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Comment

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Comment on "Probing the Thermodynamics of Competitive Ion Binding Using Minimum Energy Structures"

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We previously developed a theoretical framework to analyze ion selectivity of binding sites in macromolecules.[1] In a recent article,[2] Rogers and Rempe stated in a note added in proof that this framework ignored an important free energy contribution.[1] A closer look shows that this statement is incorrect.

To clarify the issue, let us consider a selective ion-binding site in a protein in equilibrium with bulk solvent. Assuming that ionic species i and j are present in solution, binding selectivity is governed by the relative free energy $\Delta\Delta G_{ij} = \Delta G_{ij}^{\text{site}} - \Delta G_{ij}^{\text{bulk}}$, where $\Delta G_{ij}^{\text{bulk}} = [G_i^{\text{bulk}} - G_j^{\text{bulk}}]$ is the free energy difference between ion i and j in the bulk solvent and $\Delta G_{ij}^{\text{site}} = [\Delta G_i^{\text{site}} - \Delta G_j^{\text{site}}]$ is the free energy difference between ion i and j in the binding site. The relative free energy of ion i and j in the binding site can be written as

$$e^{-\beta\Delta G_{ij}^{\text{site}}} = \frac{\int_{\text{site}} d\mathbf{X}\, e^{-\beta[U_i(\mathbf{X})+\Delta W(\mathbf{X})]}}{\int_{\text{site}} d\mathbf{X}\, e^{-\beta[U_j(\mathbf{X})+\Delta W(\mathbf{X})]}} \tag{1}$$

where $\mathbf{X}$ represents the coordinates of a reduced subsystem comprising the ion and the ligands in a reference frame relative to the protein (no global translation or rotation), U_i and U_j are the ion−ligand and ligand−ligand interactions for the system with ion types i and j, respectively, and ΔW is an effective potential of mean force (PMF) that incorporates all the influence of the rest of the system (protein, membrane, and solvent) on the local binding site subsystem. To an excellent approximation, ΔW does not depend on the ion type i and j if the charge of the ion does not change (i.e., long-range effects cancel out). Equation 1, which is apparently not disputed by Rogers and Rempe, provides a compact and valid representation of the ion selectivity problem of a binding site in terms of the three fundamental ingredients: U_i, U_j, and ΔW. This is our starting point. In ref 1, our strategy was to reason directly from the properties of the ion-independent PMF by introducing a conceptual separation of ΔW into two components of different character (see below). This enabled us to delineate two idealized sets of conditions supporting ion selectivity. In the following, the framework is introduced following a different route that makes it easier to compare with that of ref 2.

First, we introduce the ion-independent confining step-function $H_c(\mathbf{X})$, which is equal to 1 for a subset of configuration and zero otherwise. The choice of the step-function will be specified later, but the following development is of general validity. We write the relative free energy of ions of type i and j in the binding site as

$$\Delta G_{ij}^{\text{site}} = -k_B T \ln\left[\frac{\langle H_c\rangle_j}{\langle H_c\rangle_i}\right] + \Delta G_{ij}^{\text{r}} + \Delta G_{ij}^{\text{c}} \tag{2}$$

where the average of the ion-dependent confinement function is defined as

$$\langle H_c\rangle_i = \frac{\int d\mathbf{X}\, H_c e^{-\beta[U_i+\Delta W]}}{\int d\mathbf{X}\, e^{-\beta[U_i+\Delta W]}} \tag{3}$$

with a similar expression for $\langle H_c\rangle_j$, and the relative free energies are

$$\Delta G_{ij}^{\text{c}} = -k_B T \ln\left[\frac{\int d\mathbf{X}\, H_c e^{-\beta U_i}}{\int d\mathbf{X}\, H_c e^{-\beta U_j}}\right] \tag{4}$$

and

$$e^{-\beta\Delta G_{ij}^{\text{r}}} = \left[\frac{\int d\mathbf{X}\, H_c e^{-\beta[U_i+\Delta W]}}{\int d\mathbf{X}\, H_c e^{-\beta U_i}}\right] \times \left[\frac{\int d\mathbf{X}\, H_c e^{-\beta[U_j+\Delta W]}}{\int d\mathbf{X} H_c\, e^{-\beta U_j}}\right]^{-1} \tag{5}$$

The significance of the various terms will be discussed below.

So far, the formulation is general, and a wide range of mathematical forms could possibly be used for the ion-independent confinement step-function $H_c(\mathbf{X})$. To pick a particular choice for the ion-independent function $H_c(\mathbf{X})$ that will be useful for our analysis, we are motivated by the goal of clarifying the role of architectural forces on ion selectivity. For this purpose, we defined the architectural confinement for each atom k in the system as the *smallest possible spherical volume* V_k that encompasses all the dynamic excursions of that atom in the reference frame of the protein (no global translation/rotation) whether an ion of type i or j is bound in the site. Thus, a unique volume V_k for each atom k is determined by combining the information with an ion of type i or j in the binding site. In practice, the radius of the volumes V_k from all-atom MD trajectories with Na^+ or K^+ ion in the binding site is typically on the order of 1.0−1.5 Å; see the Supporting Information in ref 1. This simple choice defines a minimal default model with a probability distribution that incorporates the generic effect of architectural confinement by the surrounding protein structure in an idealized fashion (without the protein, the volume V_k

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would be unbounded and the ligands would be allowed to disperse in space). This specific choice for $H_c(\mathbf{X})$, valid for Na^+ and K^+, allows us to uniquely and unambiguously define the function $\Delta W_c(\mathbf{X}) \equiv -k_B T \ln[H_c(\mathbf{X})]$. We have referred to the latter as the "confinement" component of $\Delta W(\mathbf{X})$. The remainder, defined unambiguously by $\Delta W_g \equiv \Delta W - \Delta W_c$, was referred to as the "geometry" component. It follows that eq 1 can be rewritten as

$$e^{-\beta \Delta G_{ij}^{site}} = \frac{\int_{site} d\mathbf{X}\, H_c e^{-\beta[U_i(\mathbf{X}) + \Delta W_g(\mathbf{X})]}}{\int_{site} d\mathbf{X}\, H_c e^{-\beta[U_j(\mathbf{X}) + \Delta W_g(\mathbf{X})]}} \quad (6)$$

(the identity $H_c H_c = H_c$ was used). This is eq 8 from ref 1.

This formulation provided a simple and unambiguous prescription to construct reasonable idealized generic reduced subsystems from the information extracted from all-atom MD simulations of complete biomolecular systems. According to the above prescription, $\langle H_c \rangle_j = \langle H_c \rangle_i = 1$ identically, by construction, and

$$\Delta G_{ij}^{site} = \Delta G_{ij}^{c} + \Delta G_{ij}^{r} \quad (7)$$

The quantity ΔG_{ij}^{c} given by eq 4 is the relative free energy governing ion selectivity arising in the subsystem confined by the function $H_c(X)$, while ΔG_{ij}^{r} accounts for the remaining (r) architectural forces from the surrounding protein. An important observation arising from this analysis is that selectivity can actually be realized via two extreme idealized mechanisms. The first case corresponds the "snug-fit" ideas familiar in host−guest chemistry in which the binding site provides a cavity of suitable size to fit one ion but is unable to adapt to other ions. Selectivity is supported by architectural forces that enforce the local geometry of the site (ΔG_{ij}^{r} is not negligible). The relative free energy ΔG_{ij} is roughly equal to the difference in the mean ion−ligand interaction energy. However, the framework also helped explain how binding sites that are inherently flexible can also display robust ion selectivity.[9] We refer to this situation as the "confined microdroplet" limit; ion selectivity emerges spontaneously at the level of the generic confinement without architectural forces imposing a precise geometry. In this case, ΔG_{ij}^{r} is negligible and ΔG_{ij}^{c} provides the dominant contribution to the relative free energy governing ion selectivity. The relative free energy of ion i and j is given, to a good approximation, by the difference in the mean ion−ligand and ligand−ligand interactions: $\Delta G_{ij}^{site} \approx \langle U_i \rangle_i - \langle U_j \rangle_j$. The trends in the confined microdroplet limit can be robust and general; in an analysis of 1077 simplified reduced models comprising typical molecular groups, 39% displayed a nontrivial selectivity in the confined microdroplet limit (i.e., $|\Delta\Delta G_{ij}| \geq 2.0$ kcal/mol).[9]

The theoretical formulation of Rogers and Rempe also relies on confinement step-functions, so-called indicator functions $I(\mathbf{X}; C_n)$, where C_n corresponds to the subset of allowed configurations of the system.[2] However, despite some similarities, there are also some important differences. In particular, the indicator functions introduced by the authors correspond to *external constraints* imposed on the system to artificially restrict its configurations. The choice of constraints narrowly centered on ion-specific energy minimum coordination states was made to enable the effective use of the harmonic oscillator approximation of primitive quasichemical theory (pQCT) that is adopted by the authors to evaluate the I-constrained free energy ΔG_{ij}^{I} (equivalent to eq 4). Of course, such external constraints introduce a bias. Nevertheless, it is well understood that valid unbiased free energies can still be obtained from computations incorporating as long as the effects of the constraints are properly accounted for. Biasing constraints and restraints are very common in the developments of binding free energy simulation methods; for example,[3−6] see ref 7 for a review. Thus, Rogers and Rempe correctly emphasized the importance of the free energy contribution arising from the configuration constraint associated with the indicator functions, $-k_B T \ln[\langle I \rangle_j / \langle I \rangle_i]$. Indeed, the magnitude of the latter is likely to be significant because the averages $\langle I \rangle_i$ and $\langle I \rangle_j$ are expected to have different values and be much smaller than 1 in the case of narrowly defined indicator functions. It is apparently within this context that our previous analysis was suggested to be incorrect because it did not include a similar free energy contribution from constraints. In this regard, however, it seems that there has been confusion. As shown above, rather than impose *external constraints* onto the system, our strategy has been to separate the PMF into two contributions, $\Delta W \equiv \Delta W_c + \Delta W_g$, to then delineate how two idealized sets of conditions can support ion selectivity. Contrary to the statement made by Rogers and Rempe in their Reply, there is nothing problematic with the concept of separating a PMF into two unambiguously defined components and using this construct for further analysis.

While one could "agree to disagree" on the relative merit of two different approaches, the Reply of Rogers and Rempe to this Comment adds little to clarify why there remains any confusion. For example, the assertion that the free energy contribution from confinement was ignored in our analysis, repeated in the Reply to this Comment, is plainly incorrect.[1] The free energy contribution $-k_B T \ln[\langle H_c \rangle_j / \langle H_c \rangle_i] = -k_B T \ln[1]$, is rigorously equal to zero *by construction* of $H_c(\mathbf{X})$; the set of spherical volumes V_k based on the maximum fluctuations of each atom k extracted from all-atom MD simulations used to define the architectural confinement were given as Supporting Information in ref 1. There seems to be more confusion about the construction of the geometry contribution, ΔW_g. In ref 1, we used a variational prescription based on a cross-entropy variational formulation previously introduced by Pratt and coworkers.[8] As explained in the Supporting Information of ref 1, this variational procedure could be carried out to any order to match ΔW_g exactly, thereby yielding a total PMF that is rigorously equivalent to the quantity ΔW appearing in eq 1. As the validity of eq 1 is not in dispute, all the results ensuing from the exact PMF, $\Delta W_c + \Delta W_g$, would also be exact. However, for the sake of simplifying the discussion, ΔW_g was truncated to lowest order and its magnitude expressed in terms of a single parameter, λ_g, related to the average quadratic fluctuations of the atoms in the binding site. This simplification allowed us to draw a fundamental and novel observation about the existence of two idealized mechanisms supporting ion selectivity: the snug-fit ($\lambda_g \to \infty$) and the confined microdroplet ($\lambda_g = 0$). While such a first order approximation to ΔW_g would be exact only if the remainder was truly harmonic, the real issue is whether our fundamental conclusion would be invalidated if we had used a higher order approximation for ΔW_g. Rogers and Rempe provide no indications suggesting that this is the case.

Ultimately, the goal of either of these approaches is to better evaluate the relative importance of ion−ligand and ligand−ligand interactions, $U_i(\mathbf{X})$ and $U_j(\mathbf{X})$, and architectural forces, $\Delta W(\mathbf{X})$, on the selectivity of ion binding sites in macromolecules. The framework formulated by Rogers and Rempe with narrowly defined indicator functions seeks to map reduced

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models onto well-defined energy minimum coordination states and enable the application of pQCT-like harmonic approximations.[2] We sought to design a theoretical framework to help clarify the construction of reduced binding site models from the information provided by the detailed MD simulations. While the approach of Rogers and Rempe is valid, it has not yet been applied to any large and complex membrane protein system. It has only been illustrated with simplified reduced models (constrained water droplets), and difficulties with binding sites in macromolecules may be anticipated.[10,11] In contrast, our framework has already been used to analyze the underlying mechanism of ion selectivity in the S2 binding site of the KcsA K^+ channel and the Na1 and Na2 binding sites of the LeuT leucine transporter[1] and to clarify the effect of protonation on the K^+ selectivity of the ion binding sites in the Na/K pump ATPase.[12]

AUTHOR INFORMATION

Notes

The authors declare no competing financial interest.

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