Behavior of 4-hydroxynonenal in Phospholipid Membranes

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Abstract

Under conditions of oxidative stress, 4-hydroxy-2-nonenal (4-HNE) is commonly present in vivo. This highly reactive and cytotoxic compound is generated by oxidation of lipids in membranes, and can be easily transferred from a membrane to both cytosol and the extracellular space. Employing time-dependent fluorescence shift (TDFS) method and molecular dynamics simulations, we found that 4-HNE is stabilized in the carbonyl region of a 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) bilayer. 4-HNE is thus able to react with cell membrane proteins and lipids. Stabilization in the membrane is, however, moderate and a transfer of 4-HNE to either extra- or intracellular space occurs on a microsecond timescale. These molecular-level details of 4-HNE behavior in the lipid membrane rationalize the experimentally observed reactivity of 4-HNE with proteins inside and outside the cell. Furthermore, these results support the view that 4-HNE may play an active role in cell signaling path-ways.

Keywords: lipids, cell membrane, oxidation, 4-hydroxynonenal, molecular dynamics, fluorescence spectroscopy

Introduction

Oxidative stress occurs in living cells due to an imbalance between production of oxidizing species and antioxidative mechanisms, and leads to the damage of biological material such as proteins, nucleic acids, and lipids.[1,2] Phospholipids building mammalian cell membranes frequently contain polyunsaturated sn-2 chains[3] which are especially prone to radical peroxidation reactions.[4] Free radical reaction mechanisms lead to the formation of lipid hydroperoxides which can subsequently decompose and generate various aldehydes. One of the most common aldehydes found upon lipid peroxide reactions of ω-6 polyunsaturated fatty acids *in vivo* is 4-hydroxy-2-nonenal (4-HNE).[5] Due to the presence of three reactive functional groups, 4-HNE is a highly reactive cytotoxic agent that can further react with various proteins inside and outside a cell.[6] For instance, 4-HNE can be effectively transferred from the cell membrane to the cytosol where it is metabolized by glutathione-S-transferases.[7] It can also react with Cytochrome C, the protein essential to cellular respiration, which is loosely bound to the inner mitochondrion membrane leaflet.[8] Furthermore, 4-HNE can readily leave the cell and enter the bloodstream where it was shown to couple with blood protein human serum albumin forming different covalently bound adducts.[9] The abundance of 4-HNE upon lipid peroxidation leads to its use as the indicator of harmful oxidation processes in the cellular membrane, forming products which can serve as efficient oxidative stress biomarkers.[10-12] A particular interest in 4-HNE stems from its involvement in a number of different neurodegenerative diseases, such as Alzheimer's disease,[13-16] Parkinson's disease[17-19] and it can induce cell apoptosis.[20,21]

As we recently demonstrated and rationalized at the molecular level, oxidative modifications of phospholipids alter membrane properties such as water permeability, transmembrane lipid

transport, and membrane stability.[22-24] In all these investigations, modifications of membrane properties were determined by both the extent and type of oxidation products present in the system. One of these studies indicated that model aldehyde products may act as stabilizing factors in massively oxidized lipid bilayers.[23] However, to the best of our knowledge, the role of short-chain aldehydes (4-HNE, in particular) in oxidized membranes has not been investigated at the molecular level for physiologically relevant model membranes yet. Specifically, despite its crucial role, it is still not known with molecular detail what is the behavior of 4-HNE inside the phospholipid bilayer.

In this work we elucidate the role of 4-HNE in phosphatidylcholine membranes by exploring localization of 4-HNE inside the 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) bilayer and the fate of 4-HNE in the membrane upon oxidation reaction by means of both fluorescence spectroscopy measurements and molecular dynamics simulations. Our goal is to answer the following questions: Where is 4-HNE located in a model POPC bilayer? How does its presence influence the bilayer properties? Finally, how easily can 4-HNE be transported out of the bilayer?

Methods

To experimentaly evaluate the interactions of 4-HNE with POPC bilayer we employed time-dependent fluorescence shift (TDFS) method,[25]capable of monitoring local lipid mobility and hydration, i.e., the parameters that are often affected by adsorption of various compounds to model lipid membranes.[26] TDFS experiments were performed on large unilamellar liposomes

of POPC with 4-HNE. The vesicles were fluorescently labeled with 6-dodecanoyl-2-

dimethylaminonaphthalene (Laurdan). The details of sample preparation and instrumentation used are provided in the Supporting Information. Steady-state emission spectra and fluorescence decays were used to reconstruct time-resolved emission spectra (TRES). The position of TRES maximum, $\nu(t)$, after electronic excitation at t=0 s, reflects dipolar relaxation of the probe microenvironment.[27] Analysis of TRES position gives two parameters: 1) $\Delta\nu = \nu(0) - \nu(\infty)$, total emission shift, which is directly proportional to the polarity of the microenvironment of Laurdan, 2) $\tau = \int_{t=0}^{\infty} \frac{\nu(t)-\nu(\infty)}{\Delta\nu}$, integrated relaxation time, which is a measure of the mobility of the hydrated carbonyls at which level the Laurdan fluorophore is known to be located.[28-30] The so-called tau-zero spectrum was experimentally estimated according to Fee and Maroncelli.[31] Its maximum was found to be at 23800 cm^{-1} and was not affected by the addition of 4-HNE. A close inspection of the recorded TRES has not revealed any signs of inhomogeneity or other artifacts that could hamper the TDFS analysis. Thus, based on our experience with Laurdan TDFS experiments[25,28] and recent simulations of Laurdan location[30] and photochemistry of the naphthalene chromophore[32] we can conclude, that the observed changes in the TDFS parameters should be related to the properties of the lipid bilayer and not to the specific interactions of the 4-HNE molecule with the probe. Time-dependent fluorescence shift (TDFS) method, often referred to as fluorescence solvent relaxation, was described in details elsewhere.[25]

The experiments were accompanied by classical molecular dynamics (MD) simulations using the atomistic-level empirical force-field for a model POPC bilayer. We explored the structural properties of the membrane in the presence of 4-HNE (<1 mol% and 10 mol%), accompanied by potential of mean-force (PMF) simulations in order to estimate the free-energy profile for 4-HNE transport across the bilayer. For comparison, we also investigated POPC bilayers containing 10

mol% of 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphatidylcholine (PLPC) which is a model polyunsaturated lipid, oxidation of which leads to 4-HNE generation. Moreover, to mimic a membrane with a high concentration of oxidation products, we investigated a POPC bilayer containing 10 mol% of both 1-palmitoyl-2-(9'-oxononanoyl)-*sn*-glycero-3-phosphocholine (PoxnoPC) and 4-HNE, the former being a byproduct of PLPC oxidation.[33,34] All studied molecules are depicted in Chart 1. Further details of both MD simulation and TDFS experiments are provided in the Supporting Information.

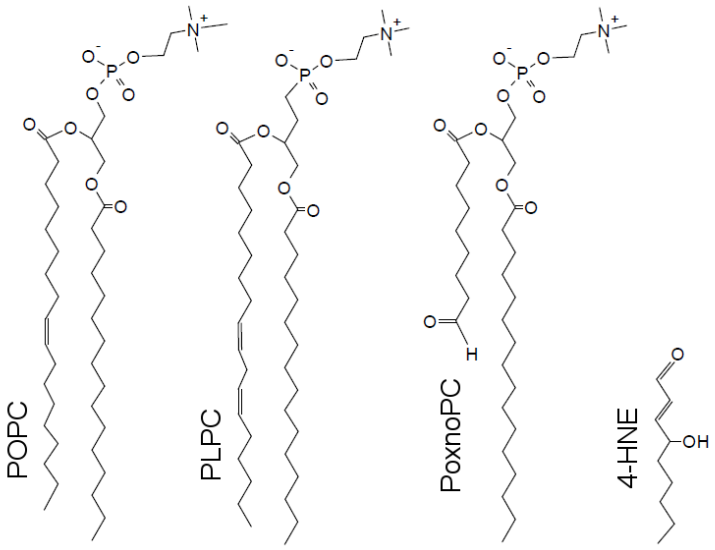

Chart 1. The structures of considered phospholipids and the 4-HNE molecule.

Results and Discussion

TDFS experiments were performed on POPC vesicles that were fluorescently labeled with 6-dodecanoyl-2-dimethylaminonaphthalene (Laurdan). We found that the fluorophore of Laurdan, precisely located at the level of lipid carbonyls,[25] was sensitive to the presence of 4-HNE in the

sample. The relaxation dynamics was slowed down upon addition of 4-HNE into the liposomal suspension. A gradual increase of the mean relaxation time τ was observed for increasing 4-HNE concentratrions (Table 1). This means that the local mobility of lipids at the level of their carbonyls is restricted. The second measured parameter, Δν, describing local hydration/polarity, was not affected. We can conclude that 4-HNE easily accomodates into the carbonyl region of lipid membrane, where it associates with the lipid molecules restricting their mobility. The changes are moderate and the bilayer structure and hydration is preserved.

Table 1. Time-dependent fluorescence shift of Laurdan in POPC vesicles in the presence of 4-HNE.

4-HNE/POPC molar ratio	integrated relaxation time, τ (ns)	total emission shift, Δν (cm^{-1})
0/100	3.10±0.05	4025±50
10/90	3.17±0.05	4025±50
35/65	3.27±0.05	4000±50
50/50	3.43±0.05	4050±50

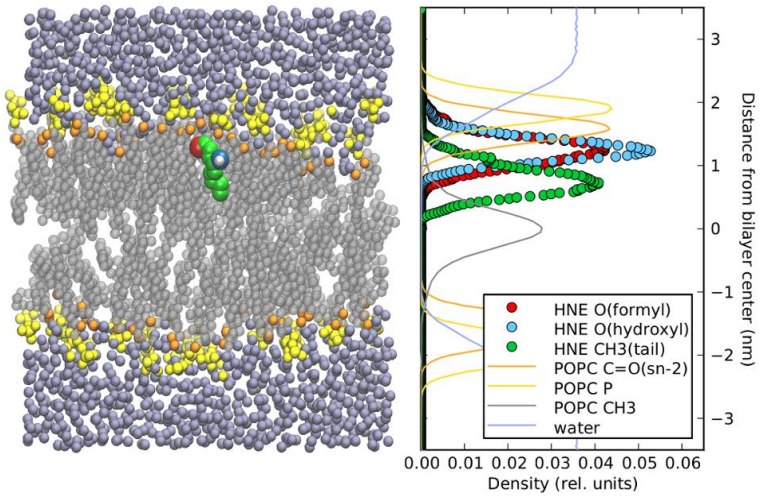

Figure 1. Typical snapshot of 4-HNE molecule in the lipid membrane for the system with a single molecule of 4-HNE in POPC bilayer (left panel), and partial density profiles of selected atoms and groups as a function of the distance from bilayer center (right panel). Color coding in the snapshot: violet, water; yellow, phosphate groups; orange, oxygen atoms of carbonyl groups; gray, lipid chains; green, carbon atoms in 4-HNE; red, formyl oxygen; blue, hydroxyl oxygen; white, hydroxyl hydrogen.

The behavior of 4-HNE in the bilayer was investigated with MD simulations. We found that 4-HNE accommodates inside the POPC bilayer. Localization of 4-HNE in the membrane can be determined based on the calculated density profiles and the snapshots of the MD trajectory (Figure 1). 4-HNE is located prefentially just below the carbonyl groups of POPC lipid molecules. Noteworthy, both aldehyde and hydroxyl group reside approximately at the same depth, while the aliphatic tail of 4-HNE is located in the region occupied by aliphatic tails of POPC and oriented toward the bilayer center. Thus, the 4-HNE molecules in the bilayer are, on average, bent rather than extended. The polar groups of 4-HNE reside in the vicinity of the relatively well hydrated carbonyl groups of lipids being able to form efficient hydrogen bonds with both carbonyl groups and water. The analysis of binding preferences between the polar

groups of 4-HNE and atoms of both lipids and water (see Table S6 in the Supporting Information) revealed that 4-HNE forms hydrogen bonds predominanly between its hydroxyl group and the water molecules that reside in the carbonyl region of the bilayer. Similarly, the formyl group of 4-HNE is able to form hydrogen bonds with water. Moreover, hydroxyl groups of 4-HNE have the ability to interact with both carbonyl and phosphate groups of lipids. On top of those polar interactions, the van der Waals interactions between the lipid chains and the hydrocarbon chain of 4-HNE provide additional stabilization in the lipid bilayer. In this way, 4-HNE shows its amphiphilic character – it maximizes both hydrophobic interactions between its chain and the aliphatic tails of lipids and interactions between its own polar groups and the polar environment of the bilayer headgroup region. Localization of 4-HNE inside the bilayer, resulting in numerous interactions with carbonyl groups, is in accord with the current fluorescence spectroscopy measurements (Table 1), where the presence of 4-HNE was shown to influence the properties of the Laurdan dye located in the carbonyl region of the bilayer. No qualitative differences in localization of 4-HNE are observed in simulations of the systems with 10 mol% of 4-HNE (with either pure-POPC or 9:1 POPC:PoxnoPC bilayer), see the density profiles in Fig. S1 in the Supporting Information.

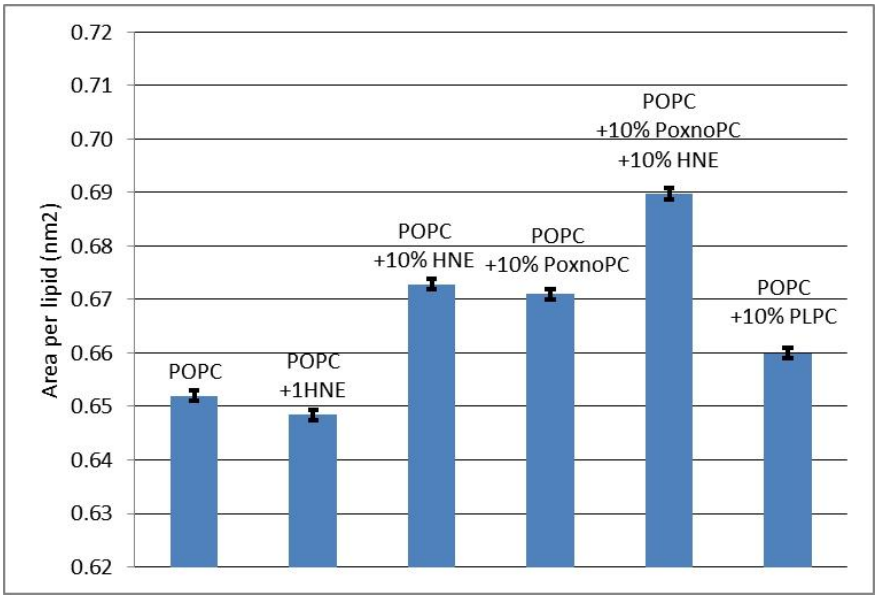

Figure 2. Area per lipid for investigated systems. In the case of POPC+PoxnoPC the data from the Ref [35] is taken.

The calculated area per lipid (APL) of the POPC bilayer at a low concentration (<1 mol %) of 4-HNE is, within the error bars, same as the APL estimated previously for pure POPC employing the same simulation method[22] (Figure 2). This implies that 4-HNE does not significantly change the lateral packing of the POPC membrane at a high dilution. Larger concentrations of 4-HNE (10 mol %) result in an enhanced APL of the bilayer, which can be rationalized by the beforehand discussed accumulation of 4-HNE between lipid molecules. In the system mimicking the lipid membrane with a high concentration of oxidation products (POPC+10 mol% 4-HNE+10 mol% PoxnoPC) a more significant increment of APL is observed. This increase originates from two effects. First, the tendency of the oxidized sn-2 chains of PoxnoPC to reorient their polar aldehyde groups toward the water phase; this reorientation is evident based on the density profile of aldyhyde groups, see Fig. S1 in the Supporting Information where the

density of aldehyde groups of PoxnoPC is located in the headgroup region of the bilayer. Such reorientation of oxidized lipid chains was previously reported based on both experimental and computational studies,[35,36] and results in enhanced APL. Second, the increase of APL due to the accommodation and stabilization of 4-HNE in the bilayer as revealed by density profile shown in Fig. S1. These two effects are additive, as the increment of APL in the POPC membrane with both 4-HNE and PoxnoPC is approximately a sum of the APL increments in the membranes containing 10 mol% of each of these species (see Fig. 2). The control system with 10 mol % of PLPC exhibits only a small increase of APL with respect to the pure POPC bilayer, which can be rationalized by the presence of non-flexible double bonds which enhance steric interactions between acyl chains of lipids inside the bilayer. In all studied systems, the presence of 4-HNE caused no change of the bilayer thickness within the error bar of 0.1 nm with regard to the pure POPC membrane where the thickness amounts to 3.8 nm.[35] It was reported previously that the thickness of the POPC membrane is reduced in the presence of 10 mol% of PoxnoPC by approximately 0.15 nm.[35] The lack of such effect in the present study indicates that the molecules of 4-HNE prevent the reduction of membrane thickness. The lateral diffusion coefficient calculated based on the mean square displacement of POPC molecules ($0.6\pm0.1\times10^{-7}$ cm^2 s^{-1}) does not depend on the amount of 4-HNE present in the bilayer. Lateral diffusion of 4-HNE is, nevertheless, significantly faster than that of lipids, with the diffusion coefficient equal to $2.3\pm0.3\times10^{-7}$ cm^2 s^{-1}.

In the light of the experimentally observed presence of 4-HNE outside the cell membrane and its ability to form there adducts with proteins,[7-9] mechanisms for a transport of 4-HNE from the membrane to the water phase must be operative. The MD simulations, supported by TDFS experiments reported above, show that 4-HNE is stabilized inside the lipid bilayer on the 100 ns

timescale. In order to quantify this stabilization and elucidate the probability of 4-HNE leaving the bilayer beyond the 100 ns timescale, the free-energy MD simulations for a transport of a molecule of 4-HNE in the POPC bilayer were performed. The calculated free energy profile for 4-HNE in the bilayer is depicted in Figure 3. 4-HNE is stabilized in the region of carbonyl groups with a free-energy of roughly 25 kJ mol^{-1} with regard to the hydrophobic membrane interior and about 17 kJ mol^{-1} with respect to the water phase. Thus, the free-energy well is relatively shallow and the cost of 4-HNE transfer from the carbonyl region of the membrane to the water phase is low in a comparison with that calculated for phospholipids. For comparison, the free-energy cost of transfer of a lipid molecule from the bilayer to the water phase, calculated employing the same method as used here, amounts to 85 kJ mol^{-1} for POPC and 80 kJ mol^{-1} for DPPC.[37] This quantity is understandably lower for phospholipids with shorter acyl chains, amounting to 39 and 48 kJ mol^{-1} for DLPC and DMPC, respectively.[37]

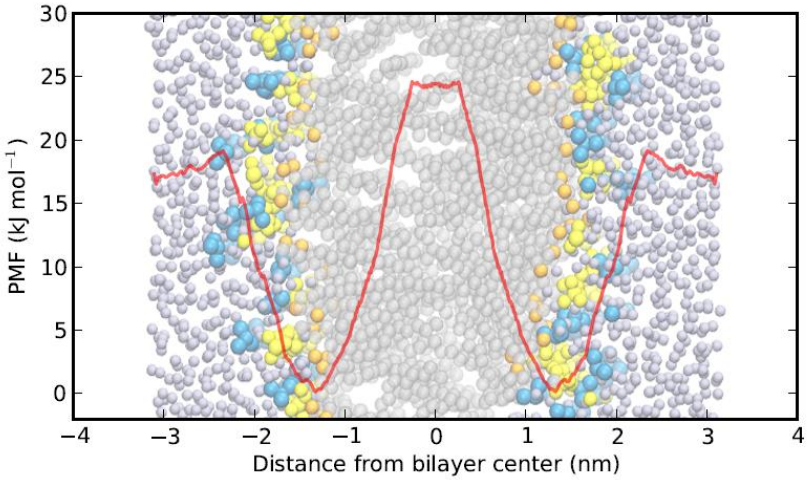

Fig. 3. Free energy profile for the transfer of a 4-HNE molecule along the bilayer normal. The distances beyond 2.5 nm correspond to the water phase. A simulation snapshot is shown in the background to guide the eye. The error of free-energy estimated employing the bootstrapping method is within ±3 kJ mol^{-1}.

The free-energy well with respect to the membrane interior, of 25 kJ mol^{-1}, corresponds to the free-energy cost of the transverse diffusion (flip-flop). A comparison with typical values calculated for phospholipids with long acyl chains, i.e., 89 and 80 kJ mol^{-1} for POPC and DPPC,[37] demonstrates that 4-HNE flip-flops more feasibly than these lipids. This difference is less pronounced with regard to the lipids with shorter chains, namely, DLPC and DMPC. Note, however, that for these latter molecules the low calculated flip-flop free-energy barriers point to possible force-field issues.[37] In the free-energy profile depicted in Fig. 3 there is a plateau in the middle of the membrane. Similar plateaus were observed in free-energy profiles of flip-flop of short lipids and were associated with formation of water pores across the bilayer.[37] We observed no water pores formation during the free-energy calculations for 4-HNE, and the plateau in Fig. 3 is rather a result of 4-HNE molecule accommodated between the two lipid leaflets which is feasible due to a relatively small size of 4-HNE. Similar behavior was observed for water and other small polar molecules crossing various lipid bilayers.[38-40] It should be noted that the free-energy calculations could be prone for artifacts mainly due to slow convergence and an ambiguous choice of the reaction coordinate. Thus, fully quantitative interpretation of the fine features observed in the free-energy profiles may be difficult.

The time-scale of lipid flip-flop, measured experimentally in lipid vesicles, is in the range of 1 to 90 hours.[41] A crude estimation, employing the transition state theory and assuming similar pre-exponential factors, leads to the timescales of 4-HNE flip-flop spanning a range from hundreds of nanosecods to tens of microseconds at the temperature of 310 K (see Supporting Information). Based on the difference between the free-energy stabilization with regard to both the water phase (17 kJ mol^{-1}) and membrane interior (25 kJ mol^{-1}), the transport of 4-HNE from the membrane to water should be approximately 20 times faster than the flip-flop. This estimate leads to the

lifetime of 4-HNE in the membrane being in the range of tens of nanoseconds to microseconds. As in our simulations no spontaneous transfer of 4-HNE from the membrane to the water phase was observed in the 100 ns trajectories, we estimate the average time before the 4-HNE molecule is transferred to the water phase to be rather in the range of microseconds. Employing this value and the calculated diffusion coefficient, a molecule of 4-HNE can laterally spread out at the distance ranging from 10 to 100 nm before leaving the membrane. The model POPC bilayers considered here do not fully account for the properties of actual cell membranes. In particular, the effects of membrane asymmetry due to a different lipid composition between the leaflets and differences in ionic content between the intra- and extracellular space are not included in the model systems. Nevertheless, as demonstrated in earlier studies,[24,41,42] the estimation of the typical timescales based on the free-energy calculations performed for simple model bilayers are typically in a qualitative agreement with the values determined for more complicated lipid membranes.

Conclusions

Based on the present experimental and computational results on stabilization of 4-HNE at the headgroup reagion of the lipid bilayer several biologically relevant conclusions can be drawn. The molecules of 4-HNE generated during lipid oxidation accommodate in the carbonyl region of lipid membranes. Under physiological conditions, concentrations of 4-HNE in a typical cell membrane are expected to be well below 10 mol %, and we anticipate that the influence of 4-HNE on the biophysical properties of lipid bilayer, such as membrane thickness and area per lipid, is minor. Nevertheless, the accomodation of 4-HNE in the bilayer opens the possibility for its reactivity with membrane proteins, as observed in the case of neurodegenerative diseases.[43-45]

The free energy of 4-HNE stablilization in the membrane is relatively small and we can estimate that after an oxidation reaction a molecule of 4-HNE will leave the membrane in a relatively short time, in the range of microseconds. This is in accord with the experimentally observed presence of 4-HNE both in the intra- and extracellular space. Molecules of 4-HNE, while in the membrane, are relatively mobile and they can flip-flop between the leaflets and laterally spread out within the range of tens of nanometers before leaving the membrane. Based on the accomodation and lateral mobility of 4-HNE inside the lipid bilayer as well as its capability to exit the membrane, the present study supports and rationalizes the view that 4-HNE is reactive in both intra- and extracellular space, and it can play an active role in cell signalling pathways.

Acknowledgments

L.C. acknowledges the Granting Agency of the Czech Republic for support (ESF EUROMEMBRANE project MEM/09/E006). Piotr J. and M.H. thank the Czech Science Foundation (via projects P203/08/0114 and P208/10/0376, respectively). Moreover, Pavel J. and M.H. acknowledge the Praemium Academie Award (Academy of Sciences of the Czech Republic). P.J. acknowledges support from the Ministry of Education (grant LH12001).

Supporting Information Available: Experimental and computational details. Density profiles. Hydrogen bonds analysis. Estimation of the timescales of flip-flop and membrane exit. This material is available free of charge via the Internet at http://pubs.acs.org.

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