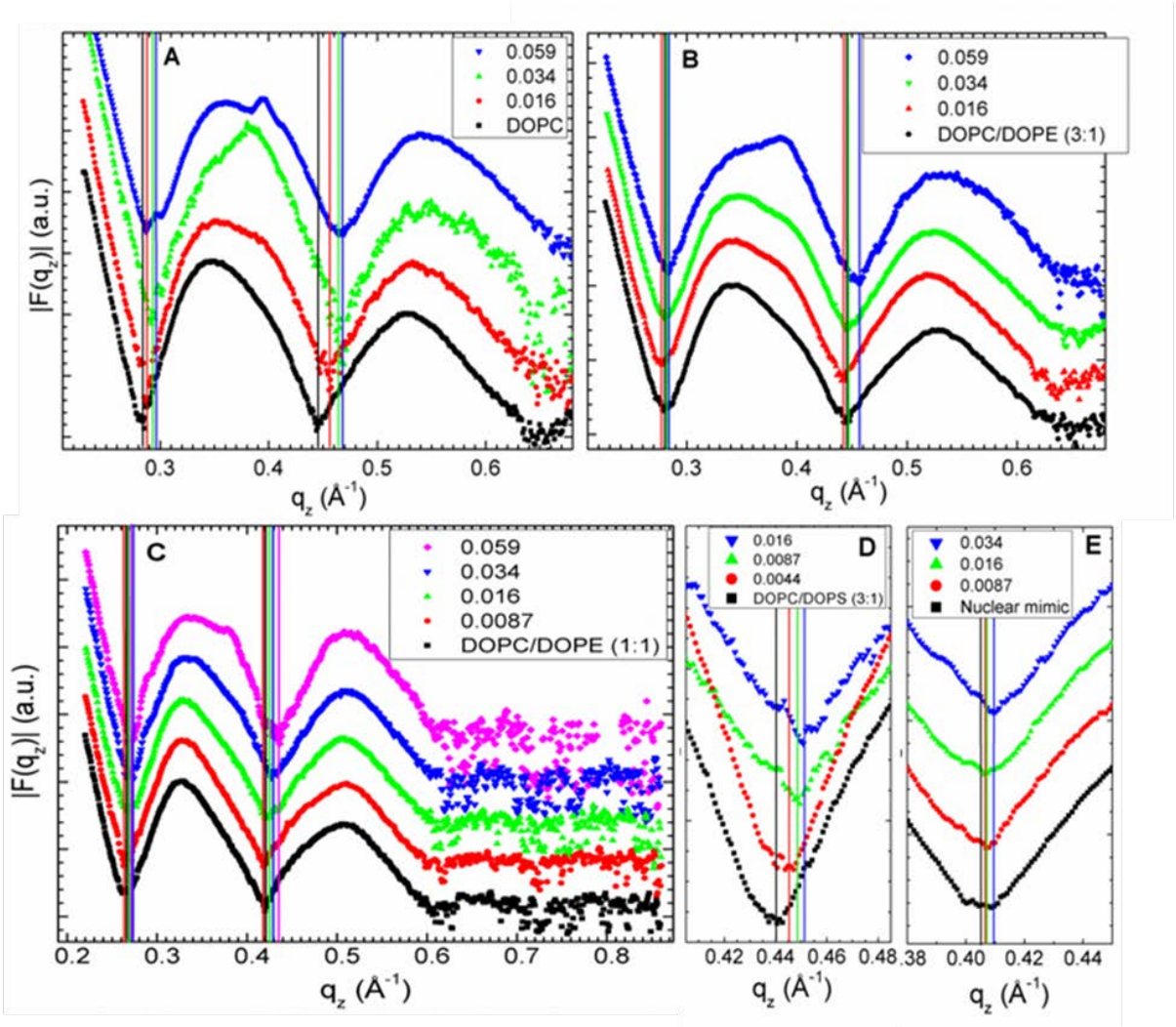

Figure 3. Form factors of lipid mixtures (arbitrarily scaled and vertically displaced) with increasing Tat mole fractions, P/(L+P), indicated on figure legends. Lipid mixtures: **A.** DOPC, **B**. DOPC/DOPE (3:1), **C.** DOPC/DOPE (1:1), **D.** DOPC/DOPS (3:1), **E.** Nuclear mimic. The entire q_z range is shown in **C**, while others show partial ranges. Solid vertical lines indicate the q_z values where the form factors equal zero between the lobes of diffuse data.

3.2 MD simulations

Equilibration of the distance Z_{Tat} of Tat from the center of the bilayer did not occur as it did not move substantially regardless of where Tat was initially placed. Consequently, our strategy ran multiple simulations, each constraining Z_{tat} to a specific value. We then compared the simulated form factor $F(q_z)$ with the experiment. **Fig. 4A** shows that good agreement was obtained when Tat was located 18 Å from the center of DOPC bilayers as shown in **Fig. 5**. The agreement worsened as Tat was constrained to be closer to the center of the bilayer as shown in **Fig. 4C** where the bilayer proceeded to bend around the fixed Tat molecules as shown in **Fig. 4D**.

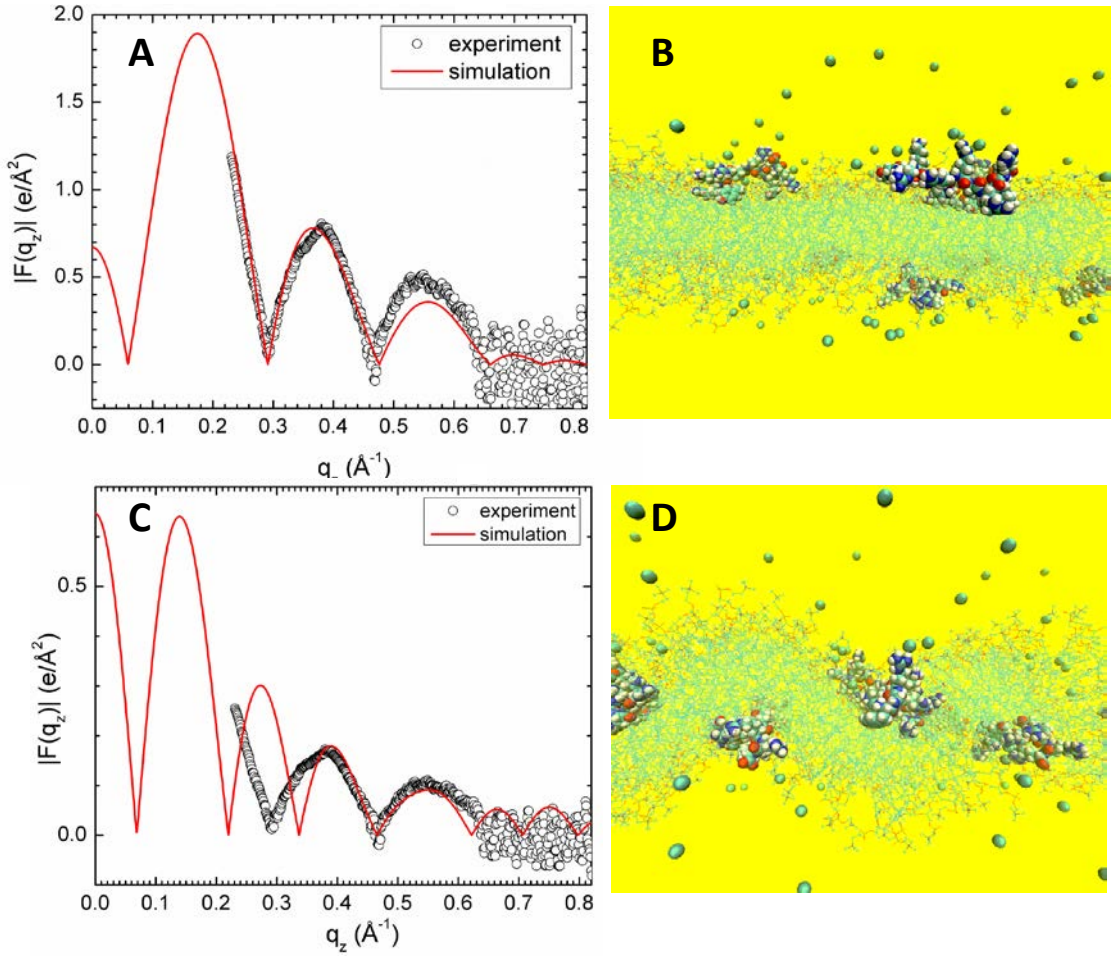

Figure 4. MD simulated form factors (red solid lines in **A** and **C)** of Tat/(DOPC+Tat), x=0.030, with Tat fixed at Z_{Tat}= 18 Å (panel **A**) and 5 Å (panel **C**) from the bilayer center compared to experimental form factors (open circles) scaled vertically to provide the best fit to the simulations. Corresponding snapshots are shown in Panels **B** and **D** in which the lipids are represented as stick models with hydrocarbon chains cyan, phosphate olive green, oxygen red and nitrogen blue. Tats are shown as space filling models with carbon cyan, oxygen red, nitrogen blue and hydrogens grey. The chloride counterions are shown as light green balls. Water is not shown for clarity.

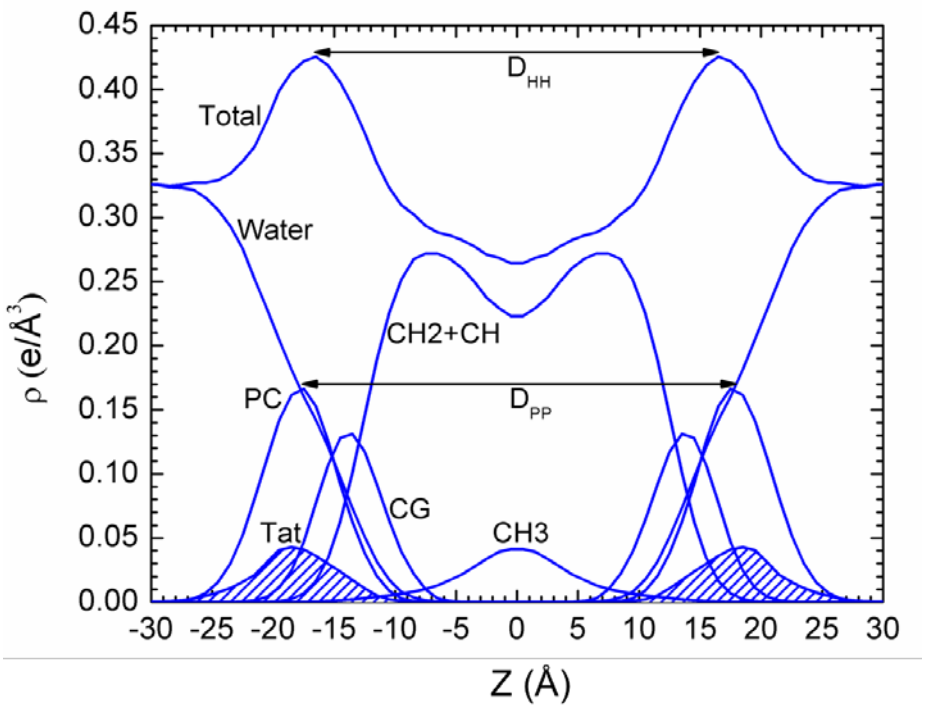

Figure 5. Symmetrized total electron density profile (EDP) from the simulation with the form factor shown in **Fig. 4A**. Also shown are the component group contributions. Component groups are labeled: PC, phosphocholine; CG, carbonyl-glycerol; CH2+CH, methylene and methine hydrocarbon region; CH3, terminal methyl; Tat peptide distribution is shaded.

3.3 SDP Modeling

We also estimate structure by fitting the experimental form factors using the SDP method [44] with the component groups identified in **Fig. 5**. The positions of these groups were free parameters and the agreement with the experimental form factors was excellent. Absolute total electron density profiles and the Tat profiles are shown for many samples in **Fig. 6 (A-C).** It must be emphasized, however, that, while the total EDP is well determined by this fitting procedure, the values of the parameters for the components are not as well determined as they would be if one had X-ray data to smaller and larger q_z and neutron data. Indeed, there are local minima in the fitting landscape, including one with Tat closer to the center of the bilayer as shown in **Fig. S5**. The simulations help to discard that result. For the results shown in **Fig. 6**, a consistent trend is that Tat moves away from the bilayer center as concentration increases. Electron density profiles for DOPC/DOPS (3:1) and the nuclear membrane mimic were not successful, due to loss of diffuse scattering by Tat's charge neutralization of these negatively charged membranes.

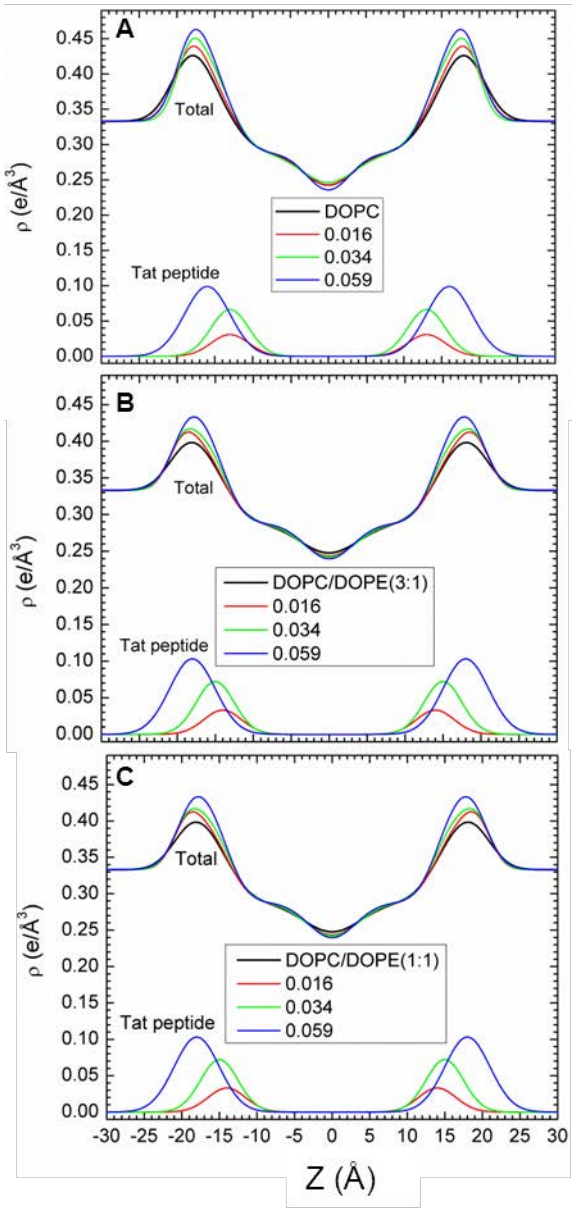

Figure 6. SDP modeling results for absolute electron density profiles (EDPs) and for the Tat location as a function of distance Z along the bilayer normal. **A.** DOPC, **B.** DOPC/DOPE (3:1) and **C.** DOPC/DOPE (1:1).

More structural detail from the modeling and from the simulations is shown in **Fig. 7**. The bilayer thickness can be described as D_{HH}, which is the distance between the maxima in the electron density profile, or as D_{PP}, which is the distance between the phosphocholines on the opposing monolayers (see **Fig. 5**). **Figs. 7A** and **7B** show that both these quantities tend to decrease with increasing Tat mole fraction (P/(L+P)), showing that Tat thins membranes, increasingly so as its concentration is increased, even though both simulation and modeling suggest that Tat moves further from the membrane center with increasing concentration as shown in **Fig. 7D.** **Fig. 7C** shows that the area per lipid A_L usually increases with increasing

mole fraction of Tat, as would be expected. The results from the simulation data plotted in **Fig. 7** were obtained by using a weighted average based on chi-square of the best fits of the simulated form factors with the experimental form factors.

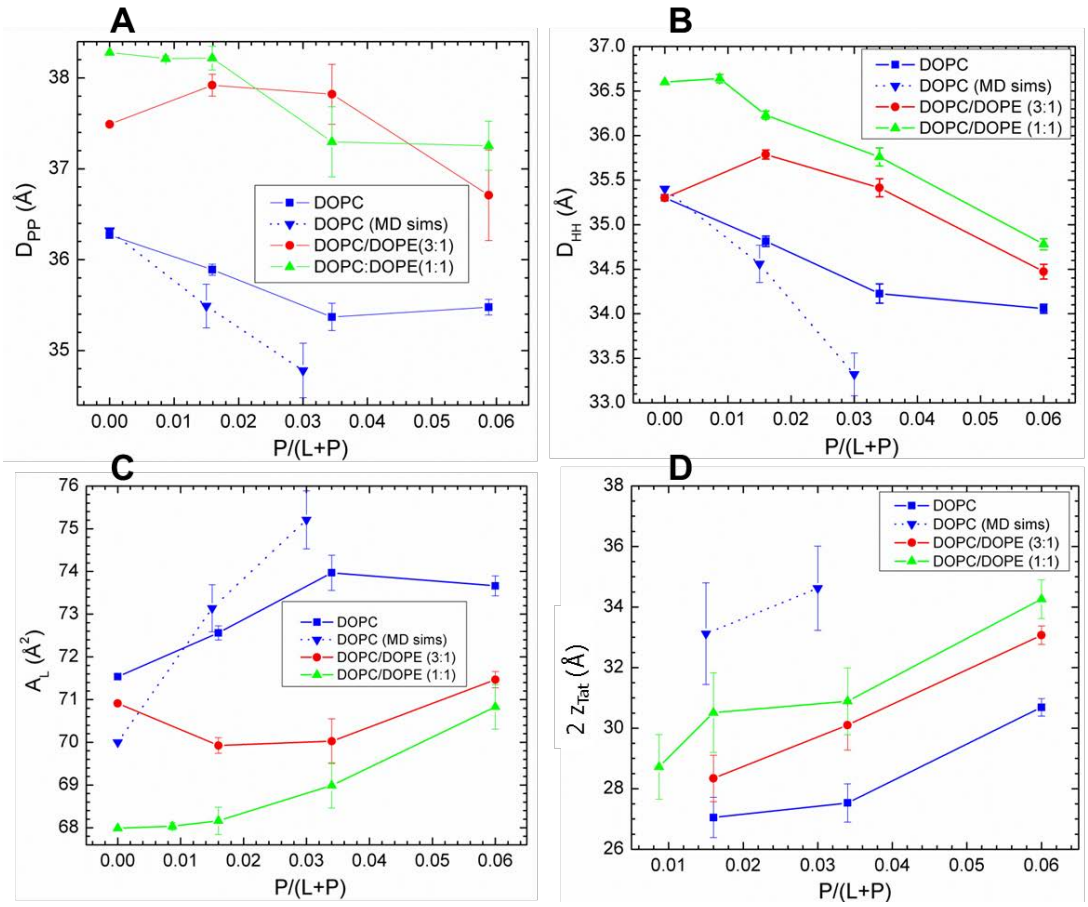

Figure 7. **A.** Bilayer thickness, D_{PP}; **B.** Bilayer thickness, D_{HH}; **C.** Area/lipid, A_L; **D.** Twice the Tat location, $2A_{Tat}$: all plotted vs. Tat mole fraction P/(L+P). Error bars are standard deviations from imposing Tat Gaussian widths, σ = 2.5, 3.0 or 3.5 Å. Inverted blue triangles connected with dotted line are results from MD simulations, averaging the best fits to the X-ray data for each parameter, with standard deviations shown.

3.4 S_{xray} order parameter from WAXS

Fig. 8 shows that the S_{xray} orientational order parameter generally decreases with increasing concentration of Tat for most of the membrane mimics studied. These decreases in membrane chain order are compatible with the increase in softening of membranes by Tat observed by a decrease in K_C in **Fig. 2**.

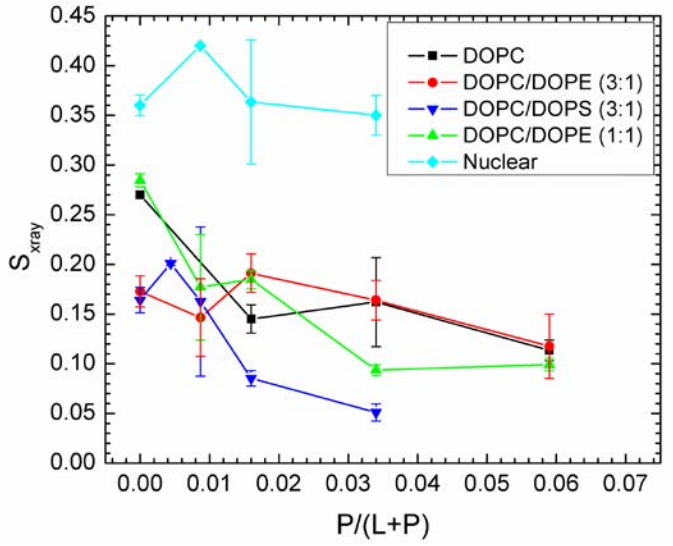

Figure 8. S_{xray}, orientational order parameter, vs. x=P/(L+P) mole fraction. Error bars are standard deviations determined by analyzing WAXS data from several lateral positions on the same sample.

3.5 CD spectroscopy

Results of the secondary structure of Tat determined using CD spectroscopy are shown in **Fig. S7** and details are given in the Supplementary data text. Basically, there was no effect of the DOPC/DOPE (3:1) membrane on the secondary structure of Tat (x=0.108) compared to Tat solubilized in water. The structure was primarily β and random coil, with <10% α-helix in both environments. The β structures include regular and distorted β-strands and β-turns. These results are summarized in **Table 1**.

Table 1. CD results

Sample	**α-Helix**	**β-Structures**	**Random Coil**
Tat in Water	0.061±0.016	0.574±0.049	0.363±0.038
Tat in Lipid Film	0.047±0.040	0.57±0.071	0.381±0.073

3.6 Volume results

Experimental and simulated volumes are given in **Table 2**. The simulated volume was obtained using the volume app in the SIMtoEXP program. The experimental Tat volume was calculated from the measured density assuming that the lipid volume was the same as with no

Tat. In general, there may be an interaction volume between the peptide and the lipid membrane as we found previously for bacteriorhodopsin [56]. As lipid was present in excess to Tat, the partial molecular volume of the lipid should be the same as with no Tat, so this way of calculating includes all the interaction volume in V_{Tat}. Comparison of V_{Tat} in water with the result for 5:1 Lipid:Tat suggests that the interaction volume may be negative, consistent with a net attractive interaction with lipid. Understandably, values of V_{Tat} were unreliable for small mole ratios of Tat:Lipid. Therefore we used simple additivity for those mimics not shown in **Table 2** for the volumes used in the SDP program. All volumes obtained from the Gromacs MD simulations were somewhat smaller than the measured volumes, but it supports the Tat volume being closer to 1822 $Å^3$ than the outlying values obtained experimentally at small Tat concentrations.

Table 2. Volume results at 37 ^oC

Tat in:	**V_{lipid} ($Å^3$)**	**Lipid:Tat**	**V_{Tat} ($Å^3$)**
Water			1877
DOPC/DOPE (3:1)	1288	5:1	1822
DOPC	1314	39.6:1	676
DOPC/DOPS (3:1)	1298	39.6:1	2613
Simulations			
DOPC	1283	128:2	1694
DOPC	1294	128:4	1699

3.7 Summary of Results

We summarize our results for how Tat affects the lipid bilayer in **Fig. 9**. The height of Tat, H_{Tat} = 8.7Å, was the full width at half maximum of the Tat electron density profiles obtained from simulations and the cylindrical radius, R_{Tat} = 8.3Å, was calculated to give the measured volume. The Z distances from the center of the bilayer were derived from weighted averages of four MD simulations of Tat:DOPC 2:128. The χ^2 obtained by comparison to experiment indicated that the best Z_{tat} lay between the simulated values of 16Å and 18Å and the best area/lipid A_L lay between the simulated values of 72$Å^2$ and 74$Å^2$, so averages were obtained from these four combinations of Z_{tat} and A_L, weighted inversely with their χ^2. The average position Z'_{Phos} of those phosphates situated under the Tats, $R'_{Phos} < R_{Tat}$, were obtained separately from the simulations. The simulation cell extended to 38Å, far enough to ensure that Z_{Phos} for most of the lipids is the same as for DOPC. Assuming a simple linear ramp in Z_{Phos}, **Fig. 9** then indicates a ring of boundary lipids that extends twice are far in R as Tat itself. Although the guanidinium electron density profile was broad (**Fig. S8**), indicating that some were pointing away from the bilayer relative to the center of Tat, more were pointing towards the bilayer center as indicated in **Fig. 9**. Numerical values are given in **Table S1**.

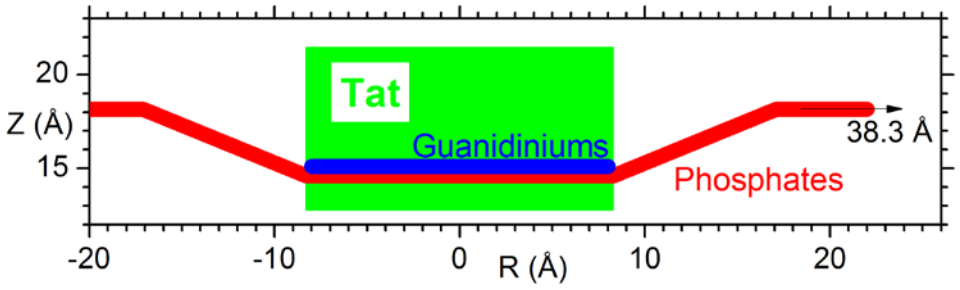

Figure 9. Location of Tat in DOPC bilayer. Tat is represented as a cylinder, Z is the distance from the bilayer center, and R is the in-plane distance from the center of Tat. The average Z of the lipid phosphates as a function of R and the arginine guanidiniums are shown in red and blue, respectively.

4. Discussion

Given that 8 of the 11 amino acids in Tat (47-57) are arginines and lysines, one would have suggested 20 years ago that highly charged Tat would partition strongly into solution rather than being associated with lipid bilayers. By contrast, but in agreement with more recent perspectives on arginine partitioning into the interfacial region [57], we find that Tat interacts with lipid bilayers, even with neutral DOPC and DOPC/DOPE mixtures, as well as with negatively charged DOPC/DOPS and nuclear membrane mimic lipid mixtures. This paper presents multiple lines of evidence for a Tat/membrane interaction. **Fig. 2** shows that Tat decreases the bending modulus. Although one could argue that such a decrease is only apparent and could instead be due to local changes in membrane spontaneous curvature [58], either interpretation supports a Tat-bilayer interaction. The changes with increasing Tat concentration in the X-ray membrane form factors in **Fig. 3** prove that Tat affects membrane structure, and the shift of the zero positions to higher q_z suggests thinning. Thinning is substantiated by quantitative analysis of the X-ray data and by MD simulations. **Fig. 7A** shows that the average membrane thickness, as measured by the distance D_{PP} between phosphocholines on opposite surfaces, decreases with increasing Tat concentration. Similar thinning is shown in **Fig. 7B** for the distance D_{HH} between the maxima in the electron density profiles of opposite surfaces. Compared to D_{PP}, D_{HH} is pulled towards both the carbonyl/glycerol groups and Tat because both have electron densities (~0.4 e/Å^3) greater than water (~0.33 e/Å^3) or hydrocarbon (~0.3 e/Å^3). Although the thinning shown in **Figs. 7A** and **7B** is not large, it obviously requires interaction of Tat with the bilayers.

It is of considerable interest to learn where Tat resides, on average, in the membrane, as this would establish a base position from which translocation would be initiated. We have combined our two main methods, MD simulations and X-ray scattering, to address this question. Our MD simulations could not be run long enough to equilibrate the location Z_{Tat} relative to the

center of the bilayer. Therefore, simulations were run with Z_{Tat} constrained and the simulated form factor was compared to the experimental X-ray form factor. As shown in **Fig. 4** this procedure placed Tat in the interfacial headgroup region close to the phosphocholine as seen by comparing the simulated $2Z_{Tat}$ in **Fig. 7D** with **Fig. 7A**. As emphasized by **Fig. 4C**, this procedure clearly eliminated placing Tat in the hydrocarbon region. This was important regarding our independent SDP modeling of the X-ray data which obtained excellent fits to the experimental form factors for a model with Tat deep in the hydrocarbon interior (see **Fig. S5**) that we can discard as a spurious local minimum in the χ^2 driven analysis. **Fig. 7D** also shows that modeling gives smaller values for Z_{Tat} than the simulation. The modeling result is supportive of the original simulation result of Herce and Garcia that Tat resides closer to the bilayer center than do the phosphocholine groups [59]. That is a base position that would be a possibly important precursor to translocation, but the current simulation is not so supportive of this. Interestingly, **Fig. 7D** indicates that Z_{Tat} increases with increasing Tat concentration, even while the bilayer becomes thinner. The main difference between the two MD simulations from the Garcia group is that the initial simulation [59] did not employ negatively charged counterions to balance the positively charged Tat, while the present simulation does. The very large (7 Å) bilayer thinning and subsequent water-filled pore observed in [59] are not observed in the present simulation. We can also compare to the second published MD simulation of Tat in DOPC membranes [36], where Tat was shown to first aggregate, then cause a large membrane deformation, similar to that seen in **Fig. 4D**. This result led those authors to suggest that micropinocytosis could be the mechanism for Tat entry into cells, and that a pore is not required. By pulling Tat through the membrane, they determined that the most likely Tat position is just outside the phosphate headgroups [36], close to what our MD simulations obtain.

Several groups have carried out calculations and MD simulations showing that the cost of moving an arginine group from water to the bilayer center is ~12-26 kcal/mol [57, 60-62] or 6-7 kcal/mol if side-chain snorkeling to the surface is taken into account [63]. This is not inconsistent with our result that Tat interacts with the membrane because, as is well known, the bilayer is not just a hydrocarbon slab, but has interfacial headgroup regions where Tat can reside. It has been suggested that the free energy cost for charged amino acids entering the headgroup region is similar to that for partitioning into octanol, about an order of magnitude smaller free energy cost than partitioning into cyclohexane [64-66]. Simulations suggest that the free energy is smaller for an arginine residing in the interfacial region than in water, roughly by 3 kcal/mole, depending upon the lipid [57, 66]. Our results therefore appear energetically reasonable.

One concern with diffraction experiments on samples consisting of adjacent bilayers in a stack or in a multilamellar vesicle is that the samples have to be partially dried to obtain conventional diffraction data. But then there is no pure water layer between adjacent bilayers, so a hydrophilic peptide is forced into the interfacial, partially hydrophilic region of the lipid bilayer. In contrast, by using diffuse scattering, we obtained structure from experimental samples that had a range of lamellar D spacings (see **Fig. 2** caption) that were considerably larger than the thickness of the bilayer in **Fig. 7A**, thereby providing an ample pure water space, typically greater than 20Å. The result that $2Z_{Tat}$ shown in **Fig. 7D** is so much smaller than our repeat spacings shows that Tat preferentially associates with the membrane rather than dissociating into water.

Consistent with Tat softening the bilayers (**Fig. 2**), it also disorders them as indicated by S_{xray} decreasing with Tat concentration shown in **Fig. 8**. Tat also increases the mosaic spread observed by X-ray and neutron scattering as shown in **Figs. S1-3**; this is a much larger scale disordering of the stack of bilayers. As shown in **Table 1** and in **Fig. S7**, Tat assumed slightly >50% β structures, both when dissolved in water and in contact with a hydrated thin film membrane. Our results were determined using the DichroWEB program, which compares the mean residue ellipticity with that from standard globular proteins, with details given in Supplementary data near **Fig. S7**. These structures include approximately equal amounts of regular β strands and turns, with ~half that amount of distorted β strands. The next most prevalent structure was random coil (~30%). Measurements in the literature (see **Section 1. Introduction**) report a primarily random structure, determined using either CD or NMR. This difference could be due to different sample preparations, or due to a different interpretation of the CD spectra. Ref. [67] reported that the CD spectra of unordered polypeptides are similar to that of the poly(Pro)II helix, and a significant fraction of the unordered conformation in globular proteins consists of poly(Pro)II helix plus distorted β strands. In an effort to better determine the secondary structure of Tat, our collaborator, Dr. Rieko Ishima, performed solution 1D and 2D-NMR at 10, 20 and 30^{o}C. Her results showed no evidence for backbone hydrogen bond formation, indicating that the peptide does not have a stable β conformation, at least on the time scale of the NMR measurement. Additionally, we have further interrogated our MD simulations by applying a computer program, Define Secondary Structure of Proteins (DSSP), which recognizes patterns of hydrogen-bonded and geometrical features [68]. DSSP found between 55 and 90% random coil structure in the MD simulations, which positioned Tat from 18 to 5 angstroms from the bilayer center, and also fixed the area/lipid from 70 to 76 Å^2. Between 0 and 32% "turn" structure was determined, where turn is a precursor to a β conformation. Therefore, both our solution NMR, and MD simulation results find primarily random coil, with a much smaller percentage of β structure, which dispute our CD findings of >50% β conformation. While the interpretation of CD spectra as β, P2 helix or coil is controversial, what is clear is that no studies have implicated Tat forming an α-helix, either in solution or in the membrane.

Given our structural and elastic moduli results, we now compare to other experiments in the literature. In 2008, the Wong group implicated Tat's ability to induce saddle-splay curvature with a potential role of bidentate hydrogen bonding as key [69]. Rhodamine-tagged Tat only entered GUVs when the PE headgroup was included with PS and PC lipids (PS/PC/PE, 20:40:40), indicating that hydrogen-bonding, and/or curvature-promoting lipids are required for Tat translocation. In PS/PE (20:80) lipids, they found Tat caused a highly curved cubic phase using X-ray diffraction [69]. In our experiments, there was little effect of adding DOPE to DOPC at either a 3:1 or 1:1 mole ratio on decrease in the bending modulus, bilayer thinning, Tat's outward movement with increasing concentration or disordering of chains (S_{xray}). Our two results are not inconsistent, however, since curvature-promotion appears not to be required for Tat's ability to lower the energy required to bend, nor to locate Tat in the bilayer, nor to disorder chains, all of which may be important for Tat translocation. Yet Tat does translocate across membranes in their experiments only with PE in the membrane, so the ability to induce saddle-splay curvature may also be required for Tat's translocation. Another study by Melikov et al. [26] found that Tat's main mechanism of action is to induce lipid mixing and membrane leakage with lipids of late endosomes. This result is consistent with our results that Tat induced a

reversible, hydration-induced increase in mosaic spread (**Figs. S1-3)** and a disordering of chains **(Fig. 8)**. Both of these could induce lipid mixing and perhaps, membrane leakage. In a recent MD simulation from the Garcia lab using umbrella sampling [70], it was found that the energy required for Tat to traverse a membrane was smaller if a water pore was first positioned in the bilayer. We cannot compare our result to this simulated result since we do not have evidence for pore formation using X-rays or neutrons. An X-ray, neutron and AFM study also did not find evidence for pore formation, but they reported thickening upon initial binding, in contradiction to our result in **Fig. 7B** that shows thinning [71]. We suggest that this difference was caused by their using stiff gel phase DPPC lipid that did not allow bound Tat to perturb the bilayer. Using a variety of techniques, including high sensitivity isothermal titration calorimetry and ^{2}H- and ^{31}P-NMR, Seelig et al. [72] presented evidence that the lipid bilayer remains intact upon Tat binding and our results confirm this. Finally, we compare our structural results to those obtained by solid state NMR, although at a lower hydration level than in our sample. Hong et al. [32] found that Tat lies parallel to the bilayer surface in the headgroup region of DMPC/DMPG (8:7) bilayers, similar to our cartoon in **Fig. 9**.

Finding a kinetically competent pathway for the interesting phenomenon of translocation of highly charged Tat through hydrophobic membranes is difficult. An energetically passive translocation likely occurs very seldom on an MD simulation time scale, and it probably happens quickly, so it would not significantly change the average structure of the membrane in which it occurs. Although our results in this paper do not reveal a kinetically competent pathway, they do show that Tat is drawn to the surface of the membrane, and is therefore ready for translocation at a region of local thinning. And they show that these interactions tend to soften (**Fig. 2**) and disorder (**Fig. 8**) the membrane, thereby likely reducing the energy barrier for passive translocation.

5. Acknowledgments: Research reported in this publication was supported by the National Institute of General Medical Sciences of the National Institutes of Health under award GM44976 (PIs JFN, STN). and GM86801 (PI AG). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health. We acknowledge the Cornell High Energy Synchrotron Source (CHESS) which is supported by the National Science Foundation and the NIH/NIGMS under NSF award DMR-0936384. We acknowledge the Center for Molecular Analysis at Carnegie Mellon University for use of the Jasco 715 and for mass spectrometry analysis and the Protein Synthesis Core of the University of Pittsburgh for peptide production. The authors also acknowledge Dr. Rieko Ishima for carrying out NMR measurements, Leah Langer for help using Matlab with the neutron scattering data and Laura Carroll for carrying out CD measurements. We would also like to acknowledge Dr. Jeffery Krzywon at NIST for technical help with our hydration chamber.

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