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Partition of Positively and Negatively Charged Tryptophan Ions in Membranes with Inverted Phospholipid Heads: Simulations and Experiments

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Abstract

A joint experimental and computational study illustrates that the partitioning of positively and negatively charged tryptophan in a phospholipid bilayer is significantly altered by a reversal in the head group dipole arrangement. Experiments were conducted by using tryptophan as a fluorescent reporter of its local environment. Based on the experimental design in a recent publication (Anderson, C. M.; Cardenas, A.; Elber, R.; Webb, L. J. *J. Phys. Chem. B* **2018**, *123*, 170–179) we were able to determine that the arrangement of the head group dipole altered the degree of partitioning of charged tryptophan in the lipid bilayer. In parallel, atomically detailed simulations were performed for the two membrane systems. The simulation results are in accord with the experimental findings and support a simple molecular partition mechanism of electrostatic interactions with the head groups, glycerol linkers and interfacial water dipoles.

Graphical Abstract

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I. Introduction

One of the main functions of a cell membrane is to hamper or block the transport of compounds or organelles in and out of the cell. Since transport of selected materials is needed for cell survival, specific protein transporters or channels facilitate the translocation of key substances. Still, some molecules can permeate the membrane via a non-facilitated diffusive mechanism. The cell must counter such a passive permeation and in some cases pumps are introduced to retain concentration gradients (e.g. proton transport¹). For charged species passive permeation is more difficult compared to uncharged species of otherwise similar properties due to the hydrophobic nature of the lipid tails. Despite the low-dielectric barrier posed by the membrane, permeation of charged species including lipophilic ions or cell penetrating peptides (CPPs), has been observed experimentally.²⁻⁵

Simulations have suggested that significant membrane distortions must occur during the permeation of charged molecules.⁶⁻⁹ These membrane defects enable partial solvation of the permeant during the transport process. A small number of water molecules enter the hydrophobic region forming transient water wires and assisting the translocation of the charged permeant. Moreover, lipid molecules in the membrane bend to facilitate interactions of the head groups and glycerol linkers with the penetrating charge. These perturbations lower the free energy barrier for permeation compared to the magnitude of the barrier estimated for unperturbed membranes. However, there is a free energy cost to create these defects that opposes permeation of charged molecules.

In recent work,¹⁰ we used a combination of atomically detailed simulations and experiments to study the insertion of a charged tryptophan (TRP) through a phosphatidylcholine lipid

bilayer, composed of 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC). We used low (pH 2.4), neutral (pH 7.2), and high (pH 10.3) pH conditions to control the charge of the tryptophan as positive, zwitterionic, or negative, respectively. By altering the pH, we were able to compare the membrane insertion of different charged states of the same molecule and avoid significant variations in other molecular parameters, such as size or hydrophobicity.

To measure the amount of tryptophan getting inside the vesicles experimentally, we recorded the fluorescence signature of tryptophan molecules inserted in the vesicles over time scales of up to 24 hours. Computationally, we simulated the potential of mean force and permeability coefficient with the inhomogeneous solubility-diffusion model.^{11,12} The main conclusion of both the experiments and the simulations was that the positively charged tryptophan permeates faster than the negatively charged and zwitterionic species. The goal of the present investigation is to examine the impact of the phospholipid head on peptide-membrane interaction by significantly altering the dipole moment of the head.

This result was consistent with previous observations about the behavior of cell penetrating peptides that are almost always positively charged.^{2,3} It was inconsistent, however, with a theory that ignores local membrane defects and interactions and predicts favorable permeation of negative charges.¹³

However, in our previous work¹⁰ it was not clear if the tryptophan ions translocated to the interior of the vesicle or if they were bound to the membrane. In a more recent paper, we experimentally addressed the difference between permeation across the lipid bilayer and partitioning of both positively and negatively charged tryptophan in DOPC vesicles.¹⁴ By adding a fraction of brominated lipids into the vesicles we were able to assess the location of the charged tryptophan species across the bilayer by measuring the tryptophan fluorescence quenching caused by the brominated lipids. Further, we were able to assign tryptophan's emission maxima (λ_{max}) to the local environment of the tryptophan molecules. For example, a blue shift in the λ_{max} is indicative of a hydrophobic environment and a red shift is indicative of tryptophan being in a more hydrophilic environment. Our results indicated that the positively charged tryptophan (W+) is partitioned into the lipid bilayer closer to the glycerol backbone region rather than completely crossing the membrane. On the other hand, negatively charged tryptophan (W-) stay mostly at the outer water-membrane interface.

In the present paper we consider the interaction of the same charged species through two different membranes, DOPC and 2-((2,3-bis(oleoyloxy)propyl)dimethylammonio)ethyl ethyl phosphate (DOCPe), where DOCPe has an inverted head group compared to DOPC (Figure 1). In DOPC, the phosphate and glycerol groups are negatively charged or polarized, and therefore support the penetration and partitioning of positively charged molecules into the membrane. The glycerol groups are embedded deeper in the membrane compared to the rest of the phospholipid head and therefore assist the insertion of cations closer to the hydrophobic core. The positively charged choline groups do not impair the insertion of cations significantly, because they are well screened by the high dielectric solvent (water) and do not penetrate significantly into the membrane.

In contrast, the negatively charged molecules that are attracted to the choline heads at the surface of the DOPC membrane, are repelled by the negatively charged groups when the anions move toward the membrane center. They are, therefore, less likely to be inserted deeply into the membrane.¹⁴

DOCPe, the inverted phospholipid we are considering in the current study, is different because the negative charge of the phosphate groups is well solvated and the contribution of the positively charged choline groups is balanced by the deeper carbonyl groups. Since the carbonyls of the glycerol group remain untouched, the impact of the head switch is not obvious.

Several related studies have been conducted on charged amino acid insertion through different phospholipid membranes.^{15–17} Recently, several inverted phosphocholine molecules were synthesized including DOCPe¹⁸ (Figure 1), the inverted head group counterpart of DOPC that we used in our previous studies. Liposomal membranes were formed and experiments showed that encapsulated anionic carboxyfluorescein is released 20 times faster from the inverted phosphocholine vesicles than the standard phosphocholine vesicles.¹⁸ However, Perttu et al.¹⁸ did not examine cationic insertion or the permeation of cationic and anionic species with similar shapes. In the present study different ions are examined to explore charge selectivity by membrane variations.

Two molecular dynamics simulations studies have been performed with inverse head group lipids. The first one compared DLPC and DLCPe lipids¹⁹ and a more recent study considered DPPC and DPCPe membranes.²⁰ Both studies considered the effects of the head group inversion on the surface binding of sodium, potassium and calcium ions in aqueous solutions. Besides differences in tail size (DOCPe has 18 carbon atoms, versus 12 and 16 for DLCPe and DPCPe lipids, respectively), and saturation (both DLCPe and DPCPe are saturated while DOCPe is monounsaturated) here we are considering more complex ions that have a side chain with some amphipathic character. The study of a tryptophan is also more relevant to the biological function of charged cell penetrating peptides.

The qualitative partitioning mechanism, summarized above, in which the relative positions and charges of the constituent groups in the phospholipid head determine the efficiency of charge insertion, was discussed in our previous study.¹⁴ The model was consistent with the reported experiments and simulations. To further test the model and expand its applicability to the design of new molecules, we examine the phospholipid molecule, DOCPe, that could impact the proposed mechanism.

II. Methods

Computational Methods:

Simulation details—The simulations were performed with the molecular dynamics package MOIL.^{21,22} In our previous simulations¹⁰ we studied tryptophan (TRP) permeation through a DOPC membrane using a membrane patch of 40 lipid molecules. To examine membrane-size effects that were suggested by others to impact diffusion and permeability estimates [19, 20], we also simulated bilayers with 128 lipid molecules and 6097 water

molecules. We compute free energy profiles and permeability for both system patches and found the size effects to be small. This is since membrane perturbation upon permeation is local and confined to the neighborhood of a few lipid molecules. Starting with a DOPC membrane patch that we computed in a previous work,²³ we conducted a 50-ns production run of the pure lipid bilayer membrane. The lipid head groups of the last configuration of that run were modified to have the corresponding inverted head groups of DOCPe molecules. We then performed an equilibration run for 1 ns to heat the system slowly to room temperature and to relax the simulation box at 1 atm. The subsequent runs were performed at constant volume and a temperature of 298 K.

We first performed a 50 ns run of the DOCPe membrane patch. We used the last 20 ns for further analysis of the pure membrane. Then we extracted out the last configuration saved in that run and we added a positively charged TRP ion (W+) to the upper water layer. To create initial configurations for the different windows of the z-constraining simulations we dragged the W+ ion toward the membrane center for 5 ns by applying a harmonic restraint on its center of mass. We repeated the procedure to create configurations for the lower water layer and, then for the negatively charged ion (W-) as well. We used sodium and chloride ions as counterions for W- and W+, respectively.

Simulations were then conducted for different positions of the tryptophan ions along the normal to the membrane plane (z). We used a harmonic potential with a force constant of 10 kcal/(mol · Å²) to constrain the position of the center of mass of the ion in 31 windows separated by 2 Å along the z axis. Each constrained window was sampled in 50 ns simulations with statistics collected only from the last 10 ns to compute the potential of mean force. For the lipids, a combination of Berger (for the acyl chain)²⁴ and OPLS (for the head group region)^{19,25} force-field parameters was used. For the amino acids OPLS force field parameters were used. Water molecules were modeled with the SPC force field²⁶ and their bond lengths and angles were constrained with a matrix version of the SHAKE algorithm.^{27,28} The particle mesh Ewald algorithm²⁹ was used to evaluate long-range electrostatic interactions with a grid resolution of 1 mesh-point/Å. The cutoffs for van der Waals interactions and for the real space component of the electrostatic forces were set to 9.5 Å. We used a 1-fs time step with the reciprocal space component of the Ewald sum evaluated every 4 fs according to a r-RESPA protocol.³⁰

Computation of the potential of mean force and permeability—We compute the potential of mean force for the permeation of the solute along the z -axis by integrating the average force needed to constrain the center of mass of the solute at fixed locations z' along the axis:^{11,12}

$$\Delta G(z) = - \int_{-L}^z \langle F_z(z') \rangle dz' \quad (1)$$

where $2L$ is the thickness of the bilayer. The mean free passage time can be estimated

$$\tau = \int_a^b dz [\exp(\Delta G(z')/RT)/D(z)] \int_a^z dz' \exp(\Delta G(z')/RT) \quad (2)$$

where $D(z)$ is a position-dependent solute diffusion coefficient. The diffusion constant is computed from an autocorrelation function of the force on the constrained solute^{31,32}:

$$D(z) = \frac{(RT)^2}{\int_0^\infty \langle \delta F_z(z, 0) \delta F_z(z, t) \rangle dt} \quad (3)$$

where $\delta F_z(z, t)$ is the instantaneous deviation of the force on the solute along the z axis at position z from the average force ($\delta F_z(z, t) = F_z(z, t) - \langle F_z(z) \rangle$). R is the gas constant and T is the absolute temperature.

Experimental Methods:

Materials and Methods.—1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC, dissolved in chloroform, 25 mg/mL) and 2-((2,3-bis(oleoyloxy)propyl)dimethylammonio)ethyl ethyl phosphate (DOCPe, dissolved in chloroform, 25 mg/mL) were purchased from Avanti Polar Lipids, Inc. and used without further purification. L-Tryptophan, Hepes, sodium bicarbonate (NaHCO_3) and sodium phosphate dibasic (Na_2HPO_4) were purchased from Sigma-Aldrich. Sodium carbonate anhydrous (Na_2CO_3), citric acid monohydrate, sodium azide (NaN_3) and HPLC grade water were purchased from Fisher Scientific. PD-10 desalting columns were purchased from GE Healthcare.

Vesicle Preparation.—Large unilamellar vesicles were prepared using the extrusion method. An aliquot of 1100 μL of DOPC or 1200 μL of DOCPe in chloroform was pipetted into a scintillation vial and dried under vacuum for at least 4 hours. The scintillation vials with the dried lipid films were then transferred to an N_2 -purged glove box for storage overnight to remove any residual solvent and to avoid possible hydration from water vapor in the air. The lipid films were hydrated with 1.5 mL of the appropriate buffer at 30 °C. For pH 2.4–0.1 M citric acid, 0.1 M NaH_2PO_4 ; for pH 10.3–0.1 M Na_2CO_3 , 0.1 M NaHCO_3 ; all buffers contained 0.02 % (w/v) NaN_3 . The hydrated lipids were then mixed using a vortex for about 5 minutes, until all of the film had been dissolved. The solution of multilamellar vesicles went through a freeze-thaw cycle 10 times, freezing the samples in liquid nitrogen and thawing in a 40 °C water bath. Samples were then passed through a mini extruder (from Avanti Polar Lipids, Inc.) with 100 nm pore size membranes 12 times to break them down into small unilamellar vesicles; the samples were then stored at 30 °C until further experimentation. The diameters of all vesicle samples were determined using dynamic light scattering (DLS) and were all in the range of 120 ± 15 nm.

A detailed characterization of the vesicles in the buffers at different pH levels was explained in previous publications.^{10,14} We determined that the concentration of the buffer salt did not affect the overall results by repeating all experiments in buffers with a lower salt

concentration and observed the same trend in samples at both pH extremes.¹⁰ Further, we characterized the shape of the vesicles using AFM, lamellarity of the vesicles using NMR, size of the vesicles using DLS and ensured there was no phase transition around room temperature using FTIR.¹⁴ Based on these results, there were no observed differences of the vesicle structure at the pH levels and buffers used for these experiments. Because of this, we assume that the change in pH itself does not affect the structure of the lipid bilayer and all interactions observed are purely from the charge of the tryptophan.

Rate of Tryptophan Associating with the Lipid Bilayer.—A 1500 μL aliquot of the prepared lipid vesicle solution and 4500 μL aliquot of a 30 mM tryptophan stock solution (in the same buffer as the vesicles) were mixed and kept at 25 °C. A 400 μL aliquot was taken at various time points (0–60 minutes and 1–12 hours) and passed through a PD-10 column that had been previously equilibrated with the appropriate buffer. The elution from the column contained the large unilamellar vesicles and no tryptophan from the exterior solution. Fluorescence measurements of tryptophan in the elution solution were taken using a Synergy plate reader with an excitation wavelength set to 280 nm and emission wavelength at 348 nm for pH 2.4 samples and 355 nm for pH 10.3 samples. The emission was background subtracted with the appropriate buffer used. The concentration of tryptophan in each sample was calculated using calibration curves for the appropriate pH, buffer, and emission wavelength.

Tryptophan Partitioning in the Lipid Bilayer.—A 1500 μL aliquot of the prepared lipid vesicle solution and 4500 μL aliquot of a 30 mM tryptophan stock solution (in the same buffer as the vesicles) were mixed and kept at 25 °C. The mixed tryptophan and vesicle solutions were then allowed to equilibrate for at least 4 hours, which was determined to be over the time necessary for equilibrium to be reached. A 400 μL aliquot of the solution was passed through a previously equilibrated (with the appropriate buffer) PD-10 column. The column volume was filled with 2100 μL of the appropriate buffer and then eluted with 3500 μL of buffer (per directions in the PD-10 column manual). The elution from the column contained vesicles with tryptophan associated with the lipid bilayer.

Immediately following elution a fluorescence spectrum of tryptophan was obtained. Subsequently, fluorescence spectra were collected of the solution every 10–20 min for 4 hours using a Fluorolog3 fluorimeter by exciting at 280 nm and collecting spectra from 300–450 nm in 1 nm increments. A 5 mm quartz cuvette (Starna Cells) was used for all samples.

As reported in our previous paper,¹⁴ to observe changes in tryptophan's environment over time, all of the fluorescence emission spectra, $F(x)$, were fit to a sum of two Gaussians I_{lip} and I_{wat} :

$$F(x) = I_{lip} + I_{wat} = a_{lip} \left\{ \exp \left[- \left(x - b_{lip} \right) / c_{lip} \right]^2 \right\} + a_{wat} \left\{ \exp \left[- \left(x - b_{wat} \right) / c_{wat} \right]^2 \right\} \quad (4)$$

where the subscripts *lip* and *wat* are representative of the environment of tryptophan in the lipid (I_{lip}) and water (I_{wat}) environment, respectively; a_i is a scaling factor, b_i is the position of the maximum intensity of I_i , c_i is the variance of I_i and x is the emission wavelength.

III. Results

III.1 Simulations

In Figure 2, we show the computed free energy profiles (or the potential of mean force) for four different translocation scenarios that include two different phospholipid membranes (DOPC and DOCPe), two different charges (positive and negative tryptophan which we denote by W^+ and W^- , respectively), and their combinations. The center of the membrane is at $z = 0$, which is also expected to be the position with the highest barrier for the permeation of the charged peptides. The solid black line, which describes translocation of the positively charged tryptophan in the inverted membrane, has the highest barrier at the center of the membrane of more than 25 kcal/mol. Conversely, the lowest central barrier for permeation is the same permeant (W^+) through the biological phospholipid DOPC. Note however that the free energy profile for W^+ in DOPC includes a minimum at about 14 Å from the membrane center. If the barrier is measured from the bottom of the well the difference between the two membranes is about 7 kcal/mol. It is also of interest that the difference in permeation between membranes for the W^- is much smaller, suggesting that retaining the glycerol groups “as are” in the modified phospholipid impact the permeation significantly.

The permeation barrier of W^- in the two membranes is quite similar and is, within the error bars of the simulations, about 2 kcal/mol. This observation on W^- suggests that the impact of the phospholipid structure is larger on W^+ translocation, an observation that is also consistent with the experimental findings (see below III.2 Experiments).

W^+ binds more than W^- in both membranes with the strongest binding for W^+ in the DOPC membrane. For the latter, the minimum is located in the glycerol region of the membrane. For the other cases, the minima are located further outside the membrane in the head group region of the bilayer.

We estimated the mean first passage time for penetration of the peptide from the water phase to the membrane center using Eq. (2). The faster time is obtained for W^+ penetration through DOPC with an estimate of 80 ± 50 h. The times are orders of magnitude larger for the other systems (10^{10} , 10^6 , and 10^4 h for W^+ in DOCPe, and W^- in DOPC and DOCPe, respectively). The penetration time ratio ($\tau(W^-)/\tau(W^+)$) is about 10^4 for DOPC and 10^{-5} for DOCPe. Therefore, there is an inversion of the preference for complete charge translocation in the membrane with the change from DOPC to DOCPe. However, according to our simulations, a complete permeation should be difficult to detect in any of these systems even for W^+ in a DOPC membrane. This is since these permeation events require very long times, times that may be longer than the lifetime of the vesicle.

III.2 Experimental Results

In Figure 3 we show the concentration of tryptophan (μM) associated with vesicles composed of DOPC (empty circles) or DOCPe (filled circles) lipid bilayers at various time points from 0–60 minutes. The concentration of tryptophan introduced to the bulk solution was 7.5 mM. It is important to note that the concentrations reported are for the solutions after being passed through the column at various time points. Once the solution is passed through the column the tryptophan outside of the vesicles is removed and we only measure

fluorescence for the tryptophan remaining partitioned in the vesicles. The charge of tryptophan, positive or negative, is controlled by the pH of the buffer solution: positive at a pH of 2.4 (pKa 2.38) and negative at a pH of 10.3 (pKa 9.39). Two conclusions are clear from these data: 1) the partitioning of positively charged tryptophan, W⁺, within the DOPC vesicles is about 2.5 times larger than that of the DOCPe lipid vesicles; and 2) the partitioning of the negatively charged tryptophan, W⁻, within both bilayers is about 3.5 times smaller than that of the positively charged species in DOPC bilayers. Both of these results are in qualitative agreement with the simulations shown in Figure 2. Moreover, the difference of the partitioning in the two membranes for W⁻ is much smaller than that for W⁺, again in accord with the simulations.

To determine whether we were observing tryptophan permeating through the lipid vesicles or partitioning into the lipid bilayer, we looked at the change in fluorescence emission energy to determine tryptophan's local environment. We previously used this method to compare the interactions of W⁺ and W⁻ with DOPC lipid vesicles.¹⁴ Because tryptophan's λ_{max} is sensitive to its local environment, we can determine if tryptophan is in a hydrophobic (lipid tail like) or hydrophilic (head group like) environment. When tryptophan is in a hydrophobic environment such as hexanes, its λ_{max} is ~340 nm; when in an aqueous solution its λ_{max} is ~375 nm.

In Figure 4 we present the change of the fluorescence emission spectra of both positively and negatively charged tryptophan with DOCPe and DOPC lipid vesicles. At pH 2.4 with W⁺ (Figure 4a), we compared the shift of λ_{max} for W⁺ in DOCPe lipid vesicles (solid lines) and W⁺ in DOPC lipid vesicles (dashed lines). There was a 7 nm shift for W⁺ in the DOCPe lipid vesicles from 10 min (λ_{max} = 354 nm) to 4 hours (λ_{max} = 361 nm) after column elution. In contrast, we observed a 12 nm shift for W⁺ in DOPC lipid vesicles from 10 min (λ_{max} = 346 nm) to 4 hours (λ_{max} = 358 nm). At pH 10.3 with W⁻ (Figure 4b), we again determined the change in λ_{max} for W⁻ in DOCPe lipid vesicles (solid lines) and W⁻ in DOPC lipid vesicles (dashed lines). In the case of W⁻ and DOCPe lipid vesicles, we observed a 3 nm shift from 10 min (λ_{max} = 366 nm) to 4 hours (λ_{max} = 369 nm) after column elution. Similarly, for W⁻ and DOPC lipid vesicles, we observed a 2 nm shift from 10 min (λ_{max} = 368 nm) to 4 hours (λ_{max} = 370 nm) after elution.

In the case of W⁺ and DOCPe lipid vesicles, there are two important observations: 1) tryptophan's λ_{max} at 10 minutes after column elution is red shifted compared to that of W⁺ and DOPC lipid vesicles; and 2) there is overall a smaller energy shift in λ_{max} from 10 min to 4 hours after column elution for W⁺ in DOCPe lipid vesicles (7 nm) compared to W⁺ in DOPC lipid vesicles (12 nm). From these two observations we can determine that W⁺ is in a more hydrophilic environment when it partitions in the DOCPe lipid bilayer compared to when it partitions in the DOPC lipid bilayer. We associate the red shifted λ_{max} of W⁺ with the DOCPe lipid bilayer at 10 min after elution with W⁺ partitioning closer to the water interface in DOCPe lipid bilayer compared to the DOPC lipid bilayer. The observed λ_{max} shifts of W⁺ provide further evidence that W⁺ is partitioned in a less hydrophobic environment within the DOCPe bilayer compared to the DOPC bilayer. We are detecting a shift in the λ_{max} because tryptophan is re-equilibrating with the surrounding bulk solution that is absent of any tryptophan after elution. When re-equilibration is reached almost all of

the tryptophan present is in bulk water because of the ratio of bulk water to lipid vesicle volume is large. Therefore, the greater the shift we detect, the more indicative it is of tryptophan being partitioned in a more hydrophobic environment in the beginning. In our previous paper¹⁴ we reported that W⁺ partitioned at or near the glycerol backbone region in the DOPC lipid bilayer; in the case of W⁺ in the DOCPe lipid bilayer, due to the flipped head group charge arrangement, it is more difficult for W⁺ to reach the glycerol backbone region and it instead partitions closer to the head group region. The different location of the partition sites for W⁺ in the two membranes agrees with the position of the wells in the PMF of Figure 2.

In the case of W⁻ and DOCPe lipid vesicles compared to W⁻ and DOPC lipid vesicles, we observed that there are not many significant differences in: 1) tryptophan's λ_{max} at 10 minutes after column elution (366 nm in DOCPe compared to 368 nm in DOPC); and 2) the observed shift in λ_{max} between 10 min and 4 hours after elution (3 nm in DOCPe and 2 nm in DOPC). These observations indicate that the inverse head group does increase the tendency of W⁻ to partition in the lipid bilayer but only slightly. This is also in agreement with the data presented in Figure 3, where we show that the amount of W⁻ partitioning in the lipid bilayer is 3.5 times less than the amount of W⁺ in DOPC, whether it is a DOCPe or DOPC lipid bilayer. The W⁻ present at the time of elution from the column must be partitioned in the lipid bilayer, but it is not as deeply partitioned as W⁺. This is indicated by the small shift in λ_{max} between 10 min and 4 hours after elution for W⁻ with DOCPe lipids (3 nm) and DOPC lipids (2 nm) compared to the 7 and 12 nm shifts for W⁺, respectively. The similarity of the partition of W⁻ in the two membranes is also in agreement with the simulations results in Figure 2.

IV Discussion

Results in Figures 2–4 show significant qualitative agreement between experimental and theoretical investigations of these systems. Both show significant membrane binding of W⁺ when the phospholipid is DOPC, as predicted by the analysis of the mechanism proposed in our previous work.¹⁴ Both simulation and experiment show the weakest binding for W⁻ in both DOCPe and DOPC membranes, followed by a slightly stronger binding for W⁺ in the DOCPe. Our experimental results only show surface binding of the charged tryptophan species without membrane crossing. These observations are in agreement with the slow permeation predicted by the simulations.

Given the agreement between experiments and simulations we used the latter to try to understand the differential binding of W⁺ and W⁻ in the two membranes.

Bilayer structure and charge distribution explained the observed membrane partition.

Figure 5 shows a comparison of the electron density profiles for pure lipid bilayers formed by both DOCPe and DOPC molecules. Figure 5(a) shows that the DOCPe bilayer is denser in the head group/water interface region and less dense at the center of the membrane than the DOPC bilayer. Figure 5(b) shows that the phosphate distribution in DOCPe is shifted by about 3 Å to the water phase region and it is more spread compared to DOPC. In the inverted lipid membrane, the phosphate groups are increasingly surrounded by water

molecules, and therefore are freer to move compared to the DOPC phosphates that are buried deeper in the membrane. The choline distribution is shifted by about 2 Å to the interior of the DOCPe membrane, and is narrower compared to that of the DOPC due to its deeper positioning toward the core. The density distributions for the glycerol groups are similar for both lipids, with slightly less penetration to the interior for the DOCPe membrane.

Figure 6 displays the charge densities along the membrane axis for the inverted DOCPe and the regular DOPC membranes. The total charge density (panel (a)) is qualitatively similar for both lipids but the DOCPe membrane shows more prominent peaks and depressions. Panel (b) demonstrates the inversion of charges due to the exchange of positions of the phosphate and choline groups in the two lipids. However, no such an inversion occurs for the charge density for glycerol (panel (c)). The charge density for the glycerol is qualitatively similar for both lipids, showing a positive density on the water side and a negative density inside the membrane (the larger error bars for DOPC indicates that this property did not fully converge in both layers). The charge density of water (panel (d)) is more complex in the DOCPe membrane, which shows two maxima and one minimum. Conversely the DOPC has only one prominent maximum and one minimum. An explanation at the molecular level for this phenomenon is given in Figure 7.

The plot of the orientation of the water dipole with respect to the membrane axis (Figure 7(a)) points to a different and more complex water structure at the DOCPe polar interface compared to the DOPC membrane. For DOPC, the oxygen atoms of the water molecules are located at the interface pointing away from the membrane center. Thus, the water's hydrogen atoms can interact with the phosphate and the glycerol carbonyl oxygen atoms without a change of orientation of the water molecules (Figure 7(b)). In contrast, the plot of the water dipole orientation (Figure 7(a)) shows a positive contribution for DOCPe with the oxygen atoms of the water molecules pointing to the membrane center when they pass the phosphate groups (the hydrogen atoms interacting with the outside phosphate and the oxygen atoms pointing to the positive choline groups). Further penetration inverts this positive contribution when the water's hydrogen atoms point to the membrane center (Figures 7 (a) and (c)). This variation of water orientations in the DOCPe membrane decreases their influence in driving the insertion of a charged particle deeper inside the membrane. Our observations are similar to those described in Perttu, E. K., et al. and Magarkar, A., et al.,^{18,19} but the molecular orientations of water in those previous simulations have additional features, probably due to the larger concentrations of monatomic ions used in those studies. Figure 7(d) shows the projections of the P-N vector (the vector from the head group phosphorus to the nitrogen) along the membrane axis. Interestingly, the distribution for DOCPe is broader, suggesting weaker electrostatic impact on charged insertion compared to the head group in DOPC.

The structural and charge configuration at the head group and glycerol regions in both membranes create an environment that favors the partition of W⁺ compared to W⁻. In the case of DOPC membrane that we consider previously,¹⁴ the presence of two neighboring groups (phosphate and glycerol) that support interaction with W⁺ enhances its membrane partition and prevents a deeper insertion of W⁻. In the case of DOCPe, such a synergistic support for a specific charge is attenuated by the less uniform arrangement of membrane

charges. In this case, the deeper polar group, glycerol, supports the insertion of W⁺ while its neighboring group, choline, favors interactions with W⁻. Both simulation (Figure 2) and experiment (Figure 4a) suggest that W⁺ does not reach the glycerol region in DOCPe due to the location of the choline groups, but it only interacts with the external phosphate groups. For W⁻, the similar location of the glycerol groups in both membranes hinders its deeper penetration inside the membrane. W⁻ only reaches the head group region in both membranes interacting with the choline groups.

The charge distribution of the DOCPe lipid membrane also modifies the orientational distribution of water molecules, inducing its flip-flop along the membrane axis (Figure 7a). This variation, combined with the broader distribution of the P-N dipole vector along the same axis (Figure 7d) has the effect of dampening any preference for the partition of a specific charge inside the DOCPe membrane, as we observe in both simulations and experiments.

Why is membrane permeation slow and not observed for these charged species?

Simulations of translocation of charged molecules usually show a high degree of membrane perturbation.^{9,15,16,33,34} Even the permeation of polar species occurs with distortion of the bilayer structure of the membrane.^{35–37} In the calculations of the PMF (Figure 2), large local perturbations are observed when the charged permeants are close to the membrane center (Figure 8). The membrane is distorted with some phosphates moving to the membrane interior for W⁺ (Figure 8(a)) and choline groups getting closer to W⁻ (Figure 8(b)). To facilitate these interactions of the head groups with the permeant buried inside the membrane's hydrophobic core, the acyl chains need to extend beyond the mid-plane of the membrane and penetrate to the other layer (Figures 8(c and d)). Deep water insertion is also observed in regions close to the charged terminal of the permeant. These membrane defects and large transversal displacements of lipids are expensive in free energy. An extreme example of membrane distortion is of the flipping of one DOPC lipid from one leaflet to the other which costs about 22 kcal/mol.³⁸ When the energy cost is that large, unassisted single charge permeation faces a large barrier and is too slow to be observed. That is the case for the systems considered in this study. Even for the fastest permeant in our simulations, W⁺ permeation through DOPC, the experiment only shows binding of this charge to the membrane. Our simulations also show the strongest binding for this pair.

A recent study of the amino acid sequences of known CPPs showed that efficient permeating peptides have a range of sequence length, charge and amphipathic character.³⁹ The average median length of residues is 14, and the average peptide charge is +5. They often contain amphipathic or hydrophobic segments. In the case of W⁺, we have a single residue with a single charge (the smallest reported CPP has three residues).⁴⁰ The tryptophan side chain gives it some amphipathic character but the size of the indole ring is too small to effectively anchor the charge amino acid deeper at the membrane core. The small size and the corresponding high charge density, and minimal amphipathic properties of W⁺ makes its permeation too slow to be observed experimentally. For W⁻ in DOPC or W⁺ and W⁻ in DOCPe, the more unfavorable membrane charge distributions prevent these charges to move beyond the head group region and make the membranes impermeable to these charges.

V. Conclusions

We present experimental and computational evidence for stronger partitioning of positively charged tryptophan (W⁺) than negatively charged tryptophan (W⁻) in both the biological relevant DOPC membrane and its inverted head group counterpart DOCPe membrane. Both experiment and simulation show that the binding of W⁺ to the DOPC is the strongest and it is located at the glycerol region of the membrane, deeper than the preferred partitioning site for W⁺ in DOCPe and for W⁻ in both membranes. We explain these results by analyzing the charge distribution of the lipid components in the two membranes that affects also the orientation of water molecules at the membrane interface and the dipole moment in the headgroup region.

The experimental results show evidence of charge partitioning without permeation. This is also in agreement with our simulations. Permeation times for all cases are too slow to be observed in the corresponding vesicular membranes. We propose that the small size of the charged amino acids (high charge density) and its lack of a large hydrophobic or amphipathic segment, prevents its deeper insertion inside the hydrophobic center with less severe distortions to the structure of the bilayer membrane.

It is expected that the qualitative understanding of the current study and our previous work¹⁴ will assist in the design of small peptides with tunable membrane permeation and partition capabilities.

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Figure 1.

A drawing of the two phospholipid molecules that are considered in the present manuscript. DOPC (top) is a phospholipid that is frequently found in biological systems, (2) DOCPe, is a synthetic molecule, not found in biological systems, in which the positions of the choline and the phosphate groups are switched.

Figure 2.

Potential of mean force for the permeation of positively (W+) and negatively (W-) charged tryptophan through a DOCPe membrane (solid lines). For comparison, the results from our previous study with a DOPC membrane are also shown (dashed lines). The error bars for the PMF of DOCPe are larger than of DOPC. This difference may be due to more significant penetration of water molecules to DOCPe (results not shown) that induces larger free energy fluctuations.

Figure 3. Concentration of charged tryptophan associated with DOCPe (filled circles) and DOPC (empty circles) vesicles as a function of time at low pH (positively charged tryptophan, black symbols) and high pH (negatively charged tryptophan, red symbols).

Figure 4.

Representative spectra from selected time points for tryptophan and DOCPe vesicles (solid lines) and DOPC vesicles (dashed lines). Spectra are shown at 10 min (blue), 1 hr (green), and 4 hr (red) after tryptophan-containing vesicles were re-equilibrated in a buffer without tryptophan. **a:** Spectra collected at pH 2.4 (W+); **b:** Spectra collected at pH 10.3 (W-).

Figure 5.

Electron density profiles of DOCPe (solid lines) and DOPC (dashed lines) bilayer membranes. They were obtained by averaging the densities of configurations from the last 20 ns of the simulations. Panel (a) shows the total densities of the membranes, along with the individual water and lipid contributions in each case. Panel (b) displays the electron density profiles of the head groups (phosphate and choline) and of the glycerol linkage.

Figure 6.

The charge density profiles for the DOCPe (solid lines) and DOPC (dashed lines) membranes as a function of the distance from the membrane center, averaged over both leaflets. (a) Total contributions of charges, (b) added contributions of choline and phosphate groups, (c) glycerol and (d) water contributions. Error bars are estimated by computing the charge densities for the two leaflets of the bilayer.

Figure 7.

(a) The average projection of the water dipole vector along the membrane axis is plotted as a function of the distance of the oxygen water from the membrane center. The projection values are weighted by the normalized population of water molecules along the membrane $P_{\text{water}}(z)$, which explains the small values. The sign of the projection indicates the orientation of the oxygen atoms (positive and negative are toward and away from the membrane center, respectively). Cartoon drawings of water molecule orientations in the headgroup/glycerol region are shown for DOPC (b) and DOPe (c) membranes. There is a uniform orientation of water in the DOPC membrane, while the orientation changes in the DOPe membrane. (d) Projection of the P-N vector along the membrane axis for both membranes. The distribution is broader for DOPe lipids, which decreases the effect of its dipole along the membrane axis. Error bars in (a) and (d) were estimated by computing the vector projections for the two leaflets of the bilayers.

Figure 8.

Two molecular snapshots extracted from molecular dynamics trajectories when the center of mass of the permeant tryptophan molecule was constrained at the center of the membrane.

(a) W+, (b) W-. At panels (a) and (b) we use van der Waals spheres to represent the phosphate (orange), choline (blue), glycerol (green), and charged tryptophan (mostly cyan). Lipids tails are shown as translucent gray lines. Panels (c) and (d) show detailed views of (a) and (b), respectively, displaying the two lipids with head groups closest to the permeant. The color labels are similar to the panels (a) and (b), but the hydrocarbon chains are shown in violet. This figure illustrates the general tendency for large local perturbation (with increased head group and water molecules penetration) when the charged tryptophan is buried in the hydrophobic core of the membrane. The figure indicates that the phospholipid heads are shifted into the middle of the membrane in the presence of a charged permeant at the center.