Comment on "Free energy simulations of single and double ion occupancy in gramicidin A" [J. Chem. Phys. **126**, 105103 (2007)]

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In a recent article, Bastug and Kuyucak[1] investigated the microscopic factors affecting double ion occupancy in the gramicidin channel. The analysis relied largely on the one-dimensional potential of mean force (PMF) of ions along the axis, z, of the channel (the so-called free energy profile of the ion along the channel axis), as well as on the calculation of the equilibrium association constant of the ions in the channel binding sites K_{eq}. The latter was estimated from the "1D" approximation

$$K_{eq} \overset{\text{Ref. 1}}{=} (\pi R_{rms}^2) \int_{site} dz \; e^{-[w(z)-w_{ref}]/k_B T}, \tag{1}$$

where w_{ref} is the asymptotic value of $w(z)$ in the bulk region and R_{rms} corresponds to the lateral x,y rms fluctuations of the bound ion in the pore (2 Å rms), k_B is the Boltzmann constant and T is the temperature. The 1D PMF $w(z)$ was computed from simulations generated in the presence of a narrow cylindrical restraining harmonic potential, $u(x,y) = k(x^2 + y^2)$. Hence, K_{eq} so defined by the 1D approximation, Eq. (1) depends on u via $w(z)$. The "true" equilibrium binding constant should, however, be independent of any restraining potential.

Such uncontrolled and artificial dependence on the choice of restraining potential is clearly undesirable as it undermines a meaningful comparison of computational results with experiments. The issue cannot be settled by simple convergence tests: monitoring the changes in K_{eq} evaluated from Eq. (1) from longer simulations cannot provide any indication about the validity of the end result. Equation (1) does not provide an *unbiased estimator* of K_{eq}. Therefore, whether or not the simulations converge is irrelevant, in this case, because there is a systematic error that has not been quantified by the authors of Ref. 1.

More generally, the formal reduction of a PMF from three-dimensional (3D) to 1D in order to extract proper statistical mechanical averages is a problem with broad implications for a wide range of computational studies. It is the purpose of this brief communication to address and clarify this issue.

In order to avoid any confusion, we now go over the mathematical steps relating K_{eq} with the 3D and 1D PMFs in detail. Fundamentally, the equilibrium single ion binding constant K_{eq} may be expressed in terms of the 3D single ion PMF, $W(\mathbf{r})$,[2]

$$K_{eq} = \frac{\int_{site} d\mathbf{r} \; e^{-W(\mathbf{r})/k_B T}}{e^{-W(\mathbf{r}')/k_B T}}, \tag{2}$$

where $\mathbf{r}'$ is a chosen reference ion position far away in the bulk where $W(\mathbf{r}')$ is equal to a constant W_{ref}. In practice, computing $W(\mathbf{r})$ in 3D represents a daunting task, and one often considers alternative routes expressing K_{eq} in terms of a 1D PMF, $w(z)$. The transformation from 3D to 1D is unambiguous: it requires integrating out the (x,y), degrees of freedom of the ion (see, for example, Refs. 2–4). To have thermodynamically well-defined nondiverging x,y integrals; however, the definition of the 1D PMF must involve some lateral restriction via the potential $u(x,y)$ [2,4]

$$e^{-w(z)/k_B T} \propto \int dx \int dy \; e^{-[W(\mathbf{r}) + u(x,y)]/k_B T} \tag{3}$$

because the ion becomes unbound once it exits the channel. By definition, $w(z')$ is equal to some constant, w_{ref}, when z' is sufficiently far away in the bulk (N.B., u acts at all z).

Obviously, different choices of restraining potential $u(x,y)$ are possible,[1,3] the fundamental issue being how to properly account for the artificial bias that such a restraint introduces. Let us rewrite Eq. (2) as

$$
\begin{aligned}
K_{eq} &= \frac{\int_{site} d\mathbf{r} \; e^{-W(\mathbf{r})/k_B T}}{\int_{site} d\mathbf{r} \; e^{-u(x,y)/k_B T} \; e^{-W(\mathbf{r})/k_B T}} \; \frac{\int_{site} d\mathbf{r} \; e^{-u(x,y)/k_B T} \; e^{-W(\mathbf{r})/k_B T}}{\int dx' \int dy' \; e^{-u(x',y')/k_B T} \; e^{-W(\mathbf{r}')/k_B T}} \; \frac{\int dx' \int dy' \; e^{-u(x',y')/k_B T} \; e^{-W(\mathbf{r}')/k_B T}}{e^{-W_{ref}/k_B T}} \\
&= e^{\Delta G_{site}/k_B T} \int_{site} dz \left[\frac{\int dx \int dy \; e^{-u(x,y)/k_B T} \; e^{-W(\mathbf{r})/k_B T}}{\int dx' \int dy' \; e^{-u(x',y')/k_B T} \; e^{-W(\mathbf{r}')/k_B T}} \right] \left(\frac{e^{-W_{ref}/k_B T}}{e^{-W_{ref}/k_B T}} \right) \int dx' \int dy' \; e^{-u(x',y')/k_B T} \\
&= e^{\Delta G_{site}/k_B T} \int_{site} dz \; e^{-[w(z)-w_{ref}]/k_B T} S_u.
\end{aligned}
\tag{4}
$$

Here, ΔG_{site} is the free energy cost introduced by the restraint $u(x,y)$ when the ion is in the binding site, and S_u is a constant determined by the potential $u(x,y)$ that has the dimension of a surface area. Equation (4) provides an *unbiased estimator* of K_{eq}, independent of the choice of u. Unfortunately, it is not possible to convert Eq. (4) into a form that is identical to the 1D approximation Eq. (1).

According to Eq. (3), any 1D free energy profile depends on the choice of cylindrical restraining potential u. For this reason, it becomes meaningless to compare the well depths in two different 1D PMFs computed with *different* cylindrical restraining potential. Although K_{eq} calculated from Eq. (4) is mathematically *independent* of the choice of u, $w(z)$ is not. The above analysis also explains why the 1D PMF from Refs. 1 and 3 appear to differ even though the simulations were both generated using the CHARMM force field.[5]

In the study carried out by Allen *et al.*,[3] a steep flat-bottom cylindrical potential of radius $R=8$ Å relative to the channel axis was used for the restraining potential u. An equilibrium dissociation constant of $(0.2-0.3)M$ was calculated for K^+, in good accord with experiment.[3] The development above leading to Eq. (4) makes it clear why S_u is equal to πR^2 in Ref. 3; because R was purposely chosen to be much larger than the lateral fluctuations of the bound ion in the site, the restraining potential u is not felt by the ion in the binding site, which means that $\Delta G_{\text{site}}=0$ identically. Bastug and Kuyucak[1] used a cylindrical harmonic potential to keep the ion along the channel axis. However, rather than use Eq. (4) to extract an unbiased estimate of K_{eq}, they used Eq. (1). This means that their estimates of K_{eq} have an undesirable (and as yet undetermined) dependence on u. While it is possible that Eqs. (4) and (1) yield similar numerical results, this is not obvious *a priori* and would be known only from careful comparison with an unbiased estimator of K_{eq}. Ultimately, the validity of the final results cannot be known from estimates of K_{eq} based solely upon Eq. (1). More important, such fortuitous agreement would depend the choice of u as well as on details of the system, which can only add to the confusion.

Although it may be tempting to regard Eq. (1) as an acceptable 1D approximation, as has been done by Kuyucak and co-workers,[1,6–10] the validity of such an approximation must be established independently, i.e., by using unbiased estimators like Eq. (4) that are properly derived from the expression for K_{eq} in terms of the full 3D PMF, $W(\mathbf{r})$, given by Eq. (2). Simply postulating that $w(z')=0$ in the bulk does not suffice to eliminate the dependence on the chosen restraining potential, as suggested by the authors of Ref. 1, because, as shown above, even in that case, the deduced K_{eq} would depend on the choice of restraining potential.

Simulation studies of complex biomolecular systems suffer from the burden of multiple approximations, some of which are arguably a matter of choice and compromise that are partly subjective. In particular, the accuracy of the atomic force field remains a central issue in ion channel simulations. However, there is no ambiguity about the proper theoretical treatments for calculating equilibrium binding constants, either from alchemical free energy perturbations,[11–17] or from integration of an unrestricted[18] as well as a restricted 1D PMF.[2–4,19] It is only by using unbiased estimators of experimentally observable quantities that one can assess the validity of those simulations.

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