

Designing an Optimum Guest for a Host Using Multimolecule Free Energy Calculations: Predicting the Best Ligand for Rebek's "Tennis Ball"

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Abstract: We have predicted that difluoromethane (CH₂F₂) will be the highest-affinity guest for Rebek's "tennis ball" host¹ using a new approach to multimolecule free energy calculations. The method, which we call chemical-Monte Carlo/Molecular Dynamics (CMC/MD), was first tested by calculating the relative free energies of solvation of a variety of molecules. Subsequently, we have used it to compare nine possible guests binding to the "tennis ball" host and predict that CH₂F₂ will bind more tightly to this host than CH₄, the strongest binding guest studied to date. This prediction has been supported by standard thermodynamic integration free energy calculations in which CH₄ was mutated into CH₂F₂ both in solution and in the host. Our results show the full power of such multimolecule calculations—namely, that they can be used to rapidly calculate and rank the relative binding free energies of many molecules from a single simulation, accelerating the discovery of novel ligands or guests.

Introduction

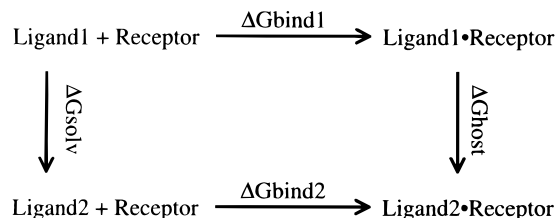
Molecular recognition is the selective, strong binding of a guest to a given host and is an essential element in biological systems, where receptor–ligand or receptor–inhibitor interactions are key to biological function. As a result, a detailed understanding of the process of molecular recognition and an ability to simulate it computationally could permit the efficient design of novel, viable drug candidates.² Thus, there are many computational approaches to ligand (or guest) design when the structure of the macromolecule (or host) is known. At one extreme of computational efficiency are approaches such as DOCK,³ which can search databases of ~100 000 potential ligands using a very simple approach to "score" compounds and qualitatively suggest which will bind most tightly to the macromolecule.

At the other extreme are free energy calculation methods such as FEP⁴ or thermodynamic integration (TI),⁵ which have proven their utility in the detailed study of protein–ligand interactions. These use a thermodynamic cycle⁶ (Figure 1) to analyze ligand binding. These methods calculate the relative free energies of the two ligands in the receptor (ΔG_{host}) and in solvent (ΔG_{solv}). The difference of these two values

$$\Delta G_{\text{host}} - \Delta G_{\text{solv}} = \Delta G_{\text{bind}}(2) -$$

$$\Delta G_{\text{bind}}(1) = \Delta \Delta G_{\text{bind}} \quad (1)$$

is the relative free energy of association, $\Delta \Delta G_{\text{bind}}$. Because the value of $\Delta \Delta G_{\text{bind}}$ defines which ligand will bind to the receptor, it is crucial data for the design of novel inhibitors or ligands.



$$\Delta \Delta G_{\text{bind}} = \Delta G_{\text{bind}}(2) - \Delta G_{\text{bind}}(1) = \Delta G_{\text{ghost}} - \Delta G_{\text{solv}}$$

Figure 1. Thermodynamic cycle for calculating the relative free energies ($\Delta \Delta G_{\text{bind}}$) for two ligands binding to a common receptor.

Free energy simulations have been successfully applied to calculate the relative binding free energy of protein–ligand complexes. Well-known examples include the binding of trimethoprim and its congeners to dihydrofolate reductase,⁷ the comparison of various HIV protease inhibitors, and the relative binding of inhibitors to thermolysin and carbonic anhydrase.⁸ However, these are expensive, pairwise comparisons between ligands. The detailed simulation of the protein–ligand complex required for just one such calculation currently requires anywhere from days to months of computer time. It is often cheaper and faster to simply carry out the relevant experiment. This has substantially limited the use of these methods in drug design or drug development applications. What, then, is the role of free energy methods in ligand design?

Free energy methods have the advantages of being thermodynamically rigorous and capable of fine distinctions between

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ligands ($\Delta G < 1$ kcal/mol) in favorable cases, so long as accurate potential functions are used.⁹ However, this accuracy is not without a price—these calculations are too slow for discovery of novel ligands. Lead discovery requires consideration and comparison of tens of thousands of compounds, a computationally prohibitive task using standard free energy methods.

In our opinion, the most efficient way to proceed in ligand design is to use a filtering strategy, where one uses rapid methods such as DOCK to first suggest 10–100 possible leads from hundred thousand- or million-compound libraries. These compounds may then be examined by methods intermediate in accuracy and detail before resorting to traditional free energy calculations. One cannot realistically do full free energy calculations on many ligands because these methods are particularly inefficient when evaluating a family of related ligands. This is due to the pairwise comparison intrinsic to standard free energy calculations—to assess the relative free energies of ligands A, B, C, and D, at least three calculations must be carried out: one to compare A and B, one to compare B and C, and finally one to compare C and D. Since the lead refinement process often involves choosing between many possible modifications of a lead compound (each of which may involve a significant amount of synthetic chemistry), computational methods are needed that retain as much as possible of the accuracy of free energy calculations but have the ability to compare many ligands at a time.

Such “multimolecule” free energy methods are actively being developed by many groups. Most notably, Kong and Brooks¹⁰ have introduced “ λ -dynamics”: by expanding the extended Hamiltonian formalism^{11,12} from one to several λ variables, they have calculated relative solvent-state free energies for many species from a single simulation and shown how expansion of the λ -variable space can accelerate the convergence of traditional pairwise free energy calculations. The use of biasing potentials to improve the convergence of such simulations is also discussed in a general way. Other multimolecule approaches include the calculation of relative free energies for many compounds by perturbation expansion from a single reference state, recently explored by Liu et al.¹³ as well as Radmer and Kollman.¹⁴

In this paper we present a new multimolecule free energy method and apply it to calculate the relative binding free energies for a series of small molecules binding to a rigid organic host. Specifically, we explored the binding of methane, ethylene, and various halomethanes to the “tennis ball” dimer described by Branda et al.¹ Our chemical Monte Carlo – Molecular Dynamics method combines molecular dynamics to sample coordinate space with Metropolis Monte Carlo¹⁵ to sample among various chemical states of the system. The use of Monte Carlo sampling in “chemical space” was originally suggested by Bennett¹⁶ and first used in a pairwise calculation of ion solvation by Tidor.¹⁷ In the CMC/MD method, the solvation free energy of each ligand can also be included as a

biasing potential in the Monte Carlo step to focus sampling toward the best binding ligands.

We have chosen the “tennis ball” host–guest system because it is experimentally well characterized and known to bind a range of ligands with varying affinity. It is also a case where theoretical calculations have complemented experiment. Specifically, Branda et al.¹ were unable to detect the binding of tetrafluoromethane (CF₄) in their initial report. Free energy calculations carried out by Fox et al.¹⁸ suggested that CF₄ should have an affinity for this host intermediate between CH₄ and CHCl₃, the best and worst known guests. This prediction was subsequently confirmed by experiment. While the “tennis ball” had been shown to bind methane, fluoromethane, ethylene, dichloromethane, and chloroform, we were interested in testing the entire range of fluoro- and chloro-substituted methanes binding to this host, an intractable series of calculations with the methods used previously. In light of the initial synergy between theory and experiment, we were excited to find our calculation predicts difluoromethane (CH₂F₂) to be an even better guest than methane.

CMC/MD is faster than the analogous thermodynamic integration calculations previously carried out by Fox et al.,¹⁸ and it converges to the same relative free energies for each ligand. In addition, our method rapidly orders the ligands according to their binding free energies, well before the precise free energy values are completely converged. A similar effect is observed with both λ -dynamics and Still’s recent work on enantioselectivity.¹⁹ All of the above properties make these multimolecule methods ideal for quickly comparing a family of related ligands and assessing their binding to a particular receptor. As such, we feel this chemical-MC/MD method will be useful in lead optimization and refinement, especially in comparison to traditional free energy methods.

Methods

The chemical Monte Carlo method is based on a derivation by Bennett.¹⁶ This derivation shows how a Monte Carlo calculation can be used to determine the relative free energy of two chemical “states” (two solutes, two ligands, etc.) by a combination of Cartesian and chemical Monte Carlo steps. It is straightforward to generalize this formalism to the case of multiple chemical “states”. The derivation and generalization are presented in Appendix I, along with a discussion of the similarities and differences between CMC/MD and other methods. It should be noted that Kong and Brooks’ λ -dynamics derivation¹⁰ is sufficiently general that it can also be extended to describe the CMC/MD approach, though both were developed independently.

Previously, combinations of Monte Carlo and molecular dynamics have primarily been used to improve the sampling of physical configurations. Notable examples are the hybrid Monte Carlo technique²⁰ and the MC(JBW)/SD method.²¹ In the hybrid Monte Carlo method, molecular dynamics is used to generate “trial move” configurations which are then evaluated with Metropolis Monte Carlo criteria to generate a thermodynamic ensemble. The MC(JBW)/SD method uses Monte Carlo steps to “jump” between conformational minima that are

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separated by free energy barriers, thus allowing a single simulation to explore a much broader set of configurations. These methods differ from the chemical-MC/MD approach described here in that they use a constant potential function. In contrast, the chemical-MC/MD method uses Monte Carlo steps to adjust the potential function, thereby representing the interaction of different ligands with the receptor. Instead of "jumping" between different Cartesian configurations and generating a Boltzmann ensemble of these configurations, we are essentially "jumping" between different ligands and generating a "Boltzmann ensemble" of ligands. In this respect, it is similar to Tidor's¹⁷ approach; however, we have extended this type of method to multiple, complex ligands in order to make it useful in the context of ligand design.

The use of Monte Carlo sampling between discrete chemical states allows us to further increase the utility of the chemical-MC/MD method. Specifically, there are two properties of interest when comparing ligands—first, a rank order of the best binders, and second, the value of $\Delta\Delta G_{\text{bind}}$ for each ligand. We want to find an optimal route to determine the relative free energy of binding, $\Delta\Delta G_{\text{bind}}$, for our ligands of interest.

Binding represents a balance between the free energies of the bound and free (solvated) states of the ligand. If we want to find the "best binders", our calculation must take into account the contributions of both these states. Drawing inspiration (and precedent) from the commonly used Monte Carlo technique of "umbrella sampling",²² we can directly determine $\Delta\Delta G_{\text{bind}}$ from our chemical-MC/MD simulation if we include the relative solvation free energies (ΔG_{solv}) as a "solvation offset" to the energy of each state. In the λ -dynamics derivation of Kong and Brooks,¹⁰ provisions are also made for the inclusion of a biasing potential associated with each λ -coordinate, though in the context of enhancing simulation convergence.

$$\Delta\Delta G_{\text{bind}} = \Delta G_{\text{ghost}} - \Delta G_{\text{solv}} \quad (7)$$

$$\Delta\Delta G_{\text{bind}} = -RT \ln \langle e^{-\Delta E_{\text{ghost}}/RT} \rangle - \Delta G_{\text{solv}} \quad (8)$$

$$\Delta\Delta G_{\text{bind}} = -RT \ln \langle e^{-(\Delta E_{\text{ghost}} - \Delta G_{\text{solv}})/RT} \rangle \quad (9)$$

Equation 9 shows that if we know or can approximate ΔG_{solv} , we can include it as a biasing potential in our chemical-MC/MD simulation of the bound state. By its nature, the chemical-MC/MD method focuses sampling on the compounds with the most favorable free energy in a given environment. In solvent, these are the compounds with the most favorable solvation free energies. In the protein or host, these are the ligands with the most favorable ΔG_{ghost} . However, the quantity of interest is $\Delta\Delta G_{\text{bind}}$, not ΔG_{solv} or ΔG_{ghost} . Including the corresponding ΔG_{ghost} for each ligand as a biasing potential in a simulation of the bound state means that the calculated value is $\Delta\Delta G_{\text{bind}}$, and the simulation spends most of its time sampling the "best binders" rather than the ligands with the lowest free energy in the bound state (lowest ΔG_{ghost}). The net result is a rapid rank-order determination of the best binding ligands and a gradually converging determination of $\Delta\Delta G_{\text{bind}}$. A useful physical analogy suggested by Kong and Brooks¹⁰ is that this process of finding the "best binder" truly corresponds to a competitive binding experiment in the laboratory, where many ligands present in solution are competing for a single binding site on a protein or host.

Computational Details

The chemical-MC/MD algorithm was implemented as part of the AMBER software package.²³ The SANDER molecular dynamics program was modified to carry out the Metropolis Monte Carlo sampling and collect, record, and report the necessary data.

During a simulation, all the solutes or ligands of interest are simultaneously included in the simulated system and their interactions calculated at every time step. However, the potential energy function is masked to reflect the chemical state of the system. At every time step, there is a single "real" ligand and the remainder are "ghosts". The "real" ligand interacts fully with the surroundings. The ghost ligands' interactions are calculated and recorded but do not affect the system energy or dynamics. In particular, the ghost ligands do not exert any forces on the surroundings. Also, no ligand ever interacts with another ligand. In effect, the "ghost" ligands are decoupled from the system surroundings. This is analogous to the "dual topology" approach to free energy calculations²⁴ except that we now have an " n -tuple topology" containing each of our n chemical species.

In the interests of simplicity and practicality, we have made a few approximations. First, the abrupt jumps between ligands mean that a newly "real" ligand does not have velocities appropriate for its surroundings. As a consequence, we randomly reassign the velocities of every particle in the simulation from a Maxwell–Boltzmann distribution whenever a Monte Carlo move occurs (Anderson temperature coupling).²⁵ In addition, a single system temperature is calculated that includes the kinetic energy of every particle in the simulation, including the ghosts. This temperature is maintained at 300 K using a Berendsen temperature coupling scheme.²⁶ The error due to these approximations is small (there are <40 ghost particles in our 9-solute, 3377 atom simulation) and should be expected to cancel when considering the relative free energies of similar ligands from a single calculation.

The system is also maintained at constant pressure by a Berendsen algorithm.²⁶ In contrast to the temperature, the virial (and the pressure) only include interactions with the "real" ligand and the surroundings. We are presently evaluating alternative temperature- and pressure-coupling algorithms to improve the rigor of our calculations.

One issue in these calculations is ensuring that the ghosts sample configurations that are appropriate for the current configuration of the surrounding "context". If the ghosts are completely decoupled from the "context", sampling of ghost configurations is essentially random. This results in poor acceptance ratios for the Monte Carlo steps, since random ghost movements often generate unrealistic situations where ghost atoms overlap atoms of the "context". We have addressed this problem in two ways. First, all of the ligands are restrained to one another by harmonic potentials between their centers of mass. Second, the ghosts are allowed to feel the influence of the "context" but not vice versa. These "ghost forces" mean that atoms of the context exert forces on the ghosts but the ghosts remain invisible to the context. Ideally, we would correct the observed free energies for these biases, but we assume that they will cancel for comparisons of similar ligands from a single

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simulation. The net result of these approximations is a substantial improvement in the acceptance ratios for Monte Carlo steps, enhancing the efficiency of the calculation.

The chemical-MC/MD protocol is as follows. The system ("context" plus "real" and "ghost" ligands) is simulated for several steps (usually 1 ps) of molecular dynamics. This generates a novel configuration of the context, the real ligand, and the ghosts. Based on this configuration, the energies of each ligand are evaluated. A ligand is chosen at random (the "trial move"). The change in energy is evaluated, and the trial move is accepted or rejected based on Metropolis Monte Carlo criteria.¹⁵

$$\Delta E \leq 0 \Rightarrow P(\text{accept}) = 1 \quad (10)$$

$$\Delta E > 0 \Rightarrow P(\text{accept}) = e^{-(\Delta E/RT)} \quad (11)$$

After the trial move is accepted or rejected, the outcome is recorded and molecular dynamics resumes, again simulating the interactions of the "context" and the currently "real" ligand. This cycle of coupled Monte Carlo and molecular dynamics steps is continued until the probability of observing each ligand converges.

While this approach is sufficient, it discards a great deal of information about each ligand. Specifically, we record the interaction energies of each ligand before selecting one for a Monte Carlo trial move. This history provides information about the "quality" of the Monte Carlo sampling and also allows us to estimate the free energy for poorly- or under-sampled states.

If an infinite number of Metropolis Monte Carlo steps were carried out on a given Cartesian configuration of the simulated system, the probabilities of each ligand would converge to the Boltzmann distribution for that configuration. That is,

$$\lim_{n \rightarrow \infty} P_j(r, \lambda_i) = \frac{e^{-\Delta E_j/RT}}{\sum_{i=1}^n e^{-\Delta E_i/RT}} \quad (12)$$

Since we only carry out one Monte Carlo step for each Cartesian configuration considered, we record this "Boltzmann" probability data over the course of our simulation as a check on our Monte Carlo sampling. The Boltzmann-based $P(\text{ligand})$ values are averaged over every Monte Carlo step to yield an optimum probability $P(\text{ligand})$ for the simulation. In our converged simulations, these Boltzmann-based probabilities mirror the observed Monte Carlo history for each state.

Simulation Specifics

1. Solvation. Relative free energies of solvation were calculated for solutes within a bath of TIP3P water molecules.²⁷ The parameters for each pair or family of compounds (including charges and geometries) were taken directly from the literature references to facilitate comparison between the chemical-MC/MD and FEP or TI calculations. Specifically the parameters for bromide and chloride were taken from Tidor's previously mentioned work.¹⁷ The anisole and benzene data were from Kuyper et al.,²⁸ and Sun and Kollman's work on hydrophobic solvation provided the parameters for methane, ethane, and

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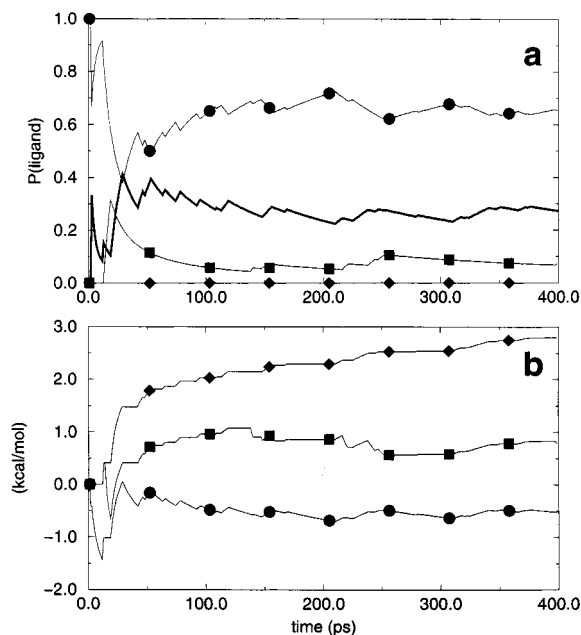


Figure 2. Population (a) and ΔGhost (b) data for the unbiased four-guest calculation. (a) shows the relative populations of each ligand in the simulation. (b) shows these population data converted to ΔGhost free energies relative to CH_4 . Solid circles show data for CH_3F ; solid squares are data for CF_4 ; and solid diamonds are the data for CHCl_3 ; and CH_4 is shown in part (a) as the heavy line. The calculation is dominated by CH_3F , the guest with the most favorable ΔGhost .

propane.²⁹ The charges, nonbonded parameters, and geometries for the substituted methanes were taken from Carlson et al.,³⁰ supplemented by bond, angle, and torsional constants from the Cornell et al. AMBER force field.³¹ In each case, the simulation system consisted of all of the solutes of interest, plus anywhere from 500 to 800 TIP3P water molecules, simulated in a rectangular periodic box.

A modified version of the SANDER module of AMBER 4.1 was used for the molecular dynamics calculation.³² A leapfrog integrator was used with a 2 fs time step. Metropolis Monte Carlo steps were evaluated every 1 ps (500 MD steps) for most systems. The system temperature was maintained at 300 K by the previously described Andersen/Berendsen temperature coupling. The Andersen temperature coupling reassigned the velocities of every atom in the system in sync with the Monte Carlo steps (every 500 steps/1 ps). The pressure was kept at 1 atm with the Berendsen coupling scheme, using the compressibility of bulk water (44.6×10^{-6} /bar) and a coupling constant of 0.2 ps^{-1} . An 8 Å cutoff was used for the nonbonded interactions, with updates to the pairlist made every 10 or 20 dynamics steps. All bonds were constrained to their equilibrium lengths using the SHAKE algorithm.³³

Since the ghosts are partially or completely decoupled from the rest of the system, something is necessary to keep them from drifting out of the vicinity of the binding cavity. For our initial test calculations, we simply constrained the analogous

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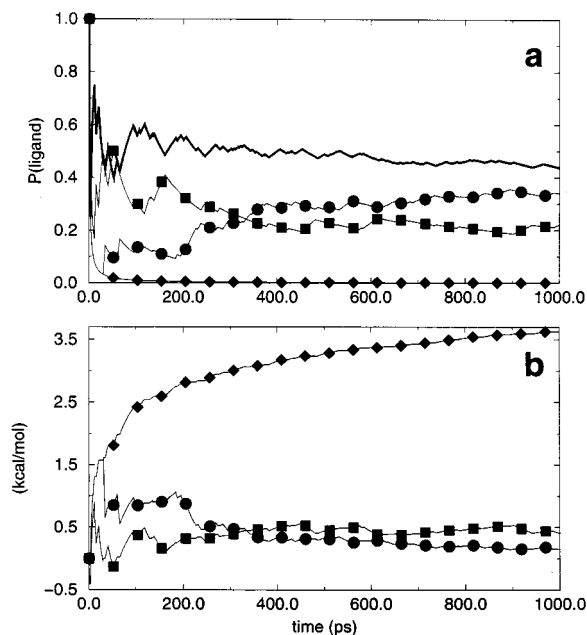


Figure 3. Population (a) and $\Delta\Delta G_{\text{bind}}$ (b) data for the "solvation offset" four-guest calculation. (a) shows the relative populations of each ligand in the simulation. (b) shows these population data converted to $\Delta\Delta G_{\text{bind}}$ free energies relative to CH_4 . Solid circles show data for CH_3F ; solid squares are data for CF_4 ; solid diamonds are the data for CHCl_3 ; and CH_4 is shown in part (a) as the heavy line. By including ΔG_{solv} as a "solvation offset" to the Monte Carlo sampling, the calculation is now dominated by CH_4 , the guest with the most favorable $\Delta\Delta G_{\text{bind}}$.

atoms (the carbon of methane and one carbon of ethane or the phenyl rings of anisole and benzene, for example) of each solute to overlap through a nonphysical "bond" of length 0.0 Å.

Each set of solutes was solvated and then equilibrated at 300 K for at least 100 ps of dynamics during which time no Monte Carlo moves were made. After the equilibration phase, Monte Carlo steps were initiated and the free energy calculation begun. Total simulation length for these calculations was anywhere from several picoseconds to 2.4 ns. Standard deviations were calculated for converged calculations by dividing the statistics from the total simulation into 4 to 8 bins depending on the simulation length and calculating a mean and standard deviation over all the bins.

2. Binding. For our binding free energy calculations, we studied the "tennis ball" host-guest system synthesized and characterized by Branda et al.¹ The host and solvent parameters were the same as described by Fox et al.¹⁸ This prior calculation also provided parameters for methane, chloroform, and tetrafluoromethane. Charges and parameters for fluoromethane were supplied by Reyes.³⁴ The values for chloromethane and dichloromethane were based on the chloroform parameters and tested as part of a new AMBER parametrization for organic solvents by Fox.³⁵ Ethylene parameters were developed by using default parameters for sp^2 carbon and associated hydrogen from the Cornell force field. All charges were determined using the RESP procedure to fit charges to electrostatic potentials from ab initio Hartree-Fock calculations using a 6-31G* basis set.³⁶

In this "tennis ball" calculation, the simulation system consisted of 2 host molecules, 631 rigid chloroform solvent molecules, and either 4 (methane, fluoromethane, tetrafluoromethane, chloroform) or 9 (methane, ethylene, fluoromethane,

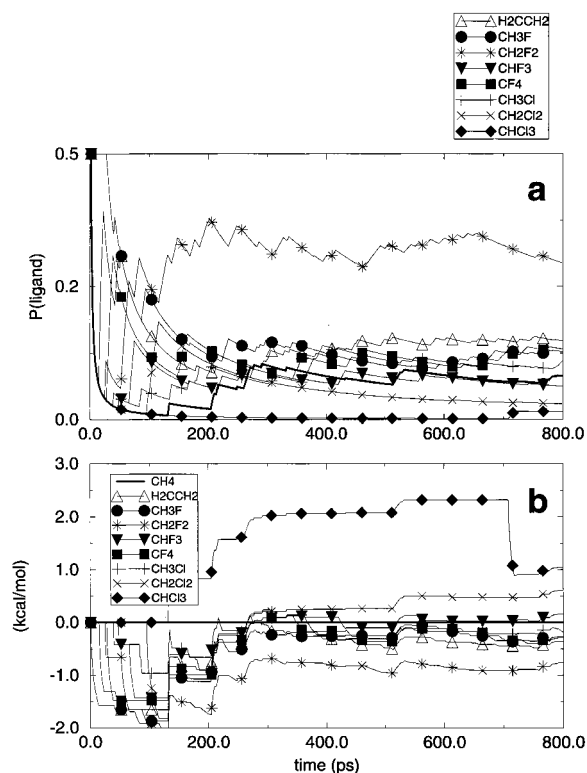


Figure 4. Population (a) and $\Delta\Delta G_{\text{bind}}$ (b) data for the "solvation offset" nine-guest calculation. (a) shows the relative populations of each ligand in the simulation. (b) shows these population data converted to $\Delta\Delta G_{\text{bind}}$ free energies relative to CH_4 . Again, solid circles show data for CH_3F ; solid squares are data for CF_4 ; solid diamonds are the data for CHCl_3 ; and CH_4 is shown in part (a) as the heavy line. In addition, data for H_2CCH_2 are indicated with open triangles pointing up, data for CH_2F_2 with stars, CHF_3 with solid triangles pointing down, CH_3Cl with plus signs and CH_2Cl_2 with X marks. These $\Delta\Delta G_{\text{bind}}$ data are clearly not converged, but the calculation readily and rapidly determines the best (CH_2F_2) and worst (CHCl_3) guests for this host.

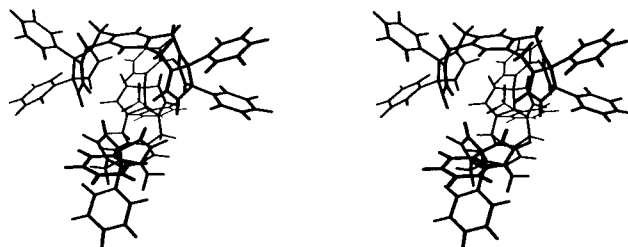


Figure 5. Single molecular dynamics snapshot of the "tennis ball" binding CH_2F_2 from the nine-guest calculation. The two halves of the tennis ball are drawn in black, and CH_2F_2 is shown in gray with both fluorines colored black. Each fluorine fits neatly into one of the major gaps between host monomers, with little strain of the host or guest molecules. Chloroform solvent molecules have been omitted for clarity.

difluoromethane, trifluoromethane, tetrafluoromethane, chloromethane, dichloromethane, and chloroform) ligands. In contrast to the solvent, all ligands were treated as having flexible angles and torsions but rigid bonds. The total system size was either 3356 or 3377 atoms and was simulated in a rectangular periodic box approximately 46 Å on a side. Figure 5 shows a stereoview of the "tennis ball" dimer with a representative configuration of difluoromethane in the binding cavity.

Again, the SANDER module of AMBER was used for the molecular dynamics calculation. A leapfrog integrator was used with a 2 fs time step. Metropolis Monte Carlo steps were evaluated every 1 ps (500 MD steps) for most systems. The

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system temperature was maintained at 300 K by the previously described Andersen/Berendsen temperature coupling. The Andersen temperature coupling reassigned the velocities of every atom in the system in sync with the Monte Carlo steps (every 500 steps/1 ps). The pressure was kept at 1 atm with the Berendsen coupling scheme, using the compressibility of bulk chloroform (108.60×10^{-6} /bar) and a coupling constant of 0.2 ps^{-1} . A 12 Å cutoff was used for the nonbonded interactions, with the pairlist update every 25 dynamics steps. A correction for the cutoff was included in the system energy and pressure.³⁷ All bonds were constrained to their equilibrium lengths using the SHAKE algorithm.³³ Aside from the chemical Monte Carlo steps and the Anderson temperature coupling, the dynamics simulation protocol is identical to that used by Fox et al. for thermodynamic integration calculations.

Since the ghosts are partially or completely decoupled from the rest of the system, something is necessary to keep them from drifting out of the vicinity of the binding cavity. We chose to constrain the center of geometry of each ligand to that of one other ligand using a flat-well restraint. This restraint was used with a force constant of 500 kcal/mol and distances $r1 = 1.0 \text{ Å}$ and $r2 = 1.5 \text{ Å}$. Since the purpose of the restraints was merely to keep the ligands in the vicinity of the binding cavity, the restraint energy was not included in the Monte Carlo calculation. Regardless, since this restraint is identical for each ligand, its contribution to the relative free energy of any two ligands largely cancels. The sum of the average restraint energy for the eight ghosts in our nine guest simulation was less than 1.0 kcal/mol, and we found that inclusion of the “ghost forces” substantially reduced the restraint energy while improving the sampling. Thus, it is highly unlikely that the restraint energy will differentially affect the calculated free energies for the different guests. This is further supported by the results of our four guest simulations, where the order of free energies is completely consistent with full TI calculations. In the future, it may be more appropriate to harmonically constrain each ligand to the center of the binding cavity, an approach that would permit analytic correction of the restraint contribution to the free energy, as outlined by Wang and Hermans,³⁸ but this idea would also be limited to relatively simple ligands and binding geometries.

Total simulation length for our binding free energy calculations was either 400 or 800 ps. This should be contrasted with the equivalent TI calculations, which required 200–800 ps to calculate ΔG_{ghost} for a single pair of ligands.¹⁸

The relative free energies of solvation in chloroform for each of our ligands were calculated using the GIBBS module of AMBER 4.1 and a simulation protocol similar to that described by Fox et al. We used the same general methodology, but instead of dividing our thermodynamic integration (TI) calculation into 101 windows of 3 ps each, we found better results from a simulation protocol of 26 larger windows each 12 ps in length. Improved convergence of the free energy value calculated for each window was seen, and the total free energy values were analogous to those determined by Fox and Reyes. Our solvation free energy data are shown in Table 1 as the average free energy for forward and reverse calculations plus or minus the hysteresis between the two runs.

Results

1. Solvation. Before applying CMC/MD to a new problem, we first tested it by calculating the relative free energies of

Table 1: Relative Free Energies of Solvation in Chloroform Calculated by Thermodynamic Integration

ligand	$\Delta G_{\text{solv}} (\text{CH}_4 \rightarrow \text{ligand})$ kcal/mol	note(s)
H ₂ CCH ₂	-0.82 ± 0.01	
CH ₃ F	-1.32 ± 0.01	<i>a</i>
CH ₂ F ₂	-1.44 ± 0.1	
CHF ₃	-1.31 ± 0.01	
CF ₄	-0.57 ± 0.04	<i>b</i>
CH ₃ Cl	-2.42 ± 0.01	
CH ₂ Cl ₂	-3.31 ± 0.25	
CHCl ₃	-3.91 ± 0.20	<i>b</i>

^a Value calculated by Reyes.³⁴ ^b Value calculated by Fox, et al.¹⁸

Table 2. Calculated and Reference Small Molecule $\Delta\Delta G_{\text{solv}}$ Values^a

system	ref ΔG_{solv} (kcal/mol)	calcd ΔG_{solv} (kcal/mol)	time (ps)
methane–methane	0	0.00	5
bromide–chloride	-3.22	-2.75	1000 [1000]
methane–ethane	0.15 ± 0.07 (-0.17)	0.03	200 [1200]
anisole–benzene	0.90 (1.1–1.6)	0.99 ± 0.48	1000 [~100]
methane, ethane and propane			
methane–ethane	0.15 ± 0.07	0.03 ± 0.07	1600
ethane–propane	0.18 ± 0.09 (0.12)	0.03 ± 0.10	[2400]
methanol and substituted methanes			
methanol–H ₃ CCN	-0.1 ± 0.2 (1.2)	0.73	400 [~5000]
methanol–H ₃ CSH	4.6 ± 0.1 (3.8)	2.95	
methanol–ethane	7.9 ± 0.2 (6.9)	4.37	

^a Reference values are from free energy perturbation calculations (no parentheses) or experiment (parentheses). Calculated values are from simulations using the chemical-MC/MD method ± 1 standard deviation. Simulation times are the total amount used to calculate ΔG_{solv} . Times in brackets are the simulation times for the reference calculation. All references are free energy perturbation calculations that include secondary references for the experimental values. Parameters for each system are taken from the references in question to facilitate direct comparison between the computational methods.

solvation in water for several families of compounds that had previously been studied by TI or FEP calculations. These results are presented in Table 2, along with the corresponding free energy data from the literature for comparison. While the data are not converged for every family of compounds studied, the CMC/MD method does a good job of determining the rank order and magnitude of the solvation free energies in each case. The relative solvation of bromide and chloride ion was studied by Tidor with the hybrid MC/MD method described previously, and our results are in reasonable agreement with his calculations. More difficult tests are the comparisons of methane versus ethane and anisole versus benzene. In particular, the comparison of anisole and benzene is significant because the steric difference between the two compounds is relatively large, yet our method gives a reasonable estimate of the free energy difference.

After these pairwise comparisons, we studied two families of compounds. Methane, ethane, and propane were studied in a single simulation that yielded quite accurate free energy estimates for all three compounds with a reasonable computational cost. Methanol and the substituted methanes formed the other family of compounds studied. They cover a broad range of polarity and free energy, yet our method rapidly gets the correct rank order and order of magnitude of the relative free energies of solvation. This latter set of molecules had been

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Table 3. Relative Binding Free Energies vs CH₄, Four-Guest Calculation^a

guest	ΔG_{ghost} (MC)	ΔG_{ghost} (TI)	ΔG_{solv} (TI)	$\Delta\Delta G_{\text{bind}}$ (MC-TI)	$\Delta\Delta G_{\text{bind}}$ (MC-offset)	$\Delta\Delta G_{\text{bind}}$ (TI-TI)	$\Delta\Delta G_{\text{bind}}$ (expt)
CH ₃ F	-0.77	-1.14	-1.32	+0.54	+0.17	+0.17	ND
CF ₄	+0.20	+0.36	-0.57	+0.77	+0.41	+0.93	+2.8
CHCl ₃	+2.50	+4.30	-3.91	+6.41	+3.57	+8.21	+5.2

^a All calculations were carried out using the parameters described in Fox et al.¹⁸ Shaded columns show chemical Monte Carlo/MD calculations carried out in this work. Unshaded columns present experimental and thermodynamic integration data for comparison. (MC): free energy from unbiased chemical-MC/MD calculation. (TI): Thermodynamic Integration data from Fox et al.¹⁸ and Reyes.³⁴ (MC-TI): $\Delta\Delta G_{\text{bind}}$ calculated as ΔG_{ghost} from unbiased chemical-MC/MD calculation - ΔG_{solv} from TI. (MC-offset): $\Delta\Delta G_{\text{bind}}$ calculated directly from a single chemical-MC/MD calculation using ΔG_{solv} from TI as a "solvation offset". (TI-TI): $\Delta\Delta G_{\text{bind}}$ calculated as ΔG_{ghost} from TI - ΔG_{solv} from TI. (expt): Experimental binding data from Branda et al.¹

studied by Kong and Brooks¹⁰ using λ -dynamics, so it was appropriate to show that our procedure could also appropriately rank the free energies of solvation of these molecules.

2. Binding. Once we had achieved these promising results on relative free energies of solvation, we then applied the CMC/MD method to study the binding of four guests to the "tennis ball" host. The guests chosen were those previously studied by Fox et al. (CH₄, CF₄, CHCl₃) and Reyes (CH₃F), so that thermodynamic integration data was readily available for comparison. As an initial test, we did not include the solvation offset in our calculation. The results of this determination of ΔG_{ghost} are shown in Figure 2. Figure 2a shows the relative populations of each ligand in the host accumulated over a 400 ps calculation. These data are converted into free energies relative to methane in Figure 2b. Clearly, our method rapidly indicates that CH₃F is the most favorably bound ligand. However, this calculation does not include the solvation free energies.

By including the solvation free energies as offsets to our Monte Carlo sampling, we can directly determine $\Delta\Delta G_{\text{bind}}$, as shown in Figure 3. This 1 ns calculation is now dominated by CH₄ instead of CH₃F, in good agreement with the actual relative binding free energies ($\Delta\Delta G_{\text{bind}}$). This is one of the major strengths of our method—most of the simulation time is spent sampling the ligands with favorable binding free energies. The calculation thus rapidly focuses on the real compounds of interest. In addition, the rank order of binding is rapidly determined (Figure 3a). Our calculation shows that guests are preferred in the order CH₄ > CH₃F > CF₄ > CHCl₃, as observed experimentally. Extended calculations converge to well-defined values of the binding free energy (Figure 3b). Our calculated values (Table 3) are in good agreement with both experimental data and earlier free energy calculations.

We subsequently decided to apply our method in a predictive fashion to a simulation that included nine guests. We chose all of the guests that had been observed experimentally (CH₄, H₂CCH₂, CH₃F, CF₄, CH₂Cl₂, CHCl₃) as well as the remaining fluoromethanes (CH₂F₂, CHF₃) and chloromethane (CH₃Cl). We did not include carbon tetrachloride (CCl₄), since we expected it to be even less favorably bound than chloroform, the worst guest observed. Using the relative solvation free energy data from Table 1, we carried out a single 800 ps simulation on this family of compounds. The population and free energy data are shown in Figure 4. The data for the nine-guest case are substantially less well converged than the simpler four-guest calculation, but several results are clear. Most importantly, the calculation quickly shows that difluoromethane is clearly the best binding compound and chloroform the worst. Our data also agree with Branda et al.¹ that methane and ethylene are approximately equally well bound by this host and that CH₂Cl₂ is preferred to CHCl₃. The predicted rank order from our calculation is CH₂F₂ >> (H₂CCH₂, CF₄, CH₃F, CH₃Cl, CH₄,

CHF₃) > CH₂Cl₂ >> CHCl₃. However, we do not think these data are perfectly converged. Particularly, we are most confident about the prediction of the best- and worst-binding compounds and less certain of the ordering of "intermediate" binders. Still, the utility of this method in rapidly sorting the compounds by approximate binding free energy is clear.

Since the CMC/MD calculation strongly suggests that CH₂F₂ is the best guest for the "tennis ball", we decided to test this prediction with a thermodynamic integration calculation. Using a protocol identical to that used for the calculation of solvation free energies in chloroform and similar to that previously used to calculate ΔG_{ghost} for other guests binding to this host, we perturbed methane to difluoromethane in the cavity of the "tennis ball". The calculated ΔG_{ghost} was -1.88 ± 0.03 kcal/mol; subtracting the previously calculated ΔG_{solv} of -1.44 kcal/mol yields the result that difluoromethane is preferred in this host by -0.44 kcal/mol. This is in good agreement with our chemical-MC/MD estimate of -0.76 kcal/mol and provides a strong internal validation of our new method. We examined the complex of difluoromethane bound to the host dimer in detail in order to understand the structural basis for its affinity. Figure 5 shows a representative configuration of the complex. The guest is slightly off-center in the host cavity and is oriented so that each fluorine projects toward one of the gaps between the two halves of the host. This arrangement appears to maximize the favorable van der Waals contacts between guest and host without straining the guest, either host monomer, or any intermonomer hydrogen bonds. Energy minimization and analysis of the electrostatic and van der Waals interactions of the complex in vacuo support this conclusion.

Comparison of the minimized CH₄/host and CH₂F₂/host complexes leads to an interaction energy difference of ~ 4 kcal/mol favoring CH₂F₂. Of this difference, 3.5 kcal/mol is due to van der Waals energy and 0.5 kcal/mol from electrostatic interactions. We can include the solvation free energy of these two guests in a qualitative way using the data in Table 1: CF₄ is more favorably solvated than CH₄ by ~ 0.6 kcal/mol, suggesting that each fluorine yields $0.6/4 = 0.15$ kcal/mol solvation due to van der Waals interactions with the chloroform solvent. Thus, the ~ 1.4 kcal/mol improved solvation of CH₂F₂ relative to CH₄ has a ~ 1 kcal/mol contribution from electrostatic energies, which makes sense given the dipolar character of CH₂F₂ and the nonpolar nature of methane. Comparing these solvation free energies with the energy minimization results suggests that the host's preference for CH₂F₂ is due to van der Waals interactions, since the favorable electrostatic contribution for CH₂F₂ versus CH₄ is even larger in solution than in the host cavity.

Of course, the above is only a qualitative analysis but is unequivocal in the predominance of van der Waals forces. As noted, CH₂F₂ can gain van der Waals attractions for its fluorines by pointing them toward the intermonomer gaps in the hosts.

Directing the fluorines toward the aromatic ring leads to unfavorable repulsion and strain in the host. Similarly, the replacement of fluorines with chlorines is also disfavored, as CH_2Cl_2 is seen to be less favorably bound than CH_4 by both theory and experiment. One can now also rationalize the weaker binding of CHF_3 because the geometry of the host and guest preclude the formation of strong van der Waals interactions for the third fluorine group.

Discussion

We have developed and applied the chemical Monte Carlo/MD (CMC/MD) method to successfully determine the relative binding free energies of several nonpolar guests binding to an organic host. With sufficient sampling, our calculation yields free energies in close agreement with previous thermodynamic integration calculations as well as experiment (Table 2, Figure 3, etc.). Our multimolecule free energy method has a great deal in common with the previously published λ -dynamics work of Brooks and Kong,¹⁰ though it was independently derived from theoretical work by Bennett¹⁶ and the subsequent coupled MC/MD work of Tidor¹⁷ as well as Radmer's work¹⁴ on other multimolecule free energy methods.

We have also shown that the solvation free energy may be included in the Monte Carlo stage of the calculation to focus sampling on the most favorably bound ligands. Application of this "solvation offset" to our four-guest calculation shifts the predominant state from CH_3F (the guest with the lowest free energy in the bound state) to CH_4 (the most favorably bound guest). In addition, our calculations rapidly yield the observed preference of the host for various guests ($\text{CH}_4 > \text{CH}_3\text{F} > \text{CF}_4 > \text{CHCl}_3$). After this paper was submitted for review, works have appeared by both Guo et al.³⁹ and Jarque and Tidor⁴⁰ which also demonstrate the feasibility and utility of sampling on the $\Delta\Delta G_{\text{solv}}$ (or $\Delta\Delta G_{\text{bind}}$) surface.

To demonstrate the real utility of multimolecule free energy methods, we have carried out a predictive calculation—the first using such techniques—comparing nine guests bound to the host. The rank order from our calculation correlates somewhat with that observed by Branda et al.,¹ for the five guests studied experimentally, and suggests that CH_2F_2 would be even more favorably bound to the host than methane. We have tested this prediction internally with a TI calculation that also finds CH_2F_2 a better guest than methane. This demonstrates the ideal application of multimolecule methods—they permit consideration of the relative binding free energy for many more compounds than could be studied otherwise and rapidly pick out promising binders for further computational or experimental study.

There are some limitations to our method. First, it is restricted to comparisons between relatively similar ligands or at least compounds of similar volume. Ligands with substantial steric differences (methyl versus phenyl derivatives, for example) are difficult to compare with the CMC/MD method, since the abrupt jumps between states do not sample large changes in volume well. However, we have applied our method to accurately calculate the relative free energies of solvation of anisole and benzene (Table 2). This change from a hydrogen to a methoxy group gives us confidence that we can apply our method to pharmacologically relevant changes.⁷ It should also be noted that free energy calculations which involve large steric changes

are still a challenging prospect for more traditional FEP and TI calculations as well.^{41,42}

Furthermore, CMC/MD appears to be much more efficient for calculating ΔG_{ghost} or $\Delta\Delta G_{\text{bind}}$ than it is for calculating ΔG_{solv} . The preorganized cavity of a host or protein binding site makes for more efficient sampling than the transient, rapidly fluctuating cavities that surround a solute in a solvent like water. Acceptance ratios are much higher for calculations of ΔG_{ghost} in our test system than they were for trial ΔG_{solv} calculations in water. In the present study, we avoided this issue by using ΔG_{solv} values from thermodynamic integration calculations. Since we expect to eventually use our chemical-MC/MD method to compare many ligands bound to a protein, we are exploring alternative, less expensive methods for calculating ΔG_{solv} , such as continuum solvent methods.⁴³ In addition, several projects are underway to use this method to compare the binding of multiple drug molecules to protein targets, with excellent initial results.⁴⁴

Both CMC/MD and λ -dynamics¹⁰ are "multimolecule" free energy methods. They provide the framework for rapid comparison of the free energy of several molecules experiencing a common environment. Kong et al.¹⁰ and Guo et al.³⁹ have both shown the power of λ -dynamics in solvation free energy calculations and in accelerating the convergence of traditional free energy simulations. In contrast to λ -dynamics, CMC/MD is a more approximate method—the rapid jumps in chemical space permit us to save time by avoiding the simulation of intermediate states, but also appear to require longer simulation times to yield converged free energy statistics. Our " n -tuple topology" approach also means that CMC/MD is more readily extensible to comparisons between ligands of arbitrary topology, an essential issue in drug design calculations. This is illustrated here by our consideration not only of substituted methanes but also ethylene as guests for the "tennis ball" host.

These multimolecule methods occupy a middle ground of detail and accuracy in the range of computational methods that are applied to structure-based drug design. At one extreme there are docking and empirical scoring methods that can examine hundreds of thousands of compounds and possibilities. Traditional free energy perturbation methods occupy the other extreme, providing a detailed assessment of only two compounds. CMC/MD and λ -dynamics both give a relatively accurate free energy assessment for 5–10 compounds. A simpler dynamics-based free energy estimation method (the linear interaction approximation, or LIA) has been introduced by Aqvist.⁴⁵ Radmer and Kollman have introduced PROFEC, a tool for optimizing ligand affinity based on extrapolations from a single dynamics calculation.¹⁴ A similar method from Liu, Mark, and van Gunsteren uses extrapolations from a simulation of a single solute to estimate free energies for a range of related compounds, with modest success.¹³ Given the range of methods available, one can imagine a funneling process, where the best compounds found by a docking method are studied in more detail by LIA or chemical-MC/MD methods, possible modifications are suggested by PROFEC, and final lead optimization is

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guided by careful CMC/MD or FEP/TI calculations. At each stage of the process, the number of compounds studied is whittled down from thousands to hundreds or tens or even pairs of compounds, while the level of detail, accuracy, and computational expense per compound is simultaneously increased.

With the development and deployment of modern parallel supercomputers and workstation clusters, we also envision a "coarse-grained" parallel implementation of our method, where one chemical Monte Carlo – MD calculation is run on each of several processors. If we can compare 5–10 ligands per processor and one "reference" ligand is common to every processor, we can expect to compare and rank hundreds of ligands at once. In addition, the chemical Monte Carlo method is intrinsically suitable for more simple applications of coarse-grained parallelism. The results from two simulations of the same family of ligands can be added together directly to yield improved (or more rapid) free energy estimates. This is a sharp contrast to traditional FEP or TI calculations, where the need to smoothly integrate along the "reaction coordinate" means that one must either do additional preparatory simulations to divide the task among processors⁴⁶ or develop intrinsically fine-grained algorithms.

Finally, the use of computational methods to study the ideal guest for Rebek's "tennis ball" host has led to an exciting result—the prediction that CH₂F₂ would be a better guest than CH₄. Analysis of the structure and energies yielded a rationalization of this preference, based on several factors. First, fluorine groups are of the appropriate size to fit neatly in the intermonomer interface. The geometry of host and guest permit only two positions on the guest to make such favorable interactions, which may also explain some of the host's preference for CH₂Cl₂ versus CHCl₃. Finally, the greater van der Waals well depth of fluorine relative to hydrogen makes this interaction stronger for CH₂F₂ than for CH₄. Thus, this study has met the fundamental requirements for any computational method—it has qualitatively and semiquantitatively reproduced known experimental data, made a prediction for a new guest, and provided mechanistic and structural insight into the origin of the increased affinity of this guest for the host. This offers encouragement for the continued utility of CMC/MD and other "multimolecule" free energy calculations in the study of host–guest complexes, whether they be organic systems such as the one described herein or biological problems such as protein–ligand interactions.

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Appendix I

Derivation. Consider n chemical states, numbered 0 through n , described by identical coordinates (\mathbf{r}) but differing only in the potential functions (U_n) describing them. The free energy difference between any two states is the ratio of their corresponding configurational integrals

$$\Delta G(m \rightarrow n) = -kT \ln \frac{Q_n}{Q_m} \quad (13)$$

where such an integral has the form

$$Q_n = \int e^{-U_n(\mathbf{r})/kT} d\mathbf{r} \quad (14)$$

Bennett showed that this ratio of configurational integrals can be calculated by a simulation that samples various (\mathbf{r}) and simultaneously carries out a special type of Metropolis Monte Carlo move. Specifically, the Monte Carlo move does not involve a change of coordinates ($\mathbf{r} \rightarrow \mathbf{r}'$) but instead involves a change in potential function ($U_n \rightarrow U_m$). The Metropolis function

$$M(x) = \min\{1, e^{-x}\} \quad (15)$$

or more specifically

$$M(\Delta U/kT) = \min\{1, e^{-\Delta U/kT}\} \quad (16)$$

defines the acceptance probability for this potential-switching move just as in traditional Cartesian applications of Metropolis Monte Carlo, where

$$\Delta U = U(\mathbf{r}') - U(\mathbf{r}) \quad (17)$$

In our case, however, ΔU is the change in energy involved in switching the system from potential function U_m to U_n :

$$\Delta U = U_n(\mathbf{r}) - U_m(\mathbf{r}) \quad (18)$$

For any physical configuration of the system (\mathbf{r}) the acceptance probabilities for any pair of potential-switching moves ($m \rightarrow n$ and $n \rightarrow m$) are related by

$$M(U_n - U_m)/M(U_m - U_n) = e^{-(U_n - U_m)} \quad (19)$$

(where we have omitted the factor of kT from the exponential for clarity) which can be rearranged into the form

$$M(U_n - U_m) e^{-U_m} = M(U_m - U_n) e^{-U_n} \quad (20)$$

Since both potential functions apply to the same coordinate space (\mathbf{r}), one can integrate both sides of the above over all possible values of (\mathbf{r}), yielding eq 21

$$\int M(U_n - U_m) e^{-U_m} d\mathbf{r} = \int M(U_m - U_n) e^{-U_n} d\mathbf{r} \quad (21)$$

Multiplying the left side of this equation by the identity Q_m/Q_m and the right by Q_n/Q_n gives eq 22:

$$\frac{Q_m}{Q_m} \int M(U_n - U_m) e^{-U_m} d\mathbf{r} = \frac{Q_n}{Q_n} \int M(U_m - U_n) e^{-U_n} d\mathbf{r} \quad (22)$$

The terms

$$\frac{1}{Q_m} \int M(U_n - U_m) e^{-U_m} d\mathbf{r}$$

and

$$\frac{1}{Q_n} \int M(U_m - U_n) e^{-U_n} d\mathbf{r}$$

are simply canonical averages in the Q_m and Q_n ensembles, respectively. A canonical average has the form

$$\langle F(U, \mathbf{r}) \rangle = \frac{\int F(U, \mathbf{r}) e^{-U(\mathbf{r})} d\mathbf{r}}{Q} \quad (23)$$

(46) DeBolt, S. E.; Pearlman, D. A.; Kollman, P. A. *J. Comput. Chem.* 1994, 15, 351–373.

so we can rearrange eq 22 to yield the ratio of interest:

$$\frac{Q_m}{Q_n} = \frac{\langle M(U_m - U_n) \rangle_n}{\langle M(U_n - U_m) \rangle_m} \quad (24)$$

The physical interpretation of the above is that a simulation which includes potential-switching Metropolis Monte Carlo moves in addition to some form of configurational sampling will sample the potential states U_m and U_n in proportions that reflect the free energy differences between states m and n . Bennett did go on to point out that it is often more efficient to evaluate the canonical integrals $\langle M(U_m - U_n) \rangle_n$ and $\langle M(U_n - U_m) \rangle_m$ directly. However, this is only true if one knows a priori which free energy differences (and states) are of interest.

For the multistate case where we start with many states ($0..n$), the full chemical Monte Carlo process has its own advantages. Specifically, we are interested in the relative free energies of each state, but our primary goal is finding the states of lowest free energy. Consequently, we do not want to waste computational time calculating detailed free energies for states that are not of interest. In the binding free energy applications discussed in this paper, the states of lowest free energy correspond to the ligands that are the “best binders” for a given receptor.

In practice, the full chemical Monte Carlo method is implemented by adding a set of additional coordinates to the simulated system, one for each chemical state of interest. These coordinates (λ_i) are analogous to the “ λ ” coordinates used in FEP and TI calculations. For a set of chemical states 0 to n , we have λ_0 to λ_n . The potential function used is of the form

$$U(\mathbf{r}, \{\lambda_i\}) = \sum_{i=1}^n \lambda_i U_i(\mathbf{r}) \quad (25)$$

where (\mathbf{r}) includes coordinates for each chemical state of interest plus the surrounding context (solvent, protein, or host molecule).

The λ values are also subject to two constraints; first, each λ_i is either 0 or 1. Second, the sum of all λ_i is constrained to be 1. The result of these two constraints is that the calculation only simulates the end states of interest and only simulates one at a time.

Comparison of Methods. As noted in the Introduction, Tidor has previously presented an implementation of Bennett’s ideas that uses molecular dynamics to sample configuration space and Monte Carlo methods to take steps along a chemical “reaction coordinate” between two end states.¹⁷ The “reaction coordinate”, often called λ typically couples the potential functions describing the two end states in a linear fashion:

$$V = \lambda * V_a + (1 - \lambda) * V_b \quad (2)$$

where V is the simulated potential function and V_a and V_b refer to the potentials appropriate for end states A and B, respectively. Both the aforementioned approach and traditional FEP or TI methods calculate the free energy difference between states A and B by integrating the free energy along λ . Tidor’s method was successfully applied to calculate a free energy difference for two solvated ions via simulated annealing along the λ coordinate. The use of a continuous “reaction coordinate” means that this method has one of the limitations of traditional free energy calculations. Namely, much time is spent simulating nonphysical intermediate states rather than the end states of interest. This problem is compounded by allowing stochastic sampling along the reaction coordinate. Simulated annealing may be necessary since the simulation may get stuck in a free

energy minimum that lies somewhere along the coordinate but is itself a poor representative of the end states.

While it would be possible to use Monte Carlo methods for both the chemical and Cartesian steps of the calculation, we were interested in eventually applying our method to studies of protein–ligand interactions. Consequently, we chose to use molecular dynamics methods instead of Monte Carlo methods for the sampling of configurational space. This rationale is not only partly historical but also based on prior studies which showed MD was a better approach than MC for configurational sampling in proteins.⁴⁷ However, Jorgensen has recently made great strides in the application of MC techniques to proteins.⁴⁸ In contrast, Monte Carlo is a better configurational sampling tool in many simpler systems, like solutions of small molecules.⁴⁹ For such systems, one could easily imagine using a chemical-MC/MC algorithm instead of our chemical-MC/MD approach.

To avoid the difficulties associated with “hybrid” or “in-between” states, we chose to restrict our chemical sampling to jumps between the end states of interest. In the formalism presented in the Appendix

$$\sum_{i=1}^n \lambda_i = 1 \text{ and } \lambda_i = \{0,1\} \quad (3)$$

This has the advantage that we are always simulating the end states of interest. However, the efficiency of the Monte Carlo sampling is now highly dependent on whether the simulation of state A samples configurations favorable for state B, or vice versa. The results of our simulations suggest that this is not an insurmountable problem, but its severity will be system dependent, an observation supported by Radmer and Kollman’s¹⁴ work. In extreme cases, the barriers between states may be reduced by including a few carefully chosen “hybrid” chemical states to bridge between the end points of interest, but we have not needed to take that approach for any of the calculations presented here.

A further advantage of our approach is that the extraction of relative free energies is very straightforward. The ratio of “populations”—the number of times each chemical state is sampled in the calculations—is directly related to the relative free energies of the chemical states by

$$\Delta G(A \rightarrow B) = -RT \ln(P(B)/P(A)) \quad (4)$$

This contrasts with the approaches that allow partial values of the reaction coordinate. In the two-state case, a simulation that has an average $\lambda = 0.5$ may not mean that the two end states are in equilibrium. Instead, it may mean the free energy minimum of the potential is $\lambda = 0.5$. Similarly, the correct way to extract a free energy from these calculations is not

$$\Delta G(0 \rightarrow 1) = -RT \ln \lambda \quad (5)$$

but rather, as Mezei et al.⁵⁰ noted

$$\Delta G(0 \rightarrow 1) = -RT \ln(P(\lambda = 1)/P(\lambda = 0)) \quad (6)$$

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Since only those configurations where λ is fully representative of a single ligand contribute to the calculated free energy, it makes sense to avoid wasting time simulating intermediate states if that is feasible. If intermediate states are included in the calculation, however, one can calculate the potential of mean

force or free energy integral along the reaction coordinate(s) as is done in a TI or FEP calculation.

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