Pathway and Endpoint Free Energy Calculations for Cyclic Nucleotide Binding to HCN Channels

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ABSTRACT cAMP and cGMP differentially bind to and regulate a variety of proteins, including cyclic nucleotide-gated (CNG) channels and hyperpolarization-activated cyclic nucleotide-regulated (HCN) channels. Previous site-directed mutagenesis studies have isolated two conserved residues that are critical for enabling certain channels to selectively bind cGMP relative to cAMP. However, no definitive mechanism has been identified that explains the preferential activation of other channels by cAMP. Here we apply computational binding free energy methods, including thermodynamic integration, linear interaction energy, and continuum electrostatic calculations, to gain insights into the mechanisms of cyclic nucleotide selectivity. Consistent with experimental observations, computational results for the cAMP-selective HCN channels show that the binding free energy of cAMP is lower (more favorable) than that of cGMP. Surprisingly, cAMP selectivity is not due to its preferential contacts with protein, but rather reflects the greater hydration energy of cGMP relative to cAMP, resulting in a greater energetic cost for cGMP binding.

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A large family of cyclic nucleotide (cNMP) regulated proteins, ranging from protein kinases to ion channels, contain a conserved cyclic nucleotide binding domain (CNBD). The CNBD contains a fold consisting of a short α-helix (the A-helix), followed by an eight-stranded β-roll, a short B-helix, and a long C-helix. The CNBD-containing proteins differ in their absolute affinity and relative selectivity for cAMP versus cGMP. Previous studies have identified two conserved residues within the CNBD that contribute to the ability of some classes of CNBDs to select for GMP over cAMP. Thus, a threonine in the β-roll of cGMP-dependent protein kinases and cNMP-regulated ion channels was first shown to contribute to cGMP selectivity; mutation of this threonine to alanine, the residue found in cAMP-dependent protein kinases, decreases cGMP selectivity (1). In a recent study using molecular dynamics simulations, we confirmed that this threonine makes three hydrogen bonds with cGMP versus two with cAMP, explaining its role in cNMP selectivity (2,3). However, the threonine residue does not fully define the ligand selectivity of a CNBD since this residue is conserved in both cAMP-selective and cGMP-selective cyclic nucleotide-regulated channels. In the CNG channel family, an aspartic acid residue in the C-helix has been shown to be critical for cGMP selectivity (4). Channels that are selective for cAMP contain a neutral amino acid at this position; mutation of this residue to aspartate is sufficient to confer cGMP selectivity (2,5).

Thus, two key residues make a positive contribution to the preferential stabilization of cGMP relative to cAMP in certain CNBDs. What is the mechanism that enables other CNBDs to preferentially bind cAMP over cGMP? Which residues make specific contributions that stabilize cAMP and what is the atomic basis for such interactions? Despite extensive mutagenesis studies, no specific interactions have been identified that can explain cAMP selectivity. We have focused our attention on the HCN channels, whose opening in response to membrane hyperpolarization is facilitated by the binding of cAMP or cGMP. Although both cAMP and cGMP enhance gating with roughly similar efficacy, as measured by the maximal voltage shift at saturating ligand concentrations (ΔV_{max}), the channels have a 60-fold higher sensitivity to cAMP relative to cGMP, as measured by the concentration that produces a half-maximal voltage shift ($K_{1/2}$). We found that cAMP selectivity could be compromised by mutating one of several residues in the C-terminus of C-helix, including R632, R635, I636, and K638. However, despite the existence of x-ray crystal structures for the cAMP and cGMP-bound CNBDs of the HCN channels, it is not clear how these residues generate cAMP selectivity. In addition, theoretical calculations indicate that cGMP has an even stronger interaction with the HCN2 CNBD than does cAMP.

Here we address the issue of ligand selectivity by examining binding free energies using both experimental and computational approaches. We first determined experimental free energies using electrophysiological patch-clamp recordings to measure the effects of cAMP and cGMP on the gating of HCN2 channels expressed in *Xenopus* oocytes. These functional measurements yield values for ΔV_{max}, $K_{1/2}$, and the maximal increase in channel opening probability at extreme hyperpolarizations. We then used these experimental values to extract free energies of ligand binding to the closed (K_C) or open (K_O) states of the channel based on a cyclic allosteric model (see Supplementary Material, Data S1) (2). This anal-

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TABLE 1 Binding free energy estimates based on physiological experiments

	Six-state model	
	K_o (μM)	K_c (μM)
cAMP	−12.0	−0.4
cGMP	−1.2	9.9
$\Delta\Delta G$ (kJ/mol)	−13.2	−10.32

ysis yields a free energy difference between cAMP and cGMP binding of ~−10 kJ/mol—that is, the channel is cAMP-selective (Table 1).

What is the mechanism for this selectivity for cAMP relative to cGMP? To obtain a more comprehensive understanding of the ligand selectivity of HCN2, we applied computational approaches based on the x-ray crystal structures of the cAMP-bound and cGMP-bound CNBD of HCN2 to estimate the theoretical binding free energy of cAMP and cGMP. In this study, we mainly relied on the MD-based methods, partially because MD simulations sample the distribution of two natural occurring stereochemical conformations for cAMP and cGMP, *syn*- and *anti*-, as defined by the dihedral angle between the purine ring and sugar base. Our MD simulations showed that cAMP free in water has no significant preference for its two conformations. However, free cGMP preferentially adopts the *syn* conformation (90%). Both results are in good agreement with previous NMR studies (6).

First, we used the thermodynamic integration (TI) method (slow-growth method) to estimate the binding free energies of cAMP and cGMP. In this double-decoupling approach, binding free energy is the difference between two ligand decoupling processes: 1), decoupling the ligand bound to protein; and 2), decoupling the free ligand in water from its environment. In a stepwise manner, we first turned off the electrostatic potential energy between ligand and protein (and also surrounding water), through a series of parallel simulations in which the coupling parameter λ-values were reduced stepwise from 1 to 0 (see Data S1). The relatively smooth curve of $\partial U/\partial\lambda$ (U, potential energy) indicates a converged result for the electrostatic interacting energy. Next, starting from the proceeding system with a λ-value of zero, in which there is no electrostatic interaction between ligand and environment, we then slowly reduced the van der Waals (VDW) energy to zero. For the TI method, the treatment of the VDW potential energy is not a trivial issue, especially when the interaction between ligand and potential is not strong enough to ''hold'' the ligand in the binding pocket. Here we used a soft-core potential to smooth the nonbonded potential energy terms if two particles approached too closely (7). Moreover, we carried out additional simulations with different λ-values to obtain a smooth $\partial U/\partial\lambda$ curve. We did not introduce restraints to artificially hold the ligand in the binding pocket as discussed in previous studies (8–10). However, we are mainly interested in the relative free energy difference between two similar ligands and the correction term related to the double-

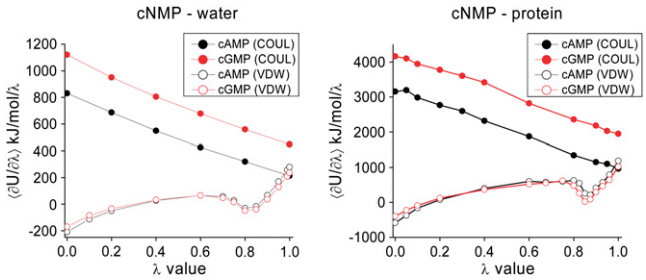

FIGURE 1 Original $\partial U/\partial\lambda$ data in the TI analysis are plotted as a function of λ. (*Left*) cAMP (*black*) or cGMP (*red*) free in water; (*right*) cAMP or cGMP bound to protein. (*Solid circles*) Electrostatic potential energy (*COUL*); (*open circles*) VDW energy (*VDW*).

decoupling method without restraints should not introduce a significant error. The well-behaved $\partial U/\partial\lambda$ curves for both cAMP and cGMP suggest a more-or-less converged sampling (Fig. 1).

The final TI results show that the binding free energy of cAMP is 51.9 kJ/mol more negative than that of cGMP (Table 2). Even though this value is higher than the estimates based on experimental data (~10 kJ/mol), the direction of change is consistent: cAMP has a lower (more favorable) binding free energy than cGMP. More importantly, this calculation revealed that the major contribution to the difference between cAMP and cGMP is not the interaction between protein and ligand (ΔG_{pro}), but the solvation energy (ΔG_{H2O}, which equals −507.2 kJ/mol for cAMP versus −762.5 for cGMP). Thus, these simulation results provide a simple answer for the question as to why the HCN channel selects cAMP: there is a higher penalty for cGMP to dehydrate.

Next we used an alternative approach, linear interaction energy (LIE), which is an endpoint approach that has less computational cost compared to the TI method. After adjusting the parameters for the LIE method based on experimental measurements (see Data S1), we calculated the absolute binding free energies of cAMP or cGMP for the wild-type HCN2 CNBD. Similar to the TI results, we were mainly interested in the difference between the binding energy of cAMP and cGMP, but not the absolute binding energy. A similar result was seen regarding the free energy difference between the two ligands: cAMP has a more favorable binding energy than cGMP due to the higher solvation energy of cGMP. Furthermore, ensemble-averaged potential energies revealed that this is mainly due to a stronger electrostatic

TABLE 2 Binding free energies by TI (kJ/mol)

	cAMP		cGMP	
	H_2O	Protein + H_2O	H_2O	Protein + H_2O
ΔG_{COUL}	499.8	2089.6	754.9	3089.4
ΔG_{VDW}	7.4	338.1	7.6	307.6
$\Delta G_{COUL} + \Delta G_{VDW}$	507.2	2427.7	762.5	3397.0
$\Delta G_{binding} = 4 \times \Delta G_{H2O} - \Delta G_{Pro}$		−398.9		−347.0
$\Delta\Delta G = \Delta G_{cAMP} - \Delta G_{cGMP}$		−51.9		

TABLE 3 Binding free energies using LIE (kJ/mol)

	cAMP		cGMP	
	H_2O	Protein + H_2O	H_2O	Protein + H_2O
$\alpha \times (V_{COUL})$	328.6	1153.9	344.1	1182.9
$\beta \times (V_{VDW})$	15.4	148.1	14.8	145.2
$\alpha \times (V_{COUL}) + \beta \times (V_{VDW})$	344.1	1302.0	358.9	1328.1
Surface area (Å^2)	401 (*anti*)		408 (*syn*)	
$\Delta G^*_{binding} = 4 \times \Delta G_{H2O} - \Delta G_{Pro}$	74.4		107.5	
$\Delta\Delta G = \Delta G_{cAMP} - \Delta G_{cGMP}$	−33.1			

*Note that the third component in the method of LIE related to the surface area is omitted due to the similar chemical structure of cAMP and cGMP and a lack of referenceable parameter for the cNMP system.

interaction between cGMP and water. The final results of LIE are shown in Table 3.

Finally, as a further theoretical approach to assessing the mechanism of cNMP selectivity, we used continuum electrostatic calculations (DELPHI and APBS) to estimate the polar solvation energy of both ligands (11–13). Disadvantages of this method include the need for two arbitrarily defined dielectric constants and ignorance of ligand flexibility due to rotation around the dihedral bond between the purine ring and sugar base. Here we used the conformation of cAMP and cGMP in the crystal structures and the partial charges and VDW radii settings from GROMACS. APBS results showed that the polar or electrostatic part of the solvation energy corresponding to the transfer of a ligand from a homogenous medium of dielectric constant 2 (protein) to a medium of dielectric constant 78 (water) is −437.2kJ/mol for cAMP (*anti*) versus −486.9 kJ/mol for cGMP (*syn*), representing a −49.7 kJ/mol more favorable energy of hydration for cGMP. An independent calculation using DELPHI showed that the polar part of the solvation energy (corrected reaction field energy) of cGMP is −45.2 kJ/mol more favorable compared to cAMP, in good agreement with the APBS results. The difference in the nonpolar solvation energy could be estimated from the solvent-accessible surface area. We ignored this term in the current study because the surface areas are within 3% of each other. Moreover, considerable uncertainty exists as to the proper value for the nonpolar energy coefficient.

In summary, we used three different theoretical methods to estimate the free energy of binding of cAMP and cGMP to the HCN2 CNBD. These different approaches differ in their complexity, from the rigorous but computationally costly pathway method (TI), to a less complex endpoint method (LIE), to continuum electrostatics. All of the approaches yielded results in good qualitative agreement with the physiological characterization of the wild-type and mutant channels, thereby providing a potential mechanistic explanation for the selectivity of HCN channels for cAMP relative to cGMP. Surprisingly, we find that the major contribution to the selectivity of the channel for cAMP is due to the higher energetic penalty of desolvation of cGMP compared to cAMP, rather than due to specific interactions of cAMP with the protein. Such results help explain the failure so far to identify experimentally specific CNBD residues that selectively stabilize cAMP versus cGMP. These results are likely to help explain cAMP selectivity in a variety of CNBDs.

SUPPLEMENTARY MATERIAL

To view all of the supplemental files associated with this article, visit www.biophysj.org.

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