The Role of Bulk Protein in Local Models of Ion-Binding to Proteins: Comparative Study of KcsA, Its Semisynthetic Analog with a Locked-in Binding Site, and Valinomycin

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ABSTRACT In studying ion-selectivity in biomaterials, it is common to study ion-protein interactions within a local neighborhood around the ion. This local system analysis for the S_2 site of KcsA, its semisynthetic analog, and valinomycin yields the free energy change in exchanging K^+ with Na^+ in quantitative agreement with the value obtained by considering ion-interactions with the entire system. But the energetics of ion binding in the local system and in the entire system differ significantly and lead to different conclusions regarding the physical basis of ion selectivity. For configurations sampled from an all-atom simulation, we show that the selectivity free energy can be decomposed into a contribution arising from interactions of the ion with its local neighborhood, $\Delta\mathcal{W}_{\text{local}}$, and a term arising from the field imposed on the ion and the binding site by the rest of the medium, $\Delta\mathcal{W}_{\phi}$. The local contribution $\Delta\mathcal{W}_{\text{local}}$ is numerically close to the actual free energy difference because the field contribution is small. The field contribution is small because of cancellation of inversely related ion-medium and site-medium interactions. Our analysis presents a rigorous foundation for the numerical success of the local system analysis and shows that its implications do not always hold for the entire protein.

INTRODUCTION

Association of metal ions with protein binding sites is crucial in catalysis, structural stability, and metal transport. Metal-protein interactions are usually highly specific, with proteins selectively binding some metal ions over other competing ions in the medium. For example, in potassium channels, the selectivity filter excludes Na^+ over K^+ (1) and in the zinc-finger system, the peptide selectively binds Zn^{2+} over Co^{2+} or Fe^{2+} (2). In these examples, the selectivity free energy, $\Delta\mu^{\text{ex}}$, is the thermodynamic metric of the preference of one metal ion over another. Specifically, the selectivity for a metal ion, 1, over any other competing ion, 2, is

$$\begin{aligned}\Delta\mu^{\text{ex}} &= \left[\mu_2^{\text{ex}}(\text{S}) - \mu_2^{\text{ex}}(\text{aq})\right] - \left[\mu_1^{\text{ex}}(\text{S}) - \mu_1^{\text{ex}}(\text{aq})\right] \\ &= \left[\mu_2^{\text{ex}}(\text{S}) - \mu_1^{\text{ex}}(\text{S})\right] - \left[\mu_2^{\text{ex}}(\text{aq}) - \mu_1^{\text{ex}}(\text{aq})\right] \quad . \quad (1) \\ &\equiv \Delta\mu^{\text{ex}}(\text{S}) - \Delta\mu^{\text{ex}}(\text{aq})\end{aligned}$$

The excess free energy of hydration is $\mu_X^{\text{ex}}(\text{aq})$ (X=1 or 2) and μ_X^{ex} (S) is the corresponding quantity in the binding site. In Eq. 1, it is understood that a common reference state is used both in the aqueous phase and in the biomaterial. The free energy change in the aqueous phase, $\Delta\mu^{\text{ex}}(\text{aq})$, is an important factor in selectivity (Eq. 1), but is a constant independent of the protein. Therefore, in this study we concentrate on $\Delta\mu^{\text{ex}}(\text{S})$, the free energy of exchanging the two ions in a protein-binding site.

The strength of interaction of a metal ion with the residues comprising the binding site is substantially strong on a thermal energy scale compared to its interactions with the surrounding protein material. This naturally encourages one to study the thermodynamics based on the local ion-medium interactions. Indeed, in a quasichemical organization of the potential distribution theorem (3–6), one takes advantage of this separation of energy scales by treating the local site in atomic detail, including at a quantum chemical level, as part of an extended system that is usually described by empirical potentials or by effective fields.

In this article, we investigate the approaches in which the outer-medium either is ignored entirely or else is described by effective fields that cannot capture the effects that arise due to dynamical correlations between site and medium atoms. Studies where one considers an energy-minimum ion-site cluster in a dielectric, or a cluster that is artificially restrained by harmonic forces, belong to this category. We identify these as "local models". Our analysis deals with K^+-over-Na^+ selectivity in the S_2 binding site in the selectivity filter (1) of the wild-type KcsA, its semisynthetic analog with glycine to d-alanine mutation at residue 77 (KcsA-G77A_D from here onwards) that locks the binding site in a conductive conformation (7), and valinomycin (8–10), a small ionophore (Fig. 1).

The thermodynamics of ion selectivity in potassium channels has been extensively probed using local models (11–18). In these studies, the outer medium is treated in one of four ways: 1), it is entirely neglected (15); 2), it is treated as a dielectric (14,16); 3), the outer-medium's role in restraining the local cluster is described by harmonic

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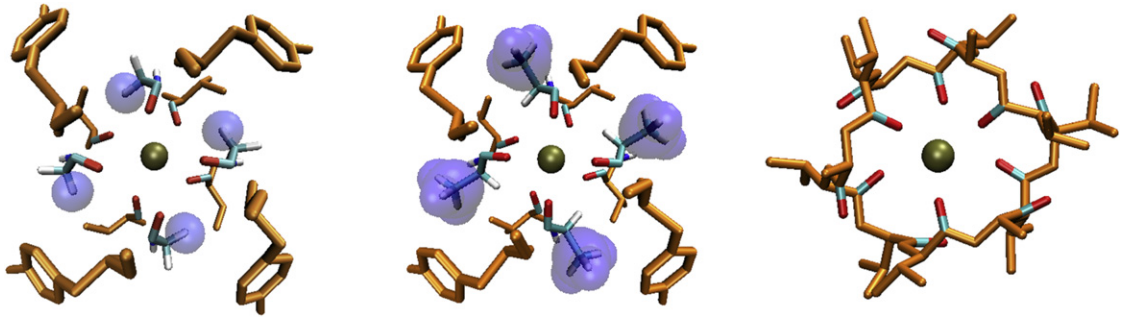

FIGURE 1 S_2 binding site of the wild-type KcsA (*left*), its semisynthetic analog, KcsA-G77A_D, with a glycine-to-d-alanine mutation at residue 77 (*middle*), and the binding site of the cyclopeptide valinomycin (*right*). The molecular surfaces of the side chains in KcsA and KcsA-G77A_D are shown; note that the d-alanine side chains (*center panel*) prevent the channel from acquiring a nonconductive conformation in the absence of K^+ (7).

forces (13,15); or 4), the outer-medium's role as a container is described by a suitable potential (11,12,18). In one of these studies (17), the outer-medium comprised all atoms outside the local site. Despite the varying choices for the field of the protein medium on the atoms comprising the local model, all these studies implicitly rely on the reproduction of the selectivity free energy as validation for the chosen local model. Subsequent discussions of the physical principles of selectivity are based on the validated local model itself.

For the systems noted above, we calculate the selectivity free energy by considering interactions between the ion and its predefined local neighborhood for configurations obtained from an all-atom MD simulation of the entire system. The local model defined to comprise the carbonyl groups in the binding site does indeed reproduce the selectivity free energy based on considering ion interaction with the entire system. Analysis of the binding energies shows that ion-medium and site-medium interactions are uncorrelated and independent of ion-type in the S_2 site of the wild-type KcsA (17), but not so for KcsA-G77A_D and valinomycin, where Na^+ interacts more favorably than K^+ with the medium outside the carbonyl groups defining the local site. Thus, in KcsA-G77A_D and valinomycin, one reaches different conclusions about the physical principles of selectivity depending on whether one considers only local ion-carbonyl interactions or all ion-system interactions.

To understand the role of the protein medium on determining the free energy of ion exchange, we formally decompose the ion-protein interaction into a local and a long-range contribution. We then show that the selectivity free energy, $\Delta\mu^{ex}(S)$, can be decomposed into a contribution, $\Delta\mathcal{W}_{local}$, that depends only on the interaction of the metal ion with the binding site, and a field contribution, $\Delta\mathcal{W}_{\phi}$, that arises from the ion interaction with the material outside the defined local model. The local models developed above implicitly assume that the field contribution is independent of ion-type. Our analysis shows that the local model appears as a good mimic in reproducing the free energy of ion exchange in the real system because of cancellation of inversely related ion-medium and site-medium interactions. A proper accounting of all interactions is shown to be vital to a correct identification of the physical basis of ion-selectivity.

The rest of the article is organized as follows. In the next section, we present Theory. In Methods, we briefly describe the simulation protocol, followed by Results and Discussion, and in the last section, we present our Conclusions.

THEORY

Potential distribution theorem and free energy decomposition

We use the potential distribution theorem (5,19) to understand the role of local and long-range effects in the thermodynamics of the ion in the protein site. The potential distribution approach can be framed in two complementary and related ways (5,6,13). In the forward approach, we imagine the protein sampling configurations in the absence of the ion in the site, whereas in the inverse approach, the protein configurations are sampled in the presence of the ion. The forward approach is more intuitive when we take an ion-centric perspective on selectivity, whereas the inverse approach is more helpful in understanding selectivity from the perspective of the ion-binding site (17). We pursue the inverse approach here.

The excess chemical potential, $\mu_i^{ex}(S)$, of the ion i in the site S is given by

$$\beta\mu_i^{ex}(S) = \log\int e^{\beta\varepsilon}P_i(\varepsilon)d\varepsilon = \log\langle e^{\beta\varepsilon}\rangle_i, \qquad (2)$$

where $P_i(\varepsilon)$ is the distribution of the interaction energy ε of the ion $i = 1, 2$ with the protein when the ion and the protein are thermally coupled (indicated by the subscript i). As usual, $\beta = 1/k_BT$, where T is the temperature and k_B is the Boltzmann constant. Equation 2 suggests that configurations that are important in understanding the thermodynamics of the ion are also the ones for which the ion-protein interaction is least favorable. As we argued recently (17), these configurations are also the ones for which the site is least energetically strained.

Formally we can write the potential energy $\mathcal{U}$ of the ion-protein system as

$$\mathcal{U} = \varepsilon_{\text{local}} + \varepsilon_{\text{m}} + U_{\text{s}} + U_{\text{m}} + U_{\text{s-m}}, \tag{3}$$

where U_{s}, U_{m}, and $U_{\text{s-m}}$ are, respectively, the site-site, medium-medium, and site-medium interactions. The ion-protein interaction energy ε is parsed into a local and a long-range contribution: $\varepsilon_{\text{local}}$ is the local ion-site interaction energy and ε_{m} is the remaining long-range, ion-medium contribution. Thus Eq. 2 is

$$\left\langle e^{\beta\varepsilon}\right\rangle_i = \frac{\int e^{\beta\varepsilon} \bullet e^{-\beta\mathcal{U}_i} d\mathcal{R}^n}{\int e^{-\beta\mathcal{U}_i} d\mathcal{R}^n}, \tag{4}$$

where $\mathcal{R}^n = \{\mathcal{R}_{\text{s}}, \mathcal{R}_{\text{m}}\}$. The values $\mathcal{R}_{\text{s}}$ are the coordinates of the site and the ion whereas the values $\mathcal{R}_{\text{m}}$ are the coordinates of the protein medium.

We identify the local contribution to the free energy by

$$\left\langle e^{\beta\varepsilon_{\text{local}}}\right\rangle_i = \frac{\int e^{\beta\varepsilon_{\text{local}}} \bullet e^{-\beta\mathcal{U}_i} d\mathcal{R}^n}{\int e^{-\beta\mathcal{U}_i} d\mathcal{R}^n} \tag{5}$$

and a field (ϕ_i) contribution by

$$\left\langle e^{\beta\varepsilon_{\text{m}}}\right\rangle_{i,\text{m}} = \frac{\int e^{\beta\varepsilon_{\text{m}}} \bullet e^{-\beta(\mathcal{U}_i - \varepsilon_{\text{local}})} d\mathcal{R}^n}{\int e^{-\beta(\mathcal{U}_i - \varepsilon_{\text{local}})} d\mathcal{R}^n}. \tag{6}$$

Combining Eqs. 4–6, we have

$$\left\langle e^{\beta\varepsilon}\right\rangle_i = \left\langle e^{\beta\varepsilon_{\text{local}}}\right\rangle_i \bullet \left\langle e^{\beta\varepsilon_{\text{m}}}\right\rangle_{i,\text{m}}. \tag{7}$$

In obtaining the local contribution (employing Eq. 5), the ion is fully coupled with the site and the medium; that is, the site and the medium reorganize in the presence of the ion, due to their mutual interaction. However, by considering only ion-site interactions in the exponential average, the effect of changes in the protein medium on the reversible work of coupling the ion with the system is ignored. This effect is gathered in the field contribution equation (Eq. 6) that is obtained in an ensemble where the ion interacts with the medium (denoted by subscript m) but not with the site. Such an ensemble can only be constructed in a computer simulation, and of course, it reflects parsing interactions into local and long-range contributions, themselves theoretical constructs. The importance of Eqs. 5–7 lies in revealing the physical features that are hidden when one considers only the local binding site in studying selectivity.

Taking the ratio of Eq. 5 for ions $i = 1, 2$, we obtain the local contribution to the free energy change, $\Delta\mathcal{W}_{\text{local}}$,

$$\Delta\mathcal{W}_{\text{local}} \equiv k_{\text{B}}T \log \frac{\left\langle e^{\beta\varepsilon_{\text{local}}}\right\rangle_2}{\left\langle e^{\beta\varepsilon_{\text{local}}}\right\rangle_1}. \tag{8}$$

Likewise defining the field contribution by $\Delta\mathcal{W}_\phi$, from Eq. 7 we obtain

$$\Delta\mu^{\text{ex}}(\text{S}) = \Delta\mathcal{W}_{\text{local}} + \Delta\mathcal{W}_\phi. \tag{9}$$

The free energy $\mu_i^{\text{ex}}(\text{S})$ can be written as a sum of mean, $\langle\varepsilon\rangle_i$, and fluctuation, $\mu_{i,\text{ fluc}}^{\text{ex}}$, contributions (17) and a similar decomposition can be sought for $\Delta\mathcal{W}_{\text{local}}$. We thus have

$$\begin{aligned} \Delta\mu^{\text{ex}}(\text{S}) &= \Delta\langle\varepsilon_{\text{local}} + \varepsilon_{\text{m}}\rangle + \Delta\mu_{\text{fluc}}^{\text{ex}} \\ \Delta\mathcal{W}_{\text{local}} &= \Delta\langle\varepsilon_{\text{local}}\rangle + \Delta\mathcal{W}_{\text{local,fluc}} \\ \Rightarrow \Delta\mathcal{W}_\phi &= \Delta\langle\varepsilon_{\text{m}}\rangle + \Delta\mu_{\text{fluc}}^{\text{ex}} - \Delta\mathcal{W}_{\text{local,fluc}}. \end{aligned} \tag{10}$$

The fluctuation contribution to $\Delta\mu_{\text{fluc}}^{\text{ex}}$ is given by

$$\begin{aligned} \Delta\mu_{\text{fluc}}^{\text{ex}} &= \Delta\langle U_{\text{s}} + U_{\text{s-m}} + U_{\text{m}}\rangle - T\Delta s^{\text{ex}}, \\ \Delta\mathcal{W}_{\text{local,fluc}} &= \Delta\langle U_{\text{s}}\rangle - T\Delta s_{\text{local}}^{\text{ex}}, \end{aligned} \tag{11}$$

where Δs^{ex} is the change in the excess entropy in exchanging ion 1 with ion 2 and $\Delta s_{\text{local}}^{\text{ex}}$ is defined as the corresponding local quantity (17). We measure the excess internal energies relative to the state where all the ligands are infinitely apart. If the energies are measured relative to an ion-free binding site, the K^+- and Na^+-bound ensembles acquire a constant energy term corresponding to the energy of the ion-free protein system. These term cancels when we take the difference of the internal energies.

The decomposition of $\Delta\mathcal{W}_{\text{local,fluc}}$ into an internal energy of the site and the rest (here called an entropic contribution) is by analogy to the decomposition of $\Delta\mu_{\text{fluc}}^{\text{ex}}$. We stress that $\Delta s_{\text{local}}^{\text{ex}}$ has other contributions besides the temperature derivative of $\Delta\mathcal{W}_{\text{local}}$ and is thus not strictly a thermodynamic entropy.

We anticipate that ion-medium and site-medium interactions are inversely correlated—i.e., if the ion interacts favorably with the medium, then the site is expected to interact less favorably with the medium. This indeed proves to be true. Equations 10 and 11 then suggest that a small $\Delta\mathcal{W}_\phi$ results from such compensating contributions.

Before closing this subsection, we emphasize some aspects of the above development. Equation 7 involves ion i and its interaction with the protein solely and leads to the conceptually simple relations Eqs. 10 and 11. For example, $\Delta\langle\varepsilon_{\text{local}}\rangle = \langle\varepsilon_{\text{local}}\rangle_2 - \langle\varepsilon_{\text{local}}\rangle_1$ is the difference in the strength of interaction of the respective ion with the site and conveys a clear physical meaning. However, the field contribution is defined in an ensemble that is difficult to implement, for in such a simulation the ion will tend to leave the site because of no ion-site interactions. ($\Delta\mathcal{W}_\phi$ measures the relative propensity for the ions to leave the site.) But we emphasize that the field contribution is never directly computed (see below).

We can avoid the ensemble implied in Eq. 6. For example, the local contribution can be defined as

$$-k_{\text{B}}T \log\left\langle e^{\beta\Delta\varepsilon_{\text{local}}}\right\rangle_1,$$

where $\Delta\varepsilon_{\text{local}}$ is the difference in the binding energy of ion 2 minus ion 1 with the averaging performed in a simulation of the protein with ion 1. The field contribution then is obtained from

$$k_B T \log\left\langle e^{\beta\Delta\varepsilon_m}\right\rangle_2,$$

where Δ has the same meaning as for the local contribution, but the averaging is now performed in a simulation of the protein with ion 2. This requirement of averaging the local and field contributions in ensembles with different ions no longer preserves the conceptual simplicity of Eqs. 10 and 11; for example, $\langle\Delta\varepsilon_{local}\rangle_1$ is not as direct and meaningful as $\Delta\langle\varepsilon_{local}\rangle$ (Eq. 10). For this reason, we do not pursue such developments here.

Finally, note that Eq. 10 is an exact decomposition—the fluctuation contribution defined in Eq. 10 collects all cumulants of order two and greater. The fluctuation contribution reduces to the second cumulant only if the interaction energies are described well by a Gaussian distribution (5,6,16,20).

Calculating free energies

The above development presents a formal and physically transparent accounting of the local and field contributions. However, a direct calculation using Eq. 5 or Eq. 8 can suffer because the high-energy tails of the binding energy distributions may not be well sampled (17). But because we are interested in the free energy change in exchanging one ion with the other, here we obtain a robust estimate of that free energy by a perturbative (stratified sampling) approach. We change the total energy $\mathcal{U}_1$ to $\mathcal{U}_2$ by creating intermediate states with potential energies $\mathcal{U}(j)$, $j = 2, \ldots, n-1$. Here $\mathcal{U}(1) = \mathcal{U}_1$ and $\mathcal{U}(n) = \mathcal{U}_2$. The free energy change is then

$$\begin{aligned}\Delta\mu^{ex}(S) &= -k_B T \log \prod_j \left\langle e^{-\beta(\mathcal{U}(j+1)-\mathcal{U}(j))}\right\rangle_j \\ &= -k_B T \log \prod_j \left\langle e^{-\beta(\varepsilon(j+1)-\varepsilon(j))}\right\rangle_j .\end{aligned} \quad (12)$$

The subscript j denotes that the thermodynamic averaging is performed in the ensemble with potential energy $\mathcal{U}(j)$, and $\varepsilon(j)$ is the interaction energy of the ion with the system in that ensemble. We use Eq. 12 for calculating $\Delta\mu^{ex}(S)$, the same quantity that appears on the left-hand side of Eq. 9.

A local version of Eq. 12 reads

$$\Delta\mathcal{W}_{local} = -k_B T \log \prod_j \left\langle e^{-\beta(\varepsilon_{local}(j+1)-\varepsilon_{local}(j))}\right\rangle_j . \quad (13)$$

The local contribution defined in Eq. 8 and the one calculated with Eq. 13, the local version of Eq. 12, are in principle different; but both are in the same spirit of parsing the ion-system interaction into a local and a long-range contribution. The field contribution, $\Delta\mathcal{W}_\phi$, is always evaluated using Eq. 9 (that is, as a difference between $\Delta\mu^{ex}(S)$ and $\Delta\mathcal{W}_{local}$).

Alternative models

The definition of the local model adopted here is by no means unique. For example, the free energy change of exchanging the ion in a site when both the ion and the site are uncoupled from the medium can be defined as the local contribution. Gas-phase calculations of ion-site clusters (6,15) represent such calculations. Appropriately, the field term will account for all the missing effects such as those due to the reorganization of the site and the medium in the presence of ion-medium and site-medium coupling. Here, for simplicity, we choose a local model in which the ion plus the site are fully coupled with the protein medium. We show that even in this case, where structural effects of the outer medium on the local model are rigorously captured, care is required in inferring the physics of selectivity from results obtained with the local model.

METHODS

We use the CHARMM27 force field (21) and the NAMD v2.6 program (22) for simulations. Construction of the simulation cell is facilitated by VMD (23). Analysis codes were developed in-house.

Simulation system: K^+ channel

The simulation procedure for KcsA-G77A$_D$ follows the procedure for KcsA outlined before (17,24). Water is modeled with the TIP3P potential (25,26). Equilibration and production are conducted at a pressure of 1 bar using a Langevin-piston barostat (27,28) and a temperature of 310 K using a Langevin thermostat. Covalent bonds to hydrogen are held rigid using the SHAKE algorithm (29). The equations of motion are integrated with a 2 fs time step. Lennard-Jones interactions are smoothly switched to zero between 10.215 Å and 10.715 Å. Nonbonded electrostatics are treated using particle-mesh Ewald summation. The grid size for the particle mesh Ewald is always $\leq$0.75 Å.

Valinomycin

Valinomycin is immersed in a bath of 225 ethyl alcohol molecules and equilibrated for 1.5 ns before a production run of 1.5 ns for both Na^+ and K^+. The temperature is 298.15 K and the pressure is 1 bar.

Energy calculations

K^+ is transformed to Na^+ in 20 steps to calculate the free energy change (Eq. 12). Each step comprises an equilibration of 5 ps followed by 20 ps of production. The ion-protein interaction energy is a sum of Lennard-Jones interactions and electrostatics evaluated using Ewald summation. We use in-house codes for the energy calculations except for the energetics of the valinomycin molecule for which we use the mdEnergy plugin within NAMD (22).

To calculate $\Delta\mu^{ex}(S)$, we consider the energetics of the ion with the entire protein and two definitions of the binding site in the case of the K^+ channels. Thus, we consider ion interactions with:

- A_1: All protein and solvent atoms.
- A_2: Eight carbonyl ligands of the S_2 site along with two water molecules in sites S_1 and S_3.
- A_3: Eight carbonyl ligands of the S_2 site (or six carbonyl ligands in the case of valinomycin).

Approaches A_2 and A_3 are the local models of ion-protein interactions. All three approaches yield quantitatively similar results for $\Delta\mathcal{W}_{local}$, yet

differ significantly with respect to $\Delta\langle\varepsilon_{local}\rangle$, for example, and hence also in the physical explanation of selectivity.

RESULTS AND DISCUSSION

Table 1 summarizes the free energies calculated with the three different schemes A_1, A_2, and A_3. Observe that the local contribution to the free energy is almost identical to the free energy change, whereas the ion-binding energies are sensitive to the definition of the binding site. We consider each of the systems below.

Valinomycin

For valinomycin, the six carbonyl ligands around the ion define the local binding site. The small size of the molecule permits an exhaustive accounting of the different contributions in Eq. 3; Table 2 collects the relevant numbers.

For the local model, $\Delta\mathcal{W}_{local} = -11$ kcal/mol (Table 2). Because the free energy change in bulk water is $\Delta\mu^{ex}(aq) =$ –20.7 kcal/mol (17), the selectivity on the basis of the local model is $-11.0 + 20.7 = 9.7$ kcal/mol. Relative to $\Delta\mu^{ex}(aq)$, the local binding energy change $\Delta\varepsilon_{local} =$ –9.4 kcal/mol is unfavorable for Na^+ and the local site is marginally strained in the presence of the smaller ion, $\Delta\langle U_s\rangle =$ –1.1 kcal/mol. Taken together, this suggests that the local, six-carbonyl site is rigid, is unable to interact well with the smaller Na^+, and hence does not compensate the dehydration of Na^+. Interestingly, a study based on energy-minimizing the entire valinomycin molecule also comes to a similar conclusion (16). However, accounting for the dynamical correlation between the local site and the remaining bulk medium leads to an entirely different conclusion.

In contrast to the local binding energy of –9.4 kcal/mol, Na^+ is more favorably bound to the entire molecule, $\Delta\langle\varepsilon\rangle =$ –17.7 kcal/mol (Table 2). In comparison, $\Delta\mathcal{W}_{local}$ differs from $\Delta\mu^{ex}(S)$ by only 0.3 kcal/mol! Selectivity emerges because the favorable interaction of Na^+ with the material outside the binding site cannot compensate for the unfavorable changes in internal energy of the medium, $\Delta\langle U_m\rangle = 11.4$ kcal/mol, and site-medium interactions, $\Delta\langle U_{s-m}\rangle = 10.3$. It is the greater energetic strain in the medium that is responsible for selectivity. The energetic strain in the molecule is not surprising given the observed deformation of the site in the presence of the smaller Na^+ ion (Fig. 2).

The fluctuation contribution to $\Delta\mu^{ex}(S)$ (Eq. 10) is $\Delta\mu^{ex}_{fluc} = 6.4$ kcal/mol; thus in a trend mirroring KcsA (17), a major part of the observed selectivity free energy (of 9.4 kcal/mol) emerges from an unfavorable fluctuation contribution. The difference between valinomycin and KcsA rests in the factors that influence the fluctuation contribution: in KcsA, the fluctuation contribution is determined by the local site properties rather than by changes in the medium (17), whereas in valinomycin the fluctuation contribution is determined by the medium properties rather than by changes in the local site.

Equation 11 and the results in Table 2 thus make it apparent that the energetic strain in the entire molecule is the observed basis of selectivity, a conclusion that is

TABLE 1 Free energy and binding energy for KcsA, KcsA-G77A$_D$, and valinomycin according to three different methods of assessing ion-protein interactions (see Methods)

	A_1	A_2	A_3
KcsA			
$\Delta\mu^{ex}(S)/\Delta\mathcal{W}_{local}$	−14.7	−15.7	−15.8
$\Delta\langle\varepsilon_{local}\rangle$	−20.9	−18.8	−19.6
KcsA-G77A$_D$			
$\Delta\mu^{ex}(S)/\Delta\mathcal{W}_{local}$	−9.0	−10.0	−9.9
$\Delta\langle\varepsilon_{local}\rangle$	−28.3	−19.3	0.8
Valinomycin			
$\Delta\mu^{ex}(S)/\Delta\mathcal{W}_{local}$	−11.3		−11.0
$\Delta\langle\varepsilon_{local}\rangle$	−17.7		−9.4

$\Delta\mathcal{W}_{local}$ is insensitive to these choices and is sufficiently close to the actual free energy change, $\Delta\mu^{ex}(S)$. The local model adequately captures binding energetics in KcsA but not in KcsA-G77A$_D$ and valinomycin. For the latter two systems, Na^+ is stabilized by interactions with medium outside the binding site. Statistical uncertainties in energy values are ~0.8 kcal/mol.

TABLE 2 Energetic decomposition for valinomycin

	$\Delta\mu^{ex}(S)$	−11.3	
	$\Delta\mathcal{W}_{local}$	−11.0	
	$T\Delta s^{ex}$	14.2	
	$T\Delta s^{ex}_{local}$	0.5	
Ion-site	$\Delta\langle\varepsilon_{local}\rangle$	−9.4	−17.7
Ion-medium	$\Delta\langle\varepsilon_m\rangle$	−8.3	
Site-site	$\Delta\langle U_s\rangle$	−1.1	
Site-medium	$\Delta\langle U_{s-m}\rangle$	10.3	20.6
Medium-medium	$\Delta\langle U_m\rangle$	11.4	

Entropic contributions are obtained from the known free energy and mean binding energies (12,17). The last column gives the net binding energy of the ion and the net excess energy of the site and medium. Notation follows the information given in the Theory section (see text). All values are in kcal/mol.

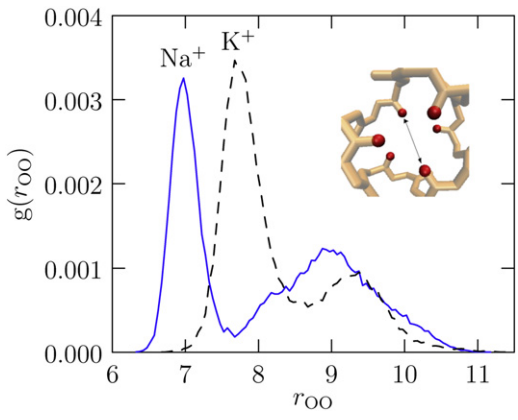

FIGURE 2 Radial distribution of the diagonally opposed (see *Inset*) carbonyl oxygens, $g(r_{OO})$, for valinomycin bound to K^+ and Na^+. Observe that the smaller Na^+ deforms the six-coordinated binding site.

consistent with one reached earlier in Noskov and Roux (12). Finally note that the ion-medium and site-medium interactions tend to balance each other (Table 2) and so do $\Delta\langle U_m\rangle$ and the entropic effects (from Eq. 11). This then leads to a small field contribution.

Our analysis also explains the observation (11,12) that artificially turning off repulsion between the carbonyl ligands in valinomycin does not render it nonselective in contrast to what is observed in the S_2 site of KcsA. Indeed this must be so, because in valinomycin selectivity is also influenced by changes outside the six-carbonyl local site.

KcsA and KcsA-G77A$_D$

For KcsA and KcsA-G77A$_D$, the eight carbonyl ligands forming the S_2 site define the local site. From Table 1, comparing the change in binding energy according to schemes A_1, A_2, and A_3 (see Methods), we find that considering the ion interaction energetics with the two adjoining water molecules accounts for a large fraction of the ion-medium interaction. As only the qualitative role of ion-medium effect is of primary interest here, we treat the two water molecules as the entire medium. (This choice is further discussed below.) Table 3 summarizes the energetics for the KcsA and KcsA-G77A$_D$ systems.

If we consider only the local eight carbonyl model, as in valinomycin, Na^+ does not bind favorably to KcsA-G77A$_D$. Further, the site is somewhat more stable in the presence of Na^+ than with K^+. Again like valinomycin, this suggests that Na^+ is unable to interact well with the site, its dehydration is not well compensated, and the site selects K^+ instead. The distribution of diagonally opposed carbonyl oxygens (13,17,30) does indeed show that the G77A$_D$ mutation prevents the site from deforming to better coordinate Na^+ (Fig. 3), as indeed was the motivation in designing this site (7). In exploring why the S_2 site in KcsA-G77A$_D$ is stable in the presence of Na^+, we find that the ion preferentially interacts with only carbonyls offered by the tetrad of Val^{76} residues (Fig. 4).

TABLE 3 Energetic decomposition for KcsA and KcsA-G77A$_D$

		KcsA	KcsA-G77AD
	$\Delta\mu^{ex}$(S)	−15.7	−10.0
	$\Delta\mathcal{W}_{local}$	−15.8	−9.9
	$T\Delta s^{ex}$	1.3	−1.1
	$T\Delta s^{ex}_{local}$	3.5	4.7
Ion-site	$\Delta\langle\varepsilon_{local}\rangle$	−19.6	0.8
Ion-medium	$\Delta\langle\varepsilon_m\rangle$	0.8	−20.1
Site-site	$\Delta\langle U_s\rangle$	7.3	−6.0
Site-medium	$\Delta\langle U_{s-m}\rangle$	−2.9	14.5
Medium-medium	$\Delta\langle U_m\rangle$	0.0	−0.3

Eight carbonyl ligands comprise the local site. We consider the water molecules in the S_1 and S_3 sites adjoining the S_2 site as the medium. Similar to valinomycin, the change in ion-medium interactions is inversely related to the change in site-medium interactions. All values are in kcal/mol.

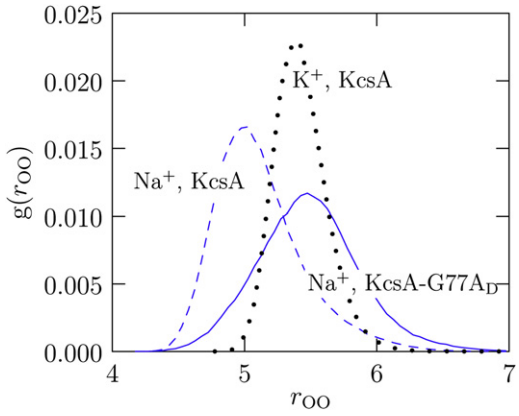

FIGURE 3 Radial distribution, $g(r_{OO})$, of the diagonally opposed carbonyl oxygens in the presence of Na^+ for KcsA (*dashed*), KcsA-G77A$_D$ (*solid*), and K^+ bound KcsA (*dotted*). Observe that the binding site cannot collapse onto the smaller Na^+ due to the side chains of d-Alanine at position 77.

However, unlike KcsA where water molecules in S_1 and S_3 do not enter the coordination sphere of the ions (17), in KcsA-G77A$_D$ water molecules bind favorably with Na^+ (Table 3). Thus the interaction that Na^+ loses with the carbonyls it makes up from the water molecules. It is interesting to note that such hydration effects were noted in the flexible but nonselective NaK channel (30).

From the above analysis, if we consider the role of ion medium interactions as well, we find that Na^+ is more favorably bound (Table 3). But the favorable ion-medium interactions are offset by unfavorable site-medium interactions. It is this energetic strain, now of the entire site-medium complex, that shifts selectivity toward K^+. In contrast, in KcsA, the energetic straining of the local site suffices to explain selectivity (17,30). Note that if we disregard the medium, selectivity in KcsA-G77A$_D$ follows the classical snug-fit model, but if we include the medium, energetic

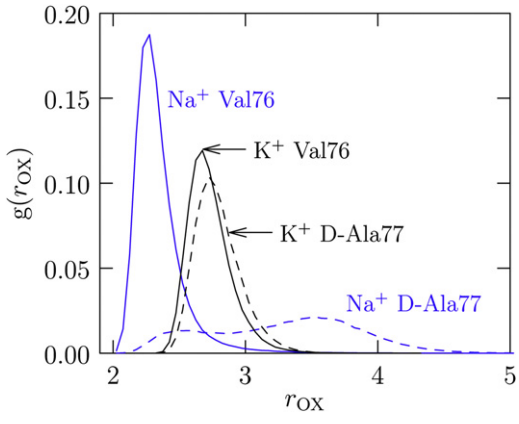

FIGURE 4 Distribution $g(r_{OX})$ of ion-carbonyl oxygen distances for oxygen ligands belonging to Val^{76} and d-Ala^{77} for Na^+ and K^+. It is seen that K^+ does not differentiate between the carbonyl ligands from Val^{76} and d-Ala^{77} whereas the smaller Na^+ prefers the more flexible Val^{76}.

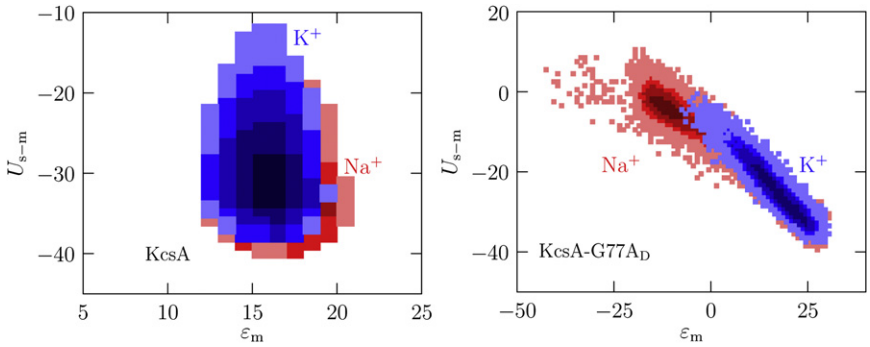

FIGURE 5 Joint probability distribution, $P(\varepsilon_m, U_{s-m})$, of the interaction of the ion with the representative medium, ε_m, versus the interaction of the site with the medium, U_{s-m}. We regard the water molecules in S_1 and S_3 sites as the medium (Table 1). The values ε_m and U_{s-m} are nearly independent of each other and similar for Na^+ and K^+ in KcsA (*left panel*) whereas they are inversely correlated in KcsA-G77A$_D$ (*right panel*).

straining of the site plus the medium is seen to determine selectivity.

The above development suggests when a local model will be appropriate. In Fig. 5, we plot the joint probability distribution, $P(\varepsilon_m, U_{s-m})$, of the interaction of the ion with medium against the interaction of the site with the medium. For KcsA, the site-medium interactions are nearly independent of ion-medium interactions and they are also insensitive to ion-type. This suggests that the medium is insensitive to the type of the ion in the binding site and the local model should be satisfactory. Observe that we can consider the site as behaving independently of the rest of the medium as far as ion-selectivity is concerned. Thus our analysis provides a rigorous basis for the success of the eight carbonyl model (11) for understanding selectivity in KcsA.

For KcsA-G77A$_D$ the ion-medium and site-medium interactions are inversely correlated, just as they are for valinomycin (Table 2). In this instance, it is clear that the medium is playing a critical role in the dynamics of the site and should not be ignored in discussing selectivity. Indeed, in such a case, the local model and a model that considers the medium lead to different conclusions about the physical basis of selectivity.

CONCLUSIONS

We decompose the free energy change in exchanging an ion in a biomolecular binding site into a local contribution and a long-range, field contribution. The local contribution to the free energy, $\Delta\mathcal{W}_{local}$, is nearly the same as the actual free energy change, $\Delta\mu^{ex}(S)$, because the field contribution is small. Investigating this aspect reveals that cancellation of the inversely related ion-medium and site-medium interactions are ultimately responsible for the small magnitude of the field contribution. This cancellation also explains the apparent success in reproducing the selectivity free energy, $\Delta\mu^{ex}(S)$, of a variety of models that typically consider the interaction of the ion with a small set of adjoining groups and neglect the role of the protein medium outside that defined local ion-binding site. However, the energetics of ion binding and also the physical conclusions about selectivity sensitively depend on the choice of the local model. When ion-medium and site-medium interactions are uncorrelated and independent of the ion-type, as happens for KcsA, the local model is entirely satisfactory for understanding selectivity. But when the ion-medium and site-medium interactions are correlated, as happens for KcsA with a glycine-to-d-alanine mutation at residue 77 and for valinomycin, the model that neglects the role of the protein medium outside the carbonyl groups comprising the defined local site leads to conclusions about the physical mechanism of selectivity that are not supported when all interactions are considered.

Our study emphasizes that in probing selectivity of a binding site, sufficient care must be taken in defining the local binding site and in representing the effect of the protein medium on the dynamics of the ion and the binding site. A satisfactory estimation of the free energy of exchanging one ion with another—a difference of large numbers—is no guarantee that the local model faithfully describes the physics of ion selectivity.

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