

# Exploring the Free Energy Landscape of Solutes Embedded in Lipid Bilayers

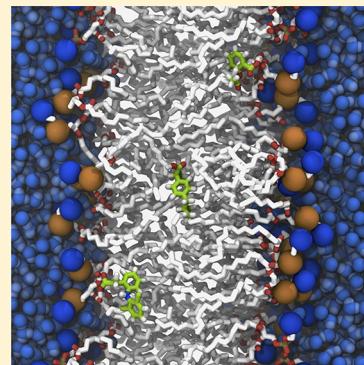
Joakim P. M. Jämbeck\* and Alexander P. Lyubartsev\*

Division of Physical Chemistry, Arrhenius Laboratory, Stockholm University, Stockholm, SE-10691, Sweden

 *Supporting Information*

**ABSTRACT:** Free energy calculations are vital for our understanding of biological processes on an atomistic scale and can offer insight to various mechanisms. However, in some cases, degrees of freedom (DOFs) orthogonal to the reaction coordinate have high energy barriers and/or long equilibration times, which prohibit proper sampling. Here we identify these orthogonal DOFs when studying the transfer of a solute from water to a model membrane. Important DOFs are identified in bulk liquids of different dielectric nature with metadynamics simulations and are used as reaction coordinates for the translocation process, resulting in two- and three-dimensional space of reaction coordinates. The results are in good agreement with experiments and elucidate the pitfalls of using one-dimensional reaction coordinates. The calculations performed here offer the most detailed free energy landscape of solutes embedded in lipid bilayers to date and show that free energy calculations can be used to study complex membrane translocation phenomena.

**SECTION:** Biophysical Chemistry and Biomolecules



Thermodynamics is one of the cornerstones in physical chemistry, and the concept of free energy is arguably the most important aspect of thermodynamics.<sup>1</sup> Many chemical and biological processes are governed by the change in free energy such as solvation phenomena,<sup>2</sup> protein–ligand association,<sup>3,4</sup> enzymatic reactions<sup>5</sup> and membrane–water partitioning.<sup>6</sup> The ability to predict free energies has been a long-term goal in several areas (pharmaceutical research to mention one), and endeavors in the molecular modeling field during three last decades<sup>1,7–11</sup> have made it possible to perform these calculations relatively routinely. Currently, due to these efforts, we can discriminate between different methods based on the problem we have at hand, and this can bring us closer to a more complete understanding of biological processes.<sup>12</sup>

Major efforts have been put into understanding the effect of drugs, toxins, anesthetics, and other solutes on cells from a molecular perspective, especially with the focus on free energies.<sup>13–17</sup> Despite this, the mechanisms for some of these widely used compounds are yet not fully understood.<sup>18</sup> Still free energy calculations offer a great deal of insight as a full understanding of the passive diffusion phenomena of these compounds over the membrane requires a detailed view of the underlying free energy surface (FES). As already mentioned, free energy calculations are used in computer-aided drug design.<sup>19</sup> Issues that often render drug candidates unfit for clinical testing are related to slow translocation of the compounds across the membranes, resulting in poor pharmacokinetic properties and poor bioavailability. Despite the fact that major efforts have been made to decrease the failure attribute of these two properties, the progress of many candidate compounds is still tampered by these factors. To

measure the permeability and partitioning of compounds is difficult from a high-throughput perspective, and therefore computer simulations can be used as an alternative.<sup>6</sup> In particular, molecular dynamics (MD) simulations have been successful when the interactions between small solutes and model membranes have been studied.<sup>14,16,17,20–23</sup> These studies offer detailed free energy profiles of transferring the solute from the surrounding water phase to the center of the membrane.<sup>24–26</sup>

As MD simulations sample parts of phase space according to a Boltzmann distribution, important regions separated by larger (free) energy barriers may therefore not be properly sampled during the simulations. Several methods have been developed with the aim to allow proper sampling of less probable parts of phase space such as umbrella sampling (US),<sup>7</sup> the Wang–Landau algorithm,<sup>8</sup> the adaptive biasing force method<sup>9,10</sup> and metadynamics.<sup>11</sup> As membrane partitioning studies goes, the US method is the most employed technique, and the reaction coordinate/collective variable (CV) is (often) trivial to define: the distance between the membrane midplane and solute along the membrane normal for which the probability distribution is biased. From these biased probability distributions, a free energy profile or potential of mean force (PMF) of the translocation can be obtained with, e.g., the weighted histogram analysis method.<sup>27</sup> However, other degrees of freedom (DOFs) that are, per definition, orthogonal to the biased DOF(s), are often of importance, and if these DOFs are not properly

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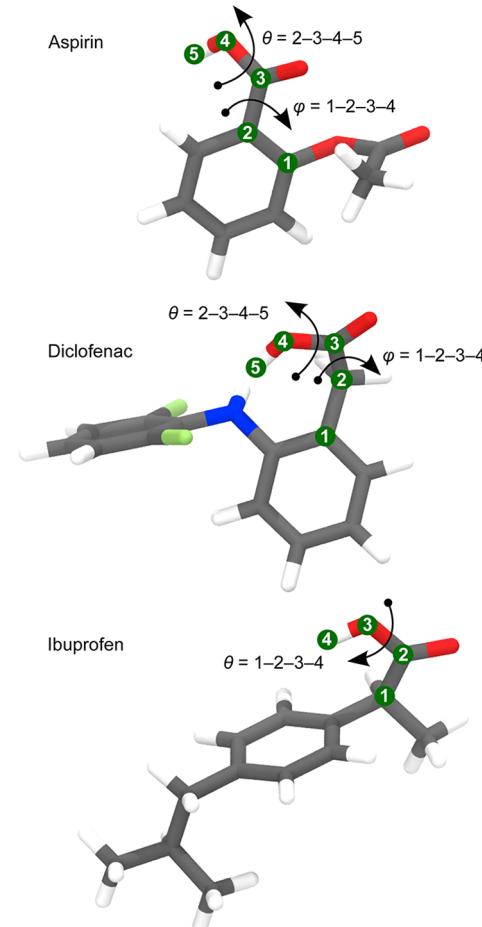
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sampled, the obtained FES will contain errors that in the worse case could lead to wrong conclusions. Often one can mistakenly assume convergence of the PMF along the reaction coordinate based on long correlation and relaxation times of other DOFs. While transferring a molecule from water to the membrane center, one important DOF orthogonal to the reaction coordinate is the membrane disruptions as the solute is transferred toward the hydrophobic core of the membrane.<sup>24</sup> If one considers the conformational freedom of the solute, the number of important DOFs that have to be properly sampled grows fast, and these DOFs can be difficult to define *a priori*. Yet they remain vital in certain cases in order for simulations to be able to reproduce and ultimately predict experiments. The ability of a solute to form intramolecular hydrogen bonds is one example of these DOFs.<sup>28–31</sup> Procedures such as Hamiltonian replica exchange US (HREX-US) can decrease the correlation between sampled conformations and speed up the convergence in some cases; however, when probable states are separated by large energetic barriers, 1D sampling often has to be abandoned for biased sampling in several dimensions. 2D HREX-US calculations are robust and can be performed,<sup>32–34</sup> however, they are extremely demanding from a computational point of view and require a lot of efforts before the simulations are even initiated. These calculations are further complicated when more than two DOFs are needed to be biased. An alternative method to this is metadynamics,<sup>11</sup> which has been demonstrated to be an efficient and potent tool in the exploration of complex FESs in many studies,<sup>3,35–38</sup> especially in the case where more than two DOFs are of interest.<sup>39–41</sup>

In the present Letter we show that the more traditional one-dimensional (1D) US approach is not able to properly describe partitioning between water and a model membrane of three typical drug compounds (aspirin, diclofenac, and ibuprofen) and that more extensive sampling is required. First, important DOFs of the solutes are identified in bulk liquids of different dielectric nature. This allows for a more complete picture of the solute's conformational space before proceeding with large scale membrane simulations were the reaction coordinates are two- (2D) or three-dimensional (3D) instead of a 1D reaction coordinate. With this scheme we are able to sample relevant regions of phase space, and the resulting PMFs and standard binding free energies are in good agreement with experiments compared to the more naive approach of running 1D US simulations without any consideration of the conformational space of the solutes.

Aspirin, diclofenac, and ibuprofen were chosen, as the two former have the possibility to form an intramolecular hydrogen bond, and they all have carboxyl groups that have a history of being difficult to sample properly.<sup>29,30</sup> In Figure 1, the intramolecular CVs are shown, and for all simulations with the membrane, an additional CV was added: the center of mass distance between the solute and lipid bilayer. The torsion angle  $\psi$  is not needed as a CV for ibuprofen, as there are no possibilities for this solute to form intramolecular hydrogen bonds. Before the membrane simulations the underlying FES of the torsion angles  $\theta$  and  $\psi$  for aspirin and diclofenac (Figure 1) were explored in gas phase, water, and *n*-hexane. As the hydrophobic core of a lipid bilayer behaves similarly to a bulk alkane, the latter solvent was used to mimic this region. These simulations were performed using the well-tempered (WT) metadynamics procedure described by Barducci et al.<sup>42</sup> For ibuprofen, the same simulations were performed but using US simulations to sample along the torsion angle  $\theta$ .

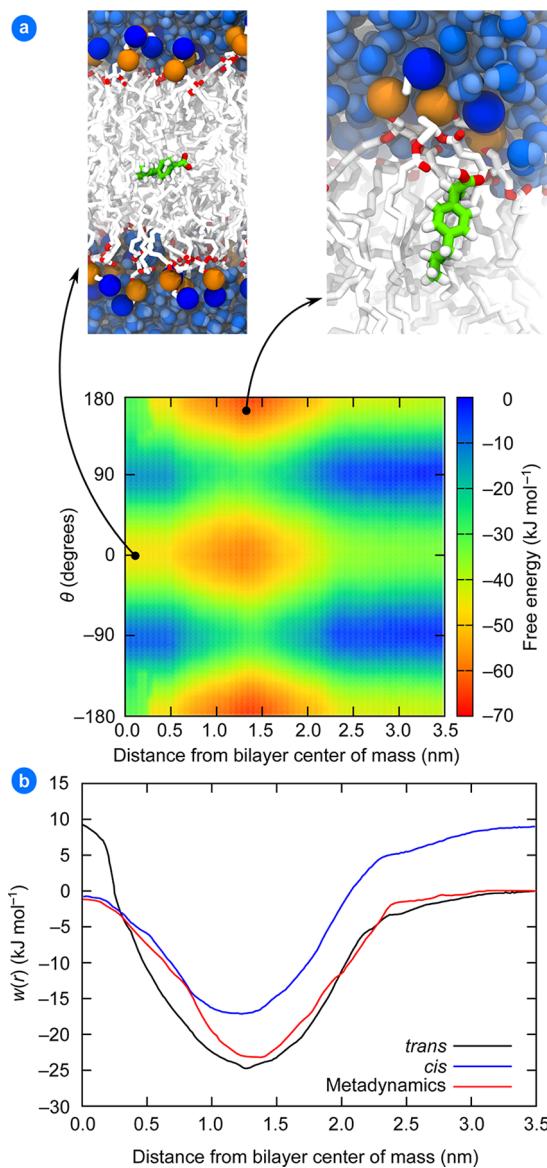


**Figure 1.** Intramolecular CVs in the present study.

For ibuprofen, the resulting FESs are shown in Figure S1 (Supporting Information, SI), and for aspirin and diclofenac, they are shown in Figure S2. When the dielectric constant of the solvent is decreased by going from water to *n*-hexane, the hydrogen of the carboxyl group prefers to be in *trans* conformation ( $\theta \sim 0$ ) with respect to the carbonyl oxygen for diclofenac and ibuprofen. In aqueous solution, the free energy minima is found when the hydroxyl hydrogen is in the *trans* conformation ( $|\theta| \sim 180$ ) meaning that the hydrogen is oriented away from the carbonyl in order to participate in a hydrogen bond network with water instead of the 1,4 intramolecular interaction. The mentioned features are similar to the findings presented by Paluch et al.<sup>30</sup> For both solutes, the FESs in *n*-hexane and gas phase are similar, with more of a shallow minimum for the former when compared to the latter. The free energy barriers between these two states are high ( $\sim 14k_B T$ ), resulting in extremely long simulations being needed in order to sample these transitions. As the conformational preference for these two molecules differ significantly between a solvent with  $\epsilon \sim 78$  (water) and  $\epsilon \sim 2$  (*n*-hexane), a transition is to be expected when the solute is transferred from water to the hydrophobic core of the membrane. By merely biasing the sampling along the distance between the solute and membrane, this transition is likely to never occur. Even if the simulations reach a microsecond time scale, it is plausible that the resulting PMF will be incorrect due to the lack of sampling, which can lead to the wrong conclusions. This is shown later on for ibuprofen. For aspirin, the FESs are more complicated and

show multiple minima in all three phases. Again the differences between gas phase and *n*-hexane are small, and for the aqueous phase the minima are slightly shallower. The intermolecular hydrogen bond between the hydroxyl hydrogen and the ether oxygen is comparable in strength to the 1,4 interaction between the hydroxyl oxygen and the carbonyl oxygen, and thus one intramolecular interaction is likely to be present during the transfer from water to membrane. This analysis of the solutes in three different environments allows us to verify our hypothesis regarding which CVs to choose besides the trivial one on the distance between the membrane and the solute. Already at this point potential issues that would occur with 1D enhanced sampling can be pointed out and thus avoided. For aspirin and diclofenac, the results show that at least three CVs are needed to sample the transfer, and for ibuprofen two CVs are needed.

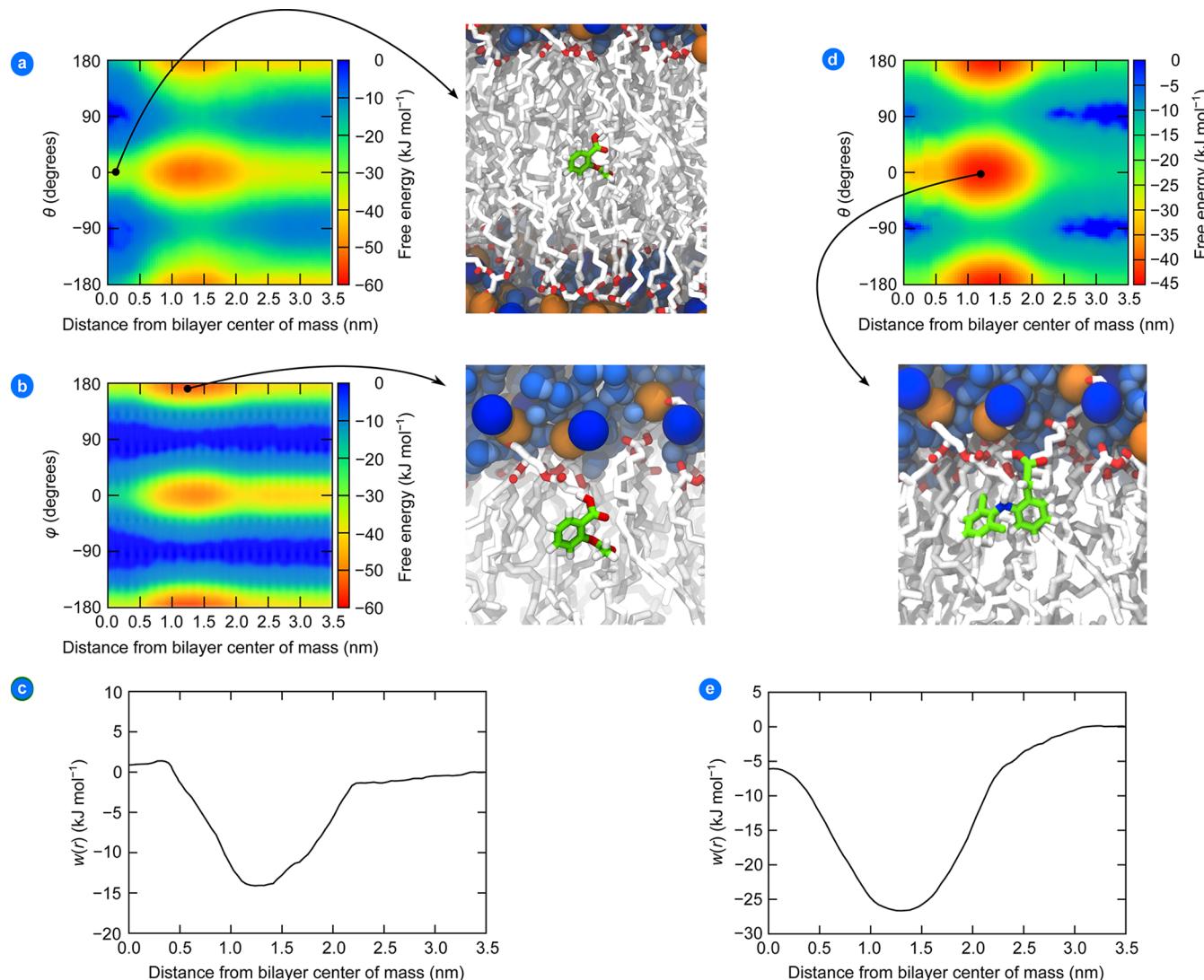
After the analysis of the conformational preferences of the solutes in different solvents, simulations of the three drug compounds in water/membrane systems were performed. The solutes were placed in simulation boxes consisting of 64 DOPC lipids hydrated by 50 water molecules/lipid. As sampling a FES with two or three CVs is considerably more time-consuming compared to sampling only one CV, the multiple walkers (MPWs) metadynamics method<sup>43</sup> was used in order to achieve faster convergence together with the WT metadynamics scheme.<sup>42</sup> In the MPW metadynamics method, a number of simulations are run in parallel, where the initial configurations of the systems can be either identical or different. A position-dependent bias in the form of a Gaussian function is added to force the system to sample other regions of phase space that were less sampled previously, and a walker (or replica) of the system feels contributions to the bias from the other walkers. This speeds up the sampling significantly and offers a way to sample the complex FESs efficiently. Consequently, 10 walkers were used for aspirin and diclofenac and five for ibuprofen. All walkers started from different configurations along all CVs and velocities. Since we employ this procedure in conjunction with WT metadynamics, the setup differs from the original implementation of metadynamics;<sup>11</sup> the bias potential is decreased as the simulation time increases according to the algorithm presented by Barducci et al.<sup>42</sup> so that the bias potential eventually converges to the free energy value. First, the simpler case with two CVs is discussed before the cases with three CVs. In Figure 2 the results from the 2D metadynamics sampling for ibuprofen are presented. In accordance with Figure S2, there are sharp barriers between the *cis* and *trans* conformations of the hydroxyl hydrogen with respect to the carbonyl oxygen in water, with a more stable *trans* conformation. Once the solute is transferred under the surface of the membrane, at a distance between 1.0 and 1.5 nm, three minima are observed. At this depth, the lowest free energy is still in the *trans* conformation, and this can be rationalized by the hydrogen bond formed by the esters of the lipids and the carboxylic group of ibuprofen. At this depth, the polar head of the solute can form these favorable interactions, whereas the hydrophobic part can partly be buried by the aliphatic lipid tails. At distances closer to the membrane center, the transition from *trans* to *cis* conformation must occur, in agreement with the predictions earlier made from the simulations with bulk solvents. Without the conformational change, the barrier for ibuprofen to cross the membrane is overestimated. In Figure 2b, the 2D FES is projected to a 1D PMF by integrating the Boltzmann factor over the angular DOF according to



**Figure 2.** Free energy landscape of ibuprofen embedded in a lipid bilayer (a) and the 1D projection of the free energy landscape compared to regular 1D US. *cis* and *trans* stand for the carboxyl acid conformation; see text for more information. In the illustrations of the simulated systems, the nitrogen and phosphorus of the lipids are rendered as blue and orange spheres, respectively, the hydrophobic tails are in white, and the solute is in green.

$$-\beta w_\theta(z) = \ln \frac{\int e^{-\beta w(\theta, z)} d\theta}{\int e^{-\beta w(\theta, z)} d\theta dz} \quad (1)$$

where  $\beta = 1/k_B T$  and  $k_B$  is the Boltzmann constant and  $T$  is the absolute temperature. The result from this Boltzmann projection is compared to a 1D US simulation that was performed with different conformations of the carboxyl group. The differences are striking toward the center of the membrane, and it is clear that the PMF obtained from the 2D sampling describes the process of transfer properly. One aim of molecular simulations is to predict membrane permeability, not only membrane partitioning.<sup>6</sup> The permeability coefficient  $P$  (or rather the resistance to permeation) is usually calculated according to



**Figure 3.** 2D FES for aspirin as a function of the torsional angle  $\theta$  and distance between the solute and the lipid bilayer (a) and the 2D free energy landscape with the torsional angle  $\psi$  and distance between the solute and the bilayer (b); projected 1D PMF for transferring aspirin (c); 2D FES for aspirin with the angle  $\theta$  and distance as CVs (d); 1D projection of the 3D FES for diclofenac (e).

$$\frac{1}{P} = \int_{z_1}^{z_2} \frac{e^{\beta w(z)}}{D(z)} dz \quad (2)$$

where  $D(z)$  is the position-dependent diffusion coefficient. As the inverse Boltzmann factor appears in the nominator, the difference between the PMFs in terms of permeability would be striking (if we assume that  $D(z)$  is the same for all the conformations, which is very likely). Assuming that  $D(z)$  does not change with the conformations, the simulation starting with a *trans* conformation would underestimate the permeability in the center of the membrane by a factor of roughly 52 compared to the more accurate metadynamics results.

For aspirin and ibuprofen, we cannot illustrate the resulting FESs, as these would be four-dimensional (4D) functions. Instead, the FESs are integrated over the Boltzmann factor of a selected DOF as in the case of ibuprofen. In Figure 3a,b,d, these 3D FESs are shown as heat maps. As with ibuprofen, the hydroxyl groups of both aspirin and diclofenac have to change conformation if the lowest free energy path is followed from water to the center of the membrane. For both molecules, this transition has to occur below the glycerol region in the

membrane. Thus, the 1,4 interaction between the hydroxyl hydrogen and the carbonyl oxygen is favored in the membrane middle due to the low polarity of the environment. Both conformations of the carboxyl group are more or less equivalent for both compounds at a distance of 1.25 nm from the bilayer center. Again, these findings are in agreement with the conformational behavior predicted from the metadynamics simulations in bulk solvents. In Figure 3c,e, the projected 1D PMFs are shown, and the shapes are similar between all the studied compounds. The clearest differences can be seen in the center of the membrane, where diclofenac shows the highest lipophilicity, followed by ibuprofen and aspirin. This trend is in agreement with experimental standard binding free energies (see below) and the hydration free energies, for which ibuprofen has the highest and aspirin the lowest. In agreement with earlier experimental and computational studies performed on anesthetics, the binding mode is identified just below the headgroup in the vicinity of the polar ester groups.<sup>13,25,26,44,45</sup>

For practical reasons, it is cumbersome to assess the accuracy of the presented FESs, and without very detailed experimental information about binding modes of the solutes, it is often

impossible. The standard binding free energy ( $\Delta G_{\text{bind}}^{\circ}$ ) can be determined from experiments where the partitioning of solutes between water and the membrane is studied, and this property can serve as an indirect measurement of the PMF accuracy. The standard binding free energies from the 2D and 3D FESs presented in the present study can be calculated according to

$$e^{-\beta \Delta G_{\text{bind}}^{\circ}} = \frac{\int_B e^{-\beta w(z)} dz}{\int_U e^{-\beta w(z)} dz} \quad (3)$$

where state  $B$  is defined as  $z \in [0.0, 3.2]$  and  $U$  is  $z \in [3.2, 6.4]$ , corresponding to the bound and unbound states, respectively. The intervals for  $z$  were chosen according to their projected 1D PMFs, at distances between 0 and 3.2 nm from the bilayer  $w(z) \neq 0$  and at distances beyond 3.2  $z = 0$ , meaning that the solutes do not interact with the membrane at distances greater than this. In Table 1 computed  $\Delta G_{\text{bind}}^{\circ}$  values are compared to

**Table 1. Standard Binding Free Energies ( $\Delta G_{\text{bind}}^{\circ}$ ) in kJ mol<sup>-1</sup> for the Studied Compounds Compared to Experiments<sup>a</sup>**

solute	method	computed	experiment
aspirin	3D metadynamics	$-12.0 \pm 1.2$	$-14.2^{47}$
diclofenac	3D metadynamics	$-22.2 \pm 1.8$	$-25.8^{48}$
ibuprofen	2D metadynamics	$-19.2 \pm 1.7$	$-22.0^{48}$
ibuprofen	1D US ( <i>cis</i> )	$-19.5 \pm 1.8$	
ibuprofen	1D US ( <i>trans</i> )	$-17.5 \pm 1.6$	

<sup>a</sup>For the US simulations, the conformation of the hydroxyl group is also stated.

available experimental work. The experimental data reported here were collected in acidic environments so that the carboxylic groups were protonated, making the comparison between simulations and experiments straightforward. Previous work has shown that the protonation state of carboxylic groups can drastically alter the binding of small solutes.<sup>16,46</sup> The agreement with experiments is good, albeit not excellent, but the errors should be assigned to the force field parameters, not the methodology, and possible errors could be the lack of explicit polarization effects that have been shown to be important when studying drug-membrane partitioning.<sup>15</sup>

The difference between simulations and experiments is around  $1k_{\text{B}}T$ , which can be considered to be small, and is about the accuracy one can expect from free energy calculations performed with current, generic force fields.<sup>2</sup> It is likely that if one or more important DOFs would have been neglected in the enhanced sampling scheme, the resulting standard binding free energies could have been in worse agreement with experiments. The opposite is also plausible: with the lack of sampling and relaxation of important DOFs, better agreement with experiments could have been obtained; however, these findings would be based on simulations that do not describe the translocation of the solutes in a fully realistic manner. This is illustrated by the values obtained from 1D US simulations for ibuprofen in Table 1. Any results regarding accuracy of the force field or even the applied methodology obtained from simulations could in the worst case scenario be nonconclusive. Moreover, if different binding modes are found when certain DOFs are neglected from the biased sampling compared to when they are considered, one has to be very cautious when analyzing and rationalizing the simulations. The convergence of the free energy profiles should also be mentioned, as it has been shown

that US simulations can take a considerable amount of time to converge.<sup>24,26</sup> In the present investigation, the convergence was tested by computing the free energy difference between two points along the projected 1D reaction coordinate, namely, when the solute is positioned in the middle of the membrane and in bulk water. This free energy difference was calculated in intervals of 10 ns during the biased simulations and plotted against time in order to illustrate the convergence. The results are shown in Figure S3 (SI) and clearly indicate that the simulations performed have converged properly.

To summarize the findings presented in the current Letter, we have performed the most detailed free energy calculations of solutes embedded in lipid bilayers, to the best of our knowledge, to date. The consequences of not identifying DOFs orthogonal to the trivial reaction coordinate along the membrane normal when studying membrane partitioning of solute showed that the resulting free energy profiles can lead to different conclusions regarding, e.g., the permeability of solutes. We also believe that in certain cases, the wrong binding modes can be identified due to the lack of sampling, which constitutes a problem of even larger proportions. Initial simulations of the solutes in bulk solvents that represent the different parts of the biphasic system important DOFs can be identified, and the results presented here show that obstacles could be circumvented by including them in the reaction coordinate. It should be mentioned that additional work is required to investigate these effects on a broader spectrum of compounds with different functional groups and a larger conformational freedom, e.g., it would be highly interesting to apply this simple analysis to Wimley–White pentapeptides,<sup>49</sup> which have been identified as difficult to simulate with atomistic models.<sup>31</sup> Further, it would be interesting to include DOFs regarding the lipids while studying partitioning phenomena. The current state of free energy methods and available computational power illustrate that they have matured to a point where the FES for complicated translocation processes can be studied in great detail. It is our belief that the findings presented here could be of use in rational drug design, toxicity studies, and for designing new reaction coordinates for more accurate free energy calculations.

## ASSOCIATED CONTENT

### S Supporting Information

Details regarding the simulations and verification of force field parameters, free energy surfaces for the solutes in water and *n*-hexane, and convergence plots for all three compounds. This material is available free of charge via the Internet at <http://pubs.acs.org/>.

## AUTHOR INFORMATION

### Corresponding Author

\*E-mail: joakim.jambeck@mmk.su.se or jambeck@me.com (J.P.M.J.); alexander.lyubartsev@mmk.su.se (A.P.L.).

### Notes

The authors declare no competing financial interest.

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Computing Center North (HPC2N), and National Supercomputer Centre (NSC).

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