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

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A B S T R A C T

We report the effect on lipid bilayers of the Tat peptide Y47GRKKRRQRRR57 from the HIV-1 virus transactivator of translation (Tat) protein. Synergistic use of low-angle X-ray scattering (LAXS) and atomistic molecular dynamic simulations (MD) indicate Tat peptide binding to neutral dioleoylphosphocholine (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 local bilayer as the locally thinned lipid phosphate groups. LAXS data for DOPC, DOPC/dioleoylphosphoethanolamine (DOPE), DOPC/dioleoylphosphoserine (DOPS), and a mimic of the membrane gave similar results. Generally, the Tat peptide decreased the bilayer bending modulus Kr and increased the area/lipid. 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 from the headgroup region.

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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 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]. 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), C48RKKRRQRRRPQ, 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 SVG/A 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 glia cells at 1–10 μM [24]. Interestingly, no hemolytic activity was used to incubate human erythrocytes with a highly toxic 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 (1:1 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 s [25]. This study suggests that negative curvature induced by the inclusion of PE facilitates translocation. In a subsequent study using much smaller unilamellar vesicles (ULVs), Tat did not release an encapsulated fluorescent probe in ULVs composed of lipids modeling the outer plasma membrane, PC/PE/SM/Chol (1:1:1:1.5), but did release the probe in ULVs composed of BMP/PC/PE (77:19:4) [26]; BMP (bis(monoacylglycerol)-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 ULVs in the absence of a negatively charged lipid. The contrasting results in these two experiments may also be due to the use of ULVs instead of GUVs since it was reported that Tat does not translocate across ULVs 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 ULVs [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 olioarginine cR9 peptide. Using single-channel conductance of gramicidin A in planar lipid membranes consisting of anionic, neutral or positively charged lipids, cR9 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 cR9 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 pellitente 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 that 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 dynamic simulations offer some insight into the molecular details of translocation. Herve and Garcia simulated the translocation of Tat (Y_{65}GRKKRRQRRR_{77}) 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 microinocytosis 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/DOPC (3:1 mole ratio), DOPC/DOPS (3:1), DOPC/DOPE (1:1) and a mimic of the nuclear membrane (POPC/POPE/POPS/SoyP/Chol, 69:15:2:4:11). Accessory techniques, densitometry, wide-angle X-ray scattering (WAXS), neutron scattering, and 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/DOPC (3:1), DOPC/DOPE (1:1), DOPC/DOPS (3:1) and nuclear membrane mimic (POPC/POPE/POPS/SoyP/Cholesterol, 69:15:2:4:11) (based on Ref. [37]). Peptide (Y_{65}GRKKRRQRRR_{77}) was purchased in three 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. The 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 h 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 was plated onto silicon wafers (15 × 30 × 1 mm) via the rocking and roll method [38] to produce stacks of ~1800 well-aligned bilayers; membranes were removed by evaporation in the fume hood followed by 2 h under vacuum. 2H NMR revealed ~1% residual solvent after these procedures. Samples were prehydrated through the vapor in polypropylene hydration chambers at 37 °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 denisimetry

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 °C and 60 °C for 10 min 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 °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(qz)| were obtained as previously described [42]. Modeling to estimate the locations of Tat and the lipid components was performed using the Scattering Density Profile (SDP) program [44].

WAXS. As described previously [45,46], wide-angle X-ray scattering (WAXS) was obtained at a fixed angle of 0.5°, background collected at −0.5° was subtracted, and these data were analyzed to obtain the Sxray 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 °C 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 dynamic simulations

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]. Tip3p water was used [53]. The number of Tats was fixed equally on each side of the bilayer to mimic experimental conditions. All systems were simulated at 310 K with a constant area in the x-y plane for and 1 atm constant pressure in the Z direction. Each system was simulated for 100 ns and the last 50 ns was used as the production run. For each Tat molecule, 8 negative chloride ions were added to the simulation.

At each DOPC/Tat mole ratio, we studied systems with three different area/lipid (A_L). For the DOPC system, we fixed A_L = 68, 70, and 72 Å²; DOPC/Tat (128:2), we fixed the A_L = 72, 74, and 76 Å²; and DOPC/Tat (128:4), we fixed the A_L = 72, 74, and 76 Å². For each DOPC/Tat system at fixed A_L, we then conducted seven independent simulations with the center of mass (COM) of each Tat constrained at different bilayer depths from the bilayer center (18, 16, 14, 12, 10, 8 and 5 Å). In total, 45 independent simulations were conducted. The goal of constrained simulations is to find the best match between experimental and MD simulation form factors. Comparison to the X-ray form factors was performed using the SIMtoEXP software [54]. Additional details concerning the MD simulation methods are in Supplementary Data Section 6.

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 Pelletier 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) 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(qz), which are the Fourier transforms of the electron density profile, through the relation I(q) = S(q)|F(qz)|^2/qz, where q = (q_z,q_r). S(q) is the structure interference factor, and q_z is the usual LAXS approximation to the Lorentz factor [39,55,56]. The first step in the analysis takes advantage of the q_z dependence of the scattering to obtain the bending modulus K_r 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.

The analysis that obtains K_r also obtains the structure factor S(q) and then the unsigned form factors |F(qz)| are obtained from the intensity I(q) by division. Results for five different membrane mimics are

Fig. 1. LAXS of DOPC/DOPE (1:1), x = 0.034 Tat mole fraction (peptide/(lipid + peptide)) at 37 °C. White lobes of diffuse scattering intensity have large gray 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.
shown in Fig. 3. Vertical lines indicate the "zero" position between the lobes of diffuse data where F(qz) change sign. In every sample, the zero positions shift to larger qz, indicating a thinning of the membranes.

3.2. MD simulations

Due to the slow relaxation in lipid bilayers and limited accuracy of the force field, a good agreement between experimental and MD simulation calculated form factors may be difficult to reach. Consequently, we carried out several constrained simulations at A0 and ZTr as described in Materials and methods. We then compared the simulated form factor F(qz) with the experimental form factor. The best match for DOPC/Tat (128:4) was found when the Tat was constrained at 18 Å away from the bilayer center (Fig. 4A,B). The other best fit results were: DOPC A0 = 70 Å2 and DOPC/Tat (128:2) A0 = 72 Å2, ZTr = 18 Å. It clearly indicates that with increasing Tat concentration, A0 increases. The agreement worsened as Tat was constrained to be closer to the center of the bilayer. When Tat was constrained at 5 Å away from the bilayer center, we observed a spontaneous formation of water pores in the MD simulation. However, as shown in Fig. 4C the corresponding form factor calculated from MD simulations does not match well with the experimental form factor. Thus, by comparing the experimental and simulated form factors, Tat's headgroup position is validated, while the hydrocarbon position is ruled out. A similar comparison of form factors from X-ray scattering and MD simulation previously allowed us to determine that the pore-forming alamethicin peptide locates in a transmembrane, not headgroup position [57].

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

![Figure 2: Bilayer bending modulus, Kc, 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 membrane mimic) to 64 Å. Estimated uncertainty in all values is ±2.](image1)

![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 qz range is shown in C, while others show partial ranges. Solid vertical lines indicate the qz values where the form factors equal zero between the lobes of diffuse data.](image2)
had X-ray data to smaller and larger q, 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.

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). Fig. 7A and B shows 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, similar to the findings from MD simulations (Section 3.2), 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 four best fits of the simulated form factors with the experimental form factors.

3.4. SAXS order parameter from WAXS

Fig. 8 shows that the $S_{xxyy}$ orientational order parameter generally decreases with increasing concentration of Tat for most of the membrane mimics studied. This indicates that the chains are more disordered as the Tat concentration in the membrane increases. 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.

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 $\beta$ and random coil, with $<10\% \alpha$-helix in both environments. The $\beta$ structures include...
Understanding, values of VTat were unreliable for small mole
fractions (P/(L + P)) are shown in the table. The experimental Tat volume was calculated from the measured
volume. The Z distances from the center of the bilayer were de-

erived from weighted averages of four MD simulations of Tat:DOPC
2:128. The χ² obtained by comparison to experiment indicated that the best ZTat lay between the simulated val-
es of 16 Å and 18 Å and the best area/lipid A₆ lay between the simulated values of 72 Å² and 74 Å², so averages were obtained from these four combinations of ZTat
and A₆, weighted inversely with their χ². The average positions, Zₚₚₜος
of phosphates situated underneath the Tats were calculated by aver-
ing over the phosphates whose in-plane distance, R, from the center of Tat is smaller than Rₜₚₜ. The simulation cell extended to 38 Å, far enough to
ensure that Zₚₚₜος for most of the lipids is the same as for DOPC. Assum-
ing a simple linear ramp in Zₚₚₜος, Fig. 9 then indicates a ring of boundary lipids that extends twice as 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 toward the bilayer center as indicated in Fig. 9. Numerical values are given in Table S1.

3.7. Summary of results

We summarize our results for how Tat affects the lipid bilayer in
Fig. 9. The height of Tat, HTat = 8.7 Å, was the full width at half maxi-
mum of the Tat electron density profiles obtained from simulations and
the cylindrical radius, RTat = 8.3 Å, was calculated to give the mea-
sured volume. The Z distances from the center of the bilayer were de-


erived from weighted averages of four MD simulations of Tat:DOPC
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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 per-
spectives on arginine partitioning into the interfacial region [59], 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 mul-
tiple 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 [60], either interpretation supports a
Tat–membrane interaction. The changes with increasing Tat concentration in the X-ray membrane form factors in Fig. 3 prove that Tat affects mem-
brane structure, and the shift of the zero positions to higher qₚ 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 Dₚₚ between phosphocholines on opposite surfaces, decreases with increasing Tat concentration. Similar thinning is shown in Fig. 7B for the distance Dₜₜ between the maxima in the electron density profiles of opposite surfaces. Compared to Dₚₚ, Dₜₜ is pulled toward both the carbonyl/glycerol groups and Tat because both have electron densities (–0.4 e/Å³) greater than water (–0.33 e/Å³) or hydrocarbon (–0.3 e/Å³). Although the thinning shown in Fig. 7A and B is not large, it obviously requires interaction of Tat with the bilayers. Fig. 7C shows that A₆ increases with increasing Tat concentration, by both model fitting and MD simulations. In a recent experimental and simulation study of the decapeptide of arginine, a
similar thinning of 10% and 12% was observed for neutral and negatively
charged bilayers, respectively [61].

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.

In general, Tat locates at the bilayer/water interface as indicated in
Section 3.2, and they are close to the phosphocholine headgroup region
by comparing the simulated 2ZTat in Fig. 7D with 7A. Although the SDP

![Fig. 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). Increasing mole fractions (P/(L + P)) are shown in the figure legends.](image-url)
modeling of the X-ray data obtains excellent fits to the experimental form factors for a model with Tat deep in the hydrocarbon interior (see Fig. S5), the corresponding MD simulation (shown in Fig. 4C) eliminates this spurious result. Fig. 7D also shows that modeling gives smaller values for \( Z_{\text{Tat}} \) 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 [35]. That is a base position that would be a possibly important precursor to translocation, as would the larger \( A_0 \). In a recent multi-scale simulation, it was found that arginines bind deeply to the glycerol-carbonyl groups as well as to the phosphate, while lysines bind only to the level of the phosphates [62]. This is in good agreement with our results, shown in Fig. S8.

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 [59,63–65] or 6–7 kcal/mol if side-

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**Table 1**

CD results.

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<th>Sample</th>
<th>( \alpha )-Helix</th>
<th>( \beta )-Structures</th>
<th>Random coil</th>
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<td>0.6</td>
<td>0.4</td>
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<tr>
<td>Tat in lipid film</td>
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<td>0.6</td>
<td>0.4</td>
</tr>
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</table>

**Table 2**

Volume results at 37 °C.

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<th>Tat in:</th>
<th>( V_{\text{lipid}} (\text{Å}^3) )</th>
<th>Lipid:Tat</th>
<th>( V_{\text{Tat}} (\text{Å}^3) )</th>
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</thead>
<tbody>
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<td></td>
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<td>5:1</td>
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<td>DOPC/DOPS (3:1)</td>
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<td>39.6:1</td>
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<td>Simulations</td>
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<td>128:2</td>
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<tr>
<td>DOPC</td>
<td>1294</td>
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</tbody>
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chain snorkeling to the surface is taken into account [66]. 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 [67–69]. 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 [59,69]. 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 20 Å. The result that Z_{Sxray} 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 induces S_{Sxray} decreasing with Tat concentration shown in Fig. 8. Tat also increases the mosaic spread observed by X-ray and neutron scattering experiments. This is because, even with a water pore, the existence of a transient water pore from our X-ray or neutron scattering experiments. This is because, even with a water pore, the existence of a transient water pore from our X-ray or neutron scattering experiments. This is because, even with a water pore, the existence of a transient water pore from our X-ray or neutron scattering experiments. This is because, even with a water pore, the existence of a transient water pore from our X-ray or neutron scattering experiments.
5. Conclusions

In this work we have used X-ray scattering to show that Tat thins membranes: 1–2 Å globally for three neutral membrane mimics (DOPC, DOPC:DOPE (3:1) and DOPC:DOPE (1:1). In addition, the X-ray form factors from DOPC:DOPS (3:1) and nuclear membrane mimics suggest a similar global thinning. MD simulations showed that Tat causes a 3 Å local thinning in DOPC membranes, with the lipid phosphate groups closer to the bilayer center than most of the guanidinium groups. By comparing the form factors generated from X-ray experiment and MD simulations, we can rule out a pore with Tat and water spanning the membrane. Our X-ray results also provide material constants; Tat caused softening and chain disordering. CD spectroscopy revealed either a β- or random coil structure that did not change upon membrane binding. Therefore, the mechanism of Tat translocation must involve a lipid perturbation and local membrane thinning. From its headgroup position, Tat must translocate quickly across the membrane, without the formation of a permanent water pore.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamac.2014.08.014.

References


