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Overcoming Dissipation in the Calculation of Standard Binding Free Energies by Ligand Extraction

Camilo Velez-Vega and Michael K. Gilson*

Skaggs School of Pharmacy and Pharmaceutical Sciences, University of California San Diego, 9500 Gilman Dr. MC 0736, La Jolla, CA 92093-0736

Abstract

This paper addresses calculations of the standard free energy of binding from molecular simulations in which a bound ligand is extracted from its binding site by steered molecular dynamics (MD) simulations or equilibrium umbrella sampling. Host-guest systems are used as test beds to examine the requirements for obtaining the reversible work of ligand extraction. We find that, for both steered MD and umbrella sampling, marked irreversibilities can occur when the guest molecule crosses an energy barrier and suddenly jumps to a new position, causing dissipation of energy stored in the stretched molecule(s). For flexible molecules, this occurs even when a stiff pulling spring is employed, and it is difficult to suppress in calculations where the spring is attached to the molecules by single, fixed attachment points. We therefore introduce and test a method, Fluctuation-Guided Pulling (FGP), which adaptively adjusts the spring's attachment points based on the guest's atomic fluctuations relative to the host. This adaptive approach is found to substantially improve the reversibility of both steered MD and umbrella sampling calculations for the present systems. The results are then used to estimate standard binding free energies within a comprehensive framework, termed attach-pull-release (APR), which recognizes that the standard free energy of binding must include not only the pulling work itself, but also the work of attaching and then releasing the spring, where the release work includes an accounting of the standard concentration to which the ligand is discharged.

Introduction

Reliable methods of computing the non-covalent binding affinities of protein-ligand and host-guest systems are needed for a variety of practical applications, such as the design of drugs, sensors, and supramolecular devices. One promising approach to the calculation of binding affinities involves the use of molecular simulations to estimate the work of computationally extracting a ligand from its receptor. This can be implemented with methods including nonequilibrium sampling by steered molecular dynamics^[19] (SMD), and equilibrium umbrella sampling (US) in successive windows along an extraction path^[20]. It has been applied in various forms to protein-ligand binding^[21–26], and may be particularly well-behaved from a numerical standpoint, especially for charged systems^[25].

In order to compute a binding affinity from an equilibrium or nonequilibrium simulation by this approach, one must compute the thermodynamically reversible work associated with the extraction process. This can be difficult even for fairly simple molecular systems. For example, in prior steered MD simulations of ligand extraction for small host-guest systems,^[30] we observed apparent irreversibilities when atoms of the guest molecule to

*Corresponding author. Phone: 858-822-0622. mgilson@ucsd.edu.

Supplementary Material: Details on the calculation of binding free energies from pulling simulations, and supporting figures S1 and S2.

which the pulling spring was not directly attached remained stuck to the host for a period of time. During this time, the guest was put under increasing tension, and the sudden release of tension when the guest eventually detached from the host led to a dissipative conformational jump. Thus, the energy stored in the guest and spring while the guest climbed an energy barrier on the way out of the host was dissipated, rather than generating a favorable contribution to the pulling work after crossing the barrier. Importantly, this mechanism can occur even if the mean force varies smoothly with distance, due to sudden jumps in hidden variables that are not directly controlled by the pulling linkage.^[30] This mechanism for irreversibility in pulling calculations recapitulates a classical molecular explanation of macroscopic friction often called the Prandtl-Tomlinson model,^[32] which has also been used to explain friction in atomic force microscopy (AFM).^[33] The connection with tribology is physically reasonable, because friction is a manifestation of irreversibility. It is also worth remarking that there is a provable relationship between frictional dissipation and the deviation of a system from equilibrium.^[34]

Here, we introduce a more robust approach to computing reversible work along ligand extraction pathways, Fluctuation-Guided Pulling (FGP), which largely mitigates such irreversibilities, as the pulling spring is adaptively attached directly to the atoms of the guest that become stuck to the host. Making this change prevents the storage and subsequent dissipative discharge of energy. We report the use of both steered MD and umbrella sampling pulling calculations, by both standard and FGP techniques, to estimate the work of ligand extraction for two host-guest complexes which offer different technical challenges. These work terms are furthermore put into the context of a straightforward scheme for computing the standard free energy of binding termed the Attach-Pull-Release (APR) method.

The first host-guest system, cucurbit[7]uril (CB[7]) with B5 (*i.e.*, 1,4-bis(methylamine) bicyclo[2.2.2]octane, Figure 1), binds in a single, well-defined conformation, with strikingly high affinity in water ($-19.5 \text{ kcal/mol} \pm 0.2 \text{ kcal/mol}$ ^[36]). The second system, cucurbit[6]uril (CB[6]) with spermine (Figure 2), binds with somewhat lower affinity (-17.2 kcal/mol ^[37]). In addition, spermine is much more flexible than B5, and because it has four ionizable ammonium groups capable of sticking to the electronegative portals of CB[6], it is expected to have two stable bound conformations, states A and B in Fig. 2, and thus to present more complex dissociation and binding pathways than B5. Note that the present study aims not to precisely reproduce the experimentally measured affinities of these systems, but instead to generate improved methods for computing the binding free energy associated with a chosen energy model. Success in this methodological aim then provides a foundation for the future development of energy models capable of reproducing experimental binding free energies.

Methods

Computing standard binding free energies from ligand extraction simulations

One may express the standard free energy of binding (ΔG^0_{bind}) as the additive inverse of the sum of three terms: the reversible work of attaching an artificial spring connecting the host and guest (W_{attach}); the reversible work of adjusting the spring to extract the host from the guest (W_{pull}); and the reversible work of releasing the spring and leaving the guest as an ideal solute at standard concentration (W_{release}). (See Supporting Information.) In the adaptive FGP method (next subsection), we consider the adjustment of the spring to include changing its attachment points to the guest. Here, we only consider increasing the equilibrium length of a spring with fixed attachment points. Thus, we have

$$\Delta G_{bind}^0 = -(W_{attach} + W_{pull} + W_{release}) \quad (1)$$

$$W_{attach} = -\frac{1}{2} \int_0^k \langle (r - r_{si})^2 \rangle_{k'} dk' \quad (2)$$

$$W_{pull} = - \int_{r_{si}}^{r_{sf}} k \langle r - r_s \rangle dr_s \quad (3)$$

$$W_{release} = -k_B T \ln \left(\frac{1}{C^0} \right) - k_B T \ln \int_0^\infty 4\pi r^2 e^{-\beta \frac{k}{2} (r - r_{sf})^2} dr \quad (4)$$

where k_b is the Boltzmann constant, T is the absolute temperature, C^0 is the standard concentration expressed in the same units as r (here $1M = 1 \text{ molecule}/[1660 \text{ \AA}^3]$), r_s is the equilibrium length of the spring with spring constant k , and r_{si} and r_{sf} are respectively the equilibrium lengths at which the spring is initially attached and finally released. Angle brackets indicate Boltzmann averages obtained as averages over simulation snapshots. Thus, W_{attach} is influenced not only by the spring constant k , but also by the interactions of the ligand with the binding site while the spring is being attached, because this affects the conformational distribution, as implicit in the angle brackets. The need to account for the work of both imposing and releasing any artificial constraints used during a free energy calculation has been discussed previously.^[38] Note that an explicit definition of the bound complex^[9] is not required so long as the ligand adequately samples its bound state and does not sample obviously unbound conformations, while the spring is being reversibly attached.

The quantity W_{pull} corresponds to the difference between the potential of mean force (PMF) on going from the initial ($r=r_{si}$) state to the final ($r=r_{sf}$) state. Suitable initial and final distances are readily chosen for the complexes studied here, because the fully-bound states display little conformational diversity, and the fully unbound states may be identified by the onset of a regime in which the pulling force becomes negligibly small. Yet, care should be taken when computing PMFs of more intricate systems, given that these may display high conformational diversity within the initial and final state ensembles. The value of $W_{release}$ may be computed analytically (Eq. 4) based on the final force constant and equilibrium length of the spring, given that the distance between the host and guest is long enough that their interactions have become negligible. It is worth remarking that this final equilibrium length of the spring may depend on the final attachment point of the spring to the guest when the adaptive FGP method is used. Note that the binding free energy can also be computed by the reverse of this procedure; *i.e.*, by reversibly attaching the spring to a guest initially at standard concentration, reversibly shortening the spring to push the guest into the host, and then reversibly releasing the spring: $\Delta G_{bind}^0 = W_{push} - (W_{attach} + W_{release})$.

Ideally, the PMF for ligand extraction or insertion will be fully equilibrated at each spring length, or host-guest distance. However, the reality of finite sampling leads to the possibility of significantly incomplete equilibration at each distance and, as a consequence, some contribution of dissipative frictional work, $W_{friction} > 0$, to the observed work, W_{pull}^{obs} or W_{push}^{obs} . Thus, an irreversible pulling process would lead to an estimate of the binding free energy, $\Delta G_{bind,obs}^0$, that corresponds to a lower limit of the free energy one would obtain from a reversible process:

$$W_{pull}^{obs} = W_{pull} + W_{friction} \quad (5a)$$

$$W_{pull}^{obs} \geq W_{pull} \quad (5b)$$

$$\Delta G_{bind}^0 \geq \Delta G_{bind,obs}^0 = - (W_{attach} + W_{pull}^{obs} + W_{release}) \quad (5c)$$

Similarly, if instead of pulling we push the guest into the host, we find that the irreversible process provides an upper limit of the reversibly computed binding free energy:

$$W_{push}^{obs} = W_{push} + W_{friction} \quad (5d)$$

$$W_{push}^{obs} \geq W_{push} \quad (5e)$$

$$\Delta G_{bind}^0 \leq \Delta G_{bind,obs}^0 = W_{push}^{obs} - (W_{attach} + W_{release}) \quad (5f)$$

Accordingly, if one computes the binding free energy by pulling out the guest and then by pushing in the guest, where both processes may be to some degree irreversible, the two results should, within the limits of statistical uncertainty, bracket the single binding free energy one would obtain via a reversible process, whether by pulling or pushing; i.e.:

$$W_{pull}^{obs} \geq W_{pull} \geq -W_{push}^{obs} \quad (6)$$

In order to simplify nomenclature, we omit the “obs” superscript in the rest of the paper; the significance of the terms should be clear from the associated text.

Calculation of W_{pull} by Steered MD Simulations with the FGP Method

In steered MD with standard fixed spring attachment points, the PMF for ligand extraction or insertion may be estimated by computing the mean spring force over short time intervals (e.g., 1ps) during the pulling process and numerically integrating this mean force over distance. The Fluctuation-Guided Pulling (FGP) method is different in that it adaptively attaches the artificial spring to that part of the guest which most strongly sticks to the host at each step of the pulling process. This is done by monitoring which guest atoms have the smallest fluctuations relative to the host, and periodically updating the attachment point of the spring to these atoms. For each new guest attachment point, the initial equilibrium length of the spring is changed to match the distance between it and the attachment point on the host; thus, given that a very stiff spring is used, the degree of compression or extension of the spring is not changed when the attachment point is updated. The equilibrium length of the spring is then gradually increased in the course of the simulation to move the guest a little further relative to the host, and the mean force is averaged just as if standard fixed spring attachments were in use. The spring is then reattached according to the adaptive FGP scheme and the process iterates until the guest is fully removed from or inserted into the host, depending upon the direction of the simulation. In the present studies, the attachment point of the spring to the host remains unchanged throughout, but there may be settings in which this, too, should be adaptively changed. Further details of the FGP method follow.

At the start of the simulation, the spring is attached to any reasonable part of the guest. After a short, user-selected time interval Δt , we identify those non-hydrogen atoms of the guest having the lowest spatial fluctuation relative to the host during the interval Δt , based on their root mean square fluctuations (RMSF) in the coordinate system of the host. To compute these RMSFs, we fit all host conformations from the interval Δt onto each other, apply the same translations and rotations to the associated guest conformations, and then compute the RMSFs of the guest atoms. The center of mass of the guest atoms with lowest fluctuations (see below) is now chosen as the guest's linkage point for the pulling spring during the subsequent pulling interval Δt . The equilibrium length of the spring is therefore reset to the distance between the updated attachment points, computed for the most recent time-step, and the guest is pulled for another Δt . This overall procedure is iterated for successive intervals Δt until the guest is fully dissociated in the case of pulling, or fully bound in the case of pushing.

The lowest-fluctuation set of guest atoms (above) at each iteration are those whose RMSF is within a cutoff (F_{cut}) of $RMSF_{min}$, the minimum RMSF found for any guest heavy atom during that Δt . That is, given $RMSF_i$, the RMSF of each guest heavy atom i during an interval Δt , and the minimum value of $RMSF_i$ across all guest heavy atoms, $RMSF_{min} = \min\{RMSF_i\}$, we include all guest heavy atoms such that

$$\frac{RMSF_i}{RMSF_{min}} < F_{cut} \quad (7)$$

The optimal value of F_{cut} is that which minimizes irreversibilities in the computed pulling process, but this value is not known *a priori*, and may depend on the system. Thus, as a general recipe, multiple FGP runs with varying values of F_{cut} (in the range 1-5 for this paper) should be performed, and in both the forward (pulling) and reverse (pushing) directions, in order to arrive at an estimate of the reversible work which is closely bracketed by the pulling run with the highest value of W_{pull} and the pushing run with the lowest value of $W_{pull} = -W_{push}$ (Eq. 6). Note that the calculation of W_{attach} and $W_{release}$ (Eqs. 2 and 4) is unaffected by the use of FGP; one simply applies the existing formulae based upon the initial and final equilibrium spring lengths, r_{si} and r_{sf} . It is also worth mentioning that the present approach was selected based on an analysis of numerous variants. For example, we found that attaching the spring to the single guest atom with the lowest RMSF often led to conformational distortion of the guest, followed by dissipative discharge of the stored energy, and hence to greater irreversibility.

Two potential sources of error in the steered MD FGP approach deserve to be considered. One is the possibility that the nonequilibrium nature of the steered dynamics process might introduce a numerically significant irreversible work contribution, despite the slow speed of the pulling. The second is the method's neglect of a possible nonzero work contribution from the repeated detachment and reattachment of the pulling spring. The following subsection therefore describes how these issues can be avoided by using FGP in the context of rigorous umbrella sampling calculations analyzed with the Bennett Acceptance Ratio method. These calculations are useful in their own right and also provide a valuable check on the steered MD FGP approach.

Calculation of W_{pull} by Umbrella Sampling/BAR

We ran US simulations for windows along ligand extraction and insertion pathways defined by prior steered MD simulations that used either fixed spring attachment points or attachment points provided by the adaptive FGP method. For each window, the umbrella restraint consisted of a spring, or artificial bond, joining a host atom or group of atoms to a

guest atom or group of atoms. All US simulations used a total of 101 overlapping windows, equilibrated for 10 ns each. For the fixed attachment point runs, the starting conformation for each US window was a snapshot automatically extracted from the respective steered MD simulation at evenly distributed milestones along the total distance. However, for FGP, this procedure occasionally led to inadequate overlap between the energy histograms of successive windows in the BAR calculations, for about 4% of neighboring US window pairs. This problem traced to overly different spring attachment points for these few neighboring windows, and was resolved by using a different snapshot from the FGP pathway as starting conformation for one of the two windows, such that the atoms defining the pulling center of this new initial conformation would be different by at most two atoms from that of its neighboring windows. For all FGP cases, a snapshot satisfying this condition was found at most ± 3 pulling intervals (Δt , see previous section) away from the snapshot that was originally selected.

Values of W_{pull} were estimated from these US runs by Bennett's Acceptance Ratio (BAR) method.^[45] Thus, the free energy difference W_i between window i and the subsequent window $i+1$ was estimated as

$$W_i = -RT \ln \frac{\left\langle \frac{1}{1 + e^{\beta(H_{i+1} - H_i - C_i)}} \right\rangle_i}{\left\langle \frac{1}{1 + e^{\beta(H_i - H_{i+1} + C_i)}} \right\rangle_{i+1}} + C \quad (8a)$$

$$W_{pull} = \sum_{i=1}^{N-1} W_i \quad (8b)$$

where H_i is the Hamiltonian for window i , R is the ideal gas constant, T is the temperature, N is the total number of windows, and C_i is the “shift constant” for window i , and we assume an equal number of conformational samples in both windows. This expression is iterated until convergence of W_i . It is worth pointing out that Hamiltonians H_{i+1} in the numerator and H_i in the denominator are evaluated for the ensemble of conformations at windows i and $i+1$, respectively, and include a contribution of the artificial spring. In fact, the only difference between H_{i+1} and H_i is in the spring part of the Hamiltonian: in the case of fixed attachment points, the equilibrium length of the spring is different between successive windows, and, for FGP calculations, the spring's attachment points can also differ. Note that Equation 8a is equally valid and rigorous for US runs with fixed spring attachment points and for those in which the spring attachment points differ between windows according to the FGP method. In particular, it implicitly includes any consequences of spring detachment and reattachment in the FGP calculations, and therefore provides an independent check of the highly efficient steered MD FGP approach examined in this paper. Note, too, that the FGP attachment points used in the US-BAR calculations are derived from a steered MD run, so the US-BAR implementation of FGP is not in itself adaptive.

For all calculations, US simulations were also performed to calculate the work of initially attaching the artificial spring (W_{attach}), according to Eq. 2. For this purpose, 34 (N_1 - C_1 pulling) or 35 (COM_g - COM_h pulling) equidistant windows were simulated at the initial complexed state with an increasingly higher force constant k' ($0 < k' < k$), and the pulling forces were numerically integrated with respect to k' across all windows. The present results used a conservative 10 ns of simulation time per window, but a retrospective analysis indicates that 1 ns/window would have sufficed.

System Setup and Computational Details

Fully bound CB[7]-B5 and CB[6]-spermine complexes were initially set up by merging energy minimized conformations of free ligand and free host, via Swiss PDB viewer.^[39] The stability of the bound states was verified by carrying out 10-ns unrestrained molecular dynamics simulations at 300 K using the Generalized Born/Surface Area^[40] (GBSA) implicit solvent model. The VC/2004 parameter set, accessible through the Vcharge program,^[41] was used to generate partial atomic charges for both complexes. All bonded and Lennard-Jones interactions were modeled via the Amber99sb^[42]/GAFF^[43] force fields. The aqueous solvent was modeled with the GBSA approach.^[40] The GROMACS^[44] molecular simulation package (*ver.* 4.5.5) was employed for all simulations. Conformations were saved every picosecond; this resulted in 10,000 structures for each unrestrained MD run, 100,000-150,000 for each steered MD simulation, and 1,010,000, 340,000 or 350,000 structures for each US run. The systems were evolved by means of the velocity Langevin dynamics algorithm with a 1 fs time step, and maintained at a constant temperature of 300 K (coupling constant $\tau_t = 10$ ps). The initial velocities for each simulation were randomly generated from a Maxwell-Boltzmann distribution at 300 K.

In the steered MD simulations, the equilibrium length of the artificial spring between the host and the guest was increased (pulled dissociation process) or decreased (pushed binding process) at a rate $v_{pull} = 0.01$ nm/ns, using a stiff spring with force constant $k = 100$ kcal/mol/ $\text{\AA}^2$. As attachment points on the host, some calculations used an equatorial carbon (C_1 in Figs. 1 and 2), while others used the center of mass of its equatorial carbons (COM_h). For conventional steered MD runs, the guests were pulled either from a terminal nitrogen (N_1 in Figs. 1 and 2), or the center of mass (COM_g). For FGP runs, we used COM_g - COM_h as the initial attachment points and then iteratively updated the attachment point on the guest (but not that of the host) every $\Delta t = 0.1$ ns. The starting conformations for all forced dissociation simulations of both complexes were chosen such that the length of the artificial bond closely matched that of the respective unrestrained complex at equilibrium; *i.e.*, the average distance between the intended initial attachment points during the unrestrained MD runs initially performed at the fully bound state. For pushed binding processes, the initial structures were arbitrarily selected from the final 100 ps of a prior dissociation run. The *pull code* routine in GROMACS was used to evolve all pulling simulations; for FGP, this is a stepwise process given that the attachment points may be modified after each interval Δt .

Results

For each complex, we begin by studying force profiles and conformational changes along pathways of ligand dissociation and binding. For both steered MD and umbrella sampling calculations, irreversibilities are observed when the attachment points of the spring are held fixed during the process, and the adaptive FGP approach largely resolves these problems. We then present the results of binding free energy calculations based on integration of force profiles for the steered MD simulations, and on the Bennett Acceptance Ratio method for the umbrella sampling calculations. For both approaches, the adaptive spring attachment points from FGP reduce irreversibility and thus provide tighter bounds on the computed binding free energy.

CB[7]-B5 system

Forces and conformations along forced dissociation and binding pathways

Steered dynamics with fixed attachment points: When the ligand is pulled from the host, using single atoms N_1 and C_1 as linkage points for the spring (Fig.1), the pulling force (green, Figure 3) ramps up from essentially zero to a rupture force of roughly 1200 pN over a distance of about 3 $\text{\AA}$, then collapses back to zero in an apparently discontinuous manner.

A prior mechanistic study of this complex [30,31] indicates that the sudden drop in force occurs when the bulky bicyclooctane moiety of the guest completes its passage through the ring of carbonyls at the exit portal of the host. Thus, despite tight control of the N_1 - C_1 distance by the stiff spring (blue, Fig. 4), there is a jump in the distance between the center of mass of the host's equatorial carbons (COM_h) and the center of mass of the guest (COM_g) (red, Fig. 4), when the bicyclooctane moiety exits the host at 46 ns of simulation time. The sudden change in the COM_g - COM_h distance, corresponding to the collapse in force seen in Figure 3 (green curve), suggests that this N_1 - C_1 pulling scheme guides B5 along a dissociation pathway that is highly irreversible. This irreversibility is demonstrated by comparing the pulling result with binding simulations in which B5 is pushed into the host from a fully unbound conformation, by gradually reducing the N_1 - C_1 distance to match the pulling scheme used during dissociation: the mean force as a function of pushing distance during this reversed process (orange, Figure 5) is clearly different from the mean force as a function of the forward pulling distance (green, Figure 5 and Figure 3).

In some cases, reversibility may be improved by restraining a ligand during a pulling calculation. [38] We tested this approach for the present system by making the guest more rigid with strong restraining potentials ($k_{tors} = 240$ kcal/mol/rad²) to hold its rotatable torsions at values virtually equivalent to those found in the initial fully bound conformation. However, the resulting force-distance curve (red, Figure 3) is essentially unchanged relative to the prior result for the flexible guest (green, Figure 3), and thus does not solve the irreversibility problem.

A possible concern with the N_1 - C_1 attachment points is that the mean spring force is not directed along the axis of symmetry of the host-guest system. However, alternative attachment points where the spring forces are on average directed along the axis of symmetry still yield significantly different force profiles for the pulling and pushing directions. For instance, when the spring is attached to the centers of mass of the guest and host (COM_g - COM_h), the force-distance curve for the pulling calculation (blue, Figure 3) has a lower peak force and includes a negative region from about 4 to 6 Å, where the spring is compressed by the two molecules as the guest is smoothly expelled from the host after crossing the peak of the energy barrier. This ejection occurred in the prior pulling simulations as well, but there it caused a conformational jump because the spring did not control of the barrier-crossing process. This jump led to dissipation of the energy stored by the tension in the molecular system, whereas here the expulsive force acts back on the artificial spring and is registered as a negative region in the force-distance graph. As detailed below, this allows the downhill part of the energy barrier to be accounted for in W_{pull} , as desired for an accurate reversible work. However, using the same COM_g - COM_h spring to push the guest back into the host yields a force profile with a marked discontinuity (Figure 6, purple), as the force peaks at -1080 pN then collapses to zero. During this process, the guest gradually tilts sideways as it approaches the host, eventually coming to lie flat across the entry portal, presumably due to electrostatic attractions between the carbonyl oxygens of the host and the ammonium groups of the guest. With increasing force, the guest suddenly rotates and threads the host. Thus, use of the COM_g - COM_h attachment points does not yield a reversible process, and this irreversibility is reflected in the computed free energies (below). An alternative set of symmetric spring attachment points, guest N_1 to host COM_h , also yielded markedly irreversible results (orange, Figure 3).

Umbrella sampling with fixed attachment points: The more extensive equilibrium sampling afforded by the umbrella sampling approach, with windows along the N_1 - C_1 or COM_g - COM_h pulling and pushing pathways, might be expected to provide a well converged value for the reversible work. This need not be the case, however, as shown by prior US calculations for this system using the N_1 - C_1 attachment points, [30] where the force profile

obtained from 10-ns US simulations closely matched that obtained from a clearly irreversible steered MD simulation. (See Fig. 4 in ref.^[30]). In order to further examine this issue, we carried out US runs based on the COM_g-COM_h attachment point pulling and pushing simulations. Figure 6 shows that the corresponding force profiles (dark gray and green, respectively), agree well with those of the corresponding steered MD runs, implying that the windowed configurations remain close to the initial states identified from the nonequilibrium steered simulations. As a consequence, the US force profiles replicate the irreversibility evident in the steered dynamics profiles, although the lower magnitude of the negative peak in the binding US profile (green) suggests some improvement relative to the corresponding steered MD result (purple). It is also worth noting that a small dip in the forces is observed between 5.1 and 5.8 Å in the dissociation US profile; this suggests that the steered MD dissociation pathway may not be fully equilibrated at a low energy state, and thus may be slightly irreversible within this range of the pulling bond length.

Steered dynamics with fluctuation-guided pulling (FGP): Initial runs of the FGP scheme with several different values of F_{cut} showed that values of 1.9 and 1.3 for the pulling and pushing calculations, respectively, led to very similar mean force profiles (Figure 7). Interestingly, these two pathways now display a dip in the forces at 51-55 ns and 25-28 ns for dissociation and binding, respectively, which was not observed during the COM_g-COM_h steered MD simulation but was observed upon further equilibration of the COM_g-COM_h pulling pathway via US; i.e., the 5.1-5.8 Å range in Fig. 6 (prior subsection). These results suggest that the FGP scheme successfully drives both the forward and reverse processes along a well-equilibrated route, including a critical region requiring longer equilibration times.

Binding free energies from steered dynamics and umbrella sampling—We now report potentials of mean force (PMFs) from the steered dynamics force profiles described above, use them to estimate W_{pull} , and compare these values with US-BAR estimates of the same quantity. Figure 8 shows the steered dynamics PMFs for N₁-C₁, COM_g-COM_h and FGP pulling and pushing calculations. Here, all results are referenced to the unbound state, so the values of $-W_{pull}$ can be read directly from the y-axis. For each spring attachment scheme, the pulling and pushing calculations are expected to bracket the desired reversible value of W_{pull} in accordance with Eq 6.

As summarized in the first three numerical rows of Table 1, the two fixed spring attachment schemes, N₁-C₁ and COM_g-COM_h, bracket the value of W_{pull} within excessively wide ranges of >10 kcal/mol, while the FGP method yields very similar values for both directions, consistent with a high degree of reversibility in this process. It is also worth noting that the steered MD FGP calculations lead to bracketing values of W_{pull} that are still in accordance with Eq. 6.

Interestingly, the results of the US-BAR calculations with windows based on COM_g-COM_h and FGP pulling and pushing simulations (last two lines of Table 1), are all within 2.2 kcal/mol of their corresponding steered MD simulations. This observation has significant implications. First, it demonstrates that apparently well-converged US-BAR calculations may yield results that deviate substantially from the desired reversible work. In particular, if one used only the five US-BAR binding calculations to estimate W_{pull} , one might interpret the narrow range of results ($-16.4 > -W_{pull} > -17.4$ kcal/mol) and small squared error as indicative of a converged result. However, this would clearly be incorrect, because its mean is 9.5 kcal/mol away from the tightly bracketed reversible value of -26.4 kcal/mol computed using FGP combined with US-BAR. Second, the agreement between the results of FGP with steered MD and FGP combined with US-BAR supports the validity of the work estimates computed directly from FGP. In particular, it suggests an imperceptible contribution to W_{pull}

arising from the work of iteratively changing spring attachment points in the course of the FGP calculations. Finally, because the US-BAR calculations based on FGP pathways make no approximation regarding the work of spring detachment and reattachment, and they provide relatively tight (1.8 kcal/mol) brackets for the value of W_{pull} , this combination of FGP with US-BAR appears to be the most reliable and rigorous of the various approaches examined here.

It is of interest to incorporate these estimates of W_{pull} into the APR formulation laid out in the Methods section (see Eq. 1), in order to obtain estimates of the standard binding free energy of this complex. The full binding free energies, provided in Table 1, account not only for the pulling work, but also the work of initially attaching (W_{attach}) and finally releasing ($W_{release}$) the spring. Interestingly, the most rigorous FGP/US-BAR results overestimate the experimental value of -19.5 ± 0.2 kcal/mol^[36] by several kcal/mol, consistent with previous analyses from our group, which indicate that the present energy model needs further adjustment in order to yield accurate results for these systems.^[46] It is also worth reiterating that the ranges provided for ΔG^0_{bind} represent the bracketing of the reversible free energy value by the results from the forward and reverse processes, in accordance with Eq. 6, and that much smaller brackets would have been obtained if one had attended only to statistical error inferred from runs in just the forward or just the reverse direction.

CB[6]-spermine system

Forces and conformations along forced dissociation and binding pathways

Steered dynamics with fixed attachment points: The force profile corresponding to the dissociation of spermine from CB[6] with N_1 - C_1 fixed attachment points has two sharp peaks of similar magnitude (Figure 9, green). These correspond to two threading transitions, first from state A to state B, and then from state B to the unbound state (see Fig. 2). Much as for the CB[7]-B5 system discussed above, the N_1 - C_1 pulling profile for CB[6]-spermine shows a sudden collapse of the force to zero after each peak and resembles the stick-slip friction regime previously observed in AFM studies of surfaces,^[33] as schematized in Figure 10. In the present case, the guest sticks to the host while climbing the energy barrier, accumulating energy which is irreversibly dissipated once it slips down to the following state. In both settings, this is a mechanism of frictional dissipation and hence thermodynamic irreversibility. Because these irreversible transitions fail to capture expulsive forces on the spermine guest, they are expected to result in an overestimate of W_{pull} and hence of the computed binding affinity, as confirmed below.

The force profile with fixed COM_g - COM_h attachment points is similar to that with fixed N_1 - C_1 points (above), but includes a region of negative forces roughly between 2.5 and 5 Å (Figure 9, blue). Here, the spring is compressed during controlled expulsion of the guest from state A to state B and thus registers the work done by the expulsive force, rather than allowing dissipation of all the stored energy. Use of the COM_g and COM_h as attachment points for the pulling spring is therefore expected to yield a better approximation to the reversible work of this barrier crossing event. However, this is not the case for the second, unbinding transition, where even the COM_g - COM_h pulling scheme permits a sudden collapse in the forces during the sudden ejection of spermine from the CB[6] cavity, as confirmed by visual inspection of the MD trajectories.

Thus, in contrast to the case of CB[7]-B5, here even the COM_g - COM_h pulling scheme allows obvious irreversible jumps in force and conformational state when the spermine guest is pulled from the CB[6] host. The difference between the behaviors of these two complexes stems from the great flexibility of spermine, whose center of mass moves outside the host before completion of the dissociation transition, leaving it only loosely linked by the guest's

flexible chain to the interfacial region, where spermine is stuck to the exit portal of CB[6]. Although we tried several additional sets of fixed spring attachment points, none succeeded in preventing sudden conformational discontinuities at both spermine transitions. For example, attaching the spring to atom N₄ of the guest (Figure 2) and COM_h prevented any obvious irreversibility during the dissociation step, but allowed a discontinuous force profile for the A→B threading transition. (Data not shown.) These observations appear to reflect a fundamental problem associated with the use of fixed spring attachment points when attempting to compute reversible work for flexible molecules. The following subsection shows that this problem persists when equilibrium umbrella sampling is used instead of nonequilibrium steered dynamics.

Umbrella sampling with fixed attachment points: As for the CB[7]-B5 system, we inquired whether extended equilibrium simulations could yield reversible processes despite the use of these fixed spring attachment points. Starting with frames drawn from evenly spaced times along the steered MD simulations with fixed N₁-C₁ and COM_g-COM_h spring attachment points, we initiated successive US windows with corresponding spring lengths, and simulated each window for 10 ns. Plots of mean force versus the mean length of the spring (Fig. 9, orange and dark gray) recapitulate the qualitative features observed in the corresponding steered MD profiles, though with peaks of somewhat lower magnitude. In particular, the US simulations show steep drops in force as a function of distance and fail to register the expulsive (negative) forces expected during conformational transitions. This observation largely recapitulates the analogous study for the CB[7]-B5 host-guest system, and further substantiates the concern that fixed spring attachment points readily generate irreversibilities.

Steered dynamics with fluctuation-guided pulling (FGP): Steered MD simulations with the FGP spring attachment method, using F_{cut} values of 3.0 and 1.9 for the pulling and pushing directions, respectively, provide similar mean force profiles for the two directions (Figure 11). The two conformational transitions (A to B, and B to dissociated) are smooth, and both transition peaks are followed on the right by regions of negative force. This result points to controlled, rather than dissipative, release of energy stored while climbing the energy barriers, and in fact, no sudden conformational jumps were observed upon inspection of the MD trajectories. Thus, the FGP scheme appears to yield non-dissipative processes for both binding and dissociation.

Binding free energies from steered dynamics and umbrella sampling—

Integration of the pulling force profiles for the fixed attachment point and the pulling and pushing FGP force profiles yields the PMFs shown in Figure 12, where distances are referenced to bound state A and the energy is referred to the dissociated state. Applying Eq. 6 yields $8.6 > W_{pull} > 8.1$ kcal/mol for these FGP calculations. The observed values of the pulling work for the N₁-C₁ and COM_g-COM_h steered MD, 14.5 and 12.8 kcal/mol, respectively, are greater than the FGP results, consistent with the dissipative nature of these processes.

As in the case of CB[7]-B5, we also performed five US simulations starting from the binding pathway identified via FGP, with different random assignments of initial velocities, and used the BAR method to estimate W_{pull} for all five US calculations. A representative PMF for these runs is shown in Fig. S2, Supporting Information. These calculations yield a mean value of -8.4 kcal/mol for W_{pull} , and all five results narrowly bracket W_{pull} in the range $8.8 - 8.0$ kcal/mol. These more rigorous US-BAR results are similar to and nicely bracket those from the nonequilibrium steered MD FGP simulations. Overall, then, FGP performs well even for this rather complex two-state transition, for both the steered MD and US-BAR approaches.

For completeness, we examine the resulting estimates of ΔG^0_{bind} for this complex. Incorporating the FGP steered MD results into the APR framework of Eq. 1 by including W_{attach} and $W_{release}$ yields $\Delta G^0_{bind} = -8.4 \pm 1.1$ kcal/mol. The small range of this estimate is pleasing, and it correctly indicates that CB[6]-spermine has lower binding affinity than CB[7]-B5, although it is far from the measured result of $\Delta G^0_{bind} = -17.2$ kcal/mol.^[37] As noted above, the deviation from experiment presumably results from problems with the force field and/or solvation model,^[46] a topic outside the scope of this study.

Discussion

The calculation of binding free energies by the integration of force over distance as a bound ligand or guest is gradually pulled from its binding site represents a potentially powerful tool for computer-aided molecular design. There are three central requirements for such a calculation to be valid. The first is a practical protocol for computing the work of the pulling process in a reversible manner, so that one obtains a state function which can be related to thermodynamics. The second is a suitable theoretical framework connecting the computed work to the experimentally observable standard binding free energy. The third is a sufficiently accurate energy model, the function that provides an estimate of the energy for each conformation of the molecular system. The present paper addresses the first and second requirements, using two host-guest complexes as tractable model systems for elucidating key issues and testing possible solutions.

Identification of low-dissipation pathways by FGP

A central result of the present study is the identification of fixed spring attachment points as a potential basis for irreversibility in both steered MD and equilibrium US calculations of the work of pulling a ligand from a binding site. The physical mechanism at work is the dissipative release of energy stored in the molecules as energy barriers are crossed, which is related to well-known microscopic explanations of macroscopic friction.^[32,47] Given the near ubiquity of frictional forces, it is perhaps not surprising that eliminating this source of irreversibility in computations can be nontrivial, as now discussed.

First, one might expect that increasing the stiffness of the pulling spring could solve this problem. However, this is not the case when it is the molecules themselves that are stretched and hence store the energy which is subsequently dissipated. Another potential solution might be to run a slower pulling calculation; but the computer time required for such a brute-force approach easily becomes prohibitive, given that reversibility is ensured only when infinitely low speeds are enforced. In particular, the problem is not necessarily solved by moving from steered MD to equilibrium simulations; *i.e.*, by using umbrella sampling to equilibrate the system in windows along a pulling or pushing process. More sophisticated equilibrium sampling methods, such as replica exchange-umbrella sampling may be a useful alternative, but may also have greater computational costs, which can pose a barrier to routine use. Jarzynski's elegant equality^[48] also deserves mention in this regard, because it can in principle yield the desired reversible work from an ensemble of irreversible pulling calculations. This approach is most useful when it is applied to an ensemble of trajectories that includes some with low work contributions, given that it involves an exponential average of the work computed for each trajectory and hence is dominated by the trajectories with the smallest work values. If such trajectories are rare, as expected in the systems investigated here – since there is a clear tendency for the guests to catch the host and then suddenly release it – then the exponential average will be hard to converge.

We have proposed to address this source of irreversibility by the Fluctuation Guided Pulling (FGP) method, which adaptively shifts the spring's attachment point to the center of mass of those guest atoms that fluctuate least with respect to the host. This is expected to be the part

of the guest that interacts most tightly with the host. Keeping the spring's guest attachment close to the point where the guest contacts the host prevents the pulling process from stretching out the guest, and thus reduces the ability of the guest to store and suddenly release energy in a dissipative manner. The FGP method is straightforward to implement and was found to substantially mitigate irreversibilities in the present applications, based on direct assessments of reversibility in which the guests were pushed into the binding site instead of being pulled out, as well as on extended equilibration of these dissociation and binding pathways via US. We furthermore demonstrate that the trajectories generated by a steered MD calculation with FGP provide an excellent starting point for rigorous and reversible free energy calculations in which the Bennett Acceptance Ratio method is combined with umbrella sampling along a binding or dissociation pathway. It is worth reiterating here that even the rigorous US-BAR approach can yield highly irreversible results when used with fixed spring attachment points, as detailed in Results.

Several directions for further development and application of the FGP approach deserve mention. For one thing, it will be of interest to extend these studies to protein-ligand systems. Because proteins can be more flexible than the host molecules studied here, it may be necessary to adaptively update the attachment point of the spring not only to the ligand, but also to the protein, so that the stretching and sudden release of parts of the protein does not lead to irreversibilities. A second direction is to explore the use of markers other than RMSF to optimize the identification of the relative strength of host-guest atomic interactions throughout the pulling process. Finally, once a highly accurate energy model has been identified for these host-guest systems, it would be interesting to explore the use Jarzynski's equality to improve bracketing of the reversible pulling work by combining the work computed from multiple forward and reverse FGP runs with optimal values of F_{cut} . Such a set of calculations has the appeal of being trivially parallelizable.

Attach-Pull-Release: A framework for calculation of ΔG^0_{bind}

For the theoretical framework, we have made use of an attach-pull-release (APR) approach, a broadly applicable and straightforward way of connecting binding free energies with pulling calculations. This approach highlights the fact that the standard binding free energy is not simply the difference in the PMF between the bound and free states. Indeed, it cannot be, because the standard binding free energy depends on standard concentration,^[9] whereas the PMF difference does not. In the APR approach, the standard concentration enters through the work of releasing the spring from the guest molecule after it has been pulled from the binding site: a lower standard concentration makes the release more favorable on an entropic basis, because the released guest in effect has more space in which to wander when it is free. (If one pushes the guest back into the binding site, rather than pulling it out, then the standard concentration appears in the work of attaching the spring to the initially free guest.) It is also worth noting that the work of attaching the spring to the bound guest at the start of a pulling procedure depends on how tightly the guest is held in the binding site. Thus, attaching the spring reduces the fluctuations of the distance between its attachment points on the guest and host, so the work of attachment will be greater if these fluctuations are large before the spring is attached.

Reversibility: a critical test for validation of forced pulling calculations

It would appear that there is currently no definitive, systematic diagnostic that can determine whether the work obtained from a unidirectional pulling study is significantly irreversible, except in the special case where one knows the reversible work *a priori*. In particular, although a sudden drop in the mean force as a function of distance or time is strongly suggestive of irreversibility, the absence of such a drop does not ensure reversibility, as sudden dissipative relaxations of parts of the system to which the spring is not attached can

generate irreversibly without producing sharp changes in the mean force profile.^[30] Visual examination may bring such conformational relaxations to light, but it is not a given that an irreversible trajectory will have a visually apparent conformational jump. Therefore, it seems essential to test explicitly for irreversibility by actually running the process in question forward and backward and comparing the additive inverse of the pushing work obtained thereby with the forward, pulling work. The closer the two values, the better. Moreover, because the irreversible work of each process must be greater (more positive) than its reversible work, one may use the inequalities in Eq. 6 to bracket the desired quantity. It is important to recognize that, if one has not definitely ascertained that the computed work is the reversible work afforded by the model, then one cannot rule out the possibility that an observed concordance between the model and experiment derives from fortuitous cancellation of errors between an irreversible computed work and an inaccurate force field. It is also interesting to note that parallel considerations would appear to apply when AFM, or similar experiments, are used to estimate free energy differences between molecular states via experimental work integrals.^[49–52]

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. CB[7]-B5 complex at its fully bound equilibrium state. The atoms are colored by type: nitrogen (blue), oxygen (red), carbon (cyan) and hydrogen (white). Atoms C₁, N₁ and N₂ are labeled (see text).

Figure 2.

CB[6]-spermine complex at one of its bound equilibrium states, termed state A in the text.

The colors of the atoms are the same as in Fig. 1, and atoms C₁, and N₁-N₄ are labeled.

Schematic diagrams showing the position of the guest relative to the host at bound states A and B are also included.

Figure 3.

Force vs. distance (length of the pulling spring) for the dissociation of CB[7]-B5, obtained from steered MD simulations with pulling schemes as noted in the legend. The data points correspond to averages over 1-ps intervals.

Figure 4. Distance between N_1-C_1 atoms and $COM_g - COM_h$ groups, averaged over 1-ps time intervals, during a forced dissociation simulation of CB[7]-B5 with the spring attached to atoms N_1 and C_1 of the guest and host, respectively.

Figure 5.

Force vs. N_1 - C_1 distance for forced dissociation and binding simulations of CB[7]-B5, using the N_1 - C_1 pulling scheme.

Figure 6.

Force vs. $\text{COM}_g - \text{COM}_h$ distance for steered MD dissociation (blue crosses) and binding (purple, filled circles) simulations of CB[7]-B5, and for US runs started from configurations along the steered MD dissociation (dark gray) and binding (green filled squares) pathways. All force profiles employed the $\text{COM}_g - \text{COM}_h$ pulling/restraining scheme.

Figure 7.

Force vs. simulation time for forced dissociation and forced binding simulations of CB[7]-B5, using FGP with $F_{cut} = 1.9$ and 1.3 for pulling and pushing, respectively. We use force versus time instead of force versus distance, because the length of the pulling spring does not increase monotonically during FGP, unlike the previous cases where the attachment points are fixed throughout the whole simulation. Accordingly, both the increasing and decreasing time scales for dissociation (exterior numbering, red tick marks) and binding (interior numbering, black tick marks), respectively, are displayed along the x -axis.

Figure 8.

Potentials of mean force computed from forced dissociation and binding simulations of CB[7]-B5 using the N_1-C_1 , $COM_g - COM_h$ and FGP ($F_{cut} = 1.9$ and 1.3 for pulling and pushing, respectively) pulling schemes. The line styles are matched between the dissociation and binding runs that were performed with an equivalent pulling scheme. The color of each curve is the same as the corresponding force profile of Figs. 5-7.

Figure 9.

Force vs. distance (length of pulling spring) for the dissociation of CB[6]-spermine, obtained from steered MD simulations with N_1-C_1 (blue) and $COM_g - COM_h$ (green) pulling schemes, and from US simulations along the N_1-C_1 (orange) and $COM_g - COM_h$ (dark gray) distance restraints, started from configurations along their corresponding steered MD pathways. The data points correspond to averages over 1-ps intervals. Note that the initial length of the pulling spring for the N_1-C_1 simulations is 9.4 Å, whereas that of the $COM_g - COM_h$ simulations is 0.35 Å.

Figure 10.

Schematic diagram of the stick-slip behavior for the transition between states A and B of CB[6]-spermine. The host's carbonyl oxygens and the guest's ammonium groups are represented in red and blue, respectively. The inset portrays a typical force profile obtained from an AFM experiment performed within a stick-slip regime.

Figure 11.

Force vs. simulation time for forced dissociation and forced binding simulations of CB[6]-spermine, using FGP with $F_{cut} = 3.0$ and 1.9 , for pulling and pushing, respectively. As in Fig. 7, the x -axis displays increasing and decreasing time scales for dissociation (exterior scale) and binding (interior scale) respectively.

Figure 12.

Potentials of mean force computed from forced dissociation and binding simulations of CB[6]-spermine using the N_1-C_1 , $COM_g - COM_h$ and FGP ($F_{cut} = 3.0$ and 1.9 , for pulling and pushing, respectively) pulling schemes. The color of each curve is the same as the corresponding force profile of Figs. 9 and 11.

Table 1

Observed values of W_{pull} and $-W_{push}$ for the CB[7]-B5 host-guest system. For steered MD, the results are obtained by integration of mean force over distance. For umbrella sampling (US), the results are from free energy differences between successive windows computed by the BAR method. Each US-BAR value derives from five independent US calculations with 10 ns simulation windows, each US calculation having been started with a different random assignment of initial atomic velocities. The table lists the highest and lowest values of W_{pull} and $-W_{push}$ found across all five runs, in each case. Application of Eq. 10a in ref. [45] yields minimal mean squared errors ranging from 0.22 to 0.49 (kcal/mol)² for the BAR calculations. A representative PMF is shown in Figure S1, Supporting Information. Estimated ranges of the standard binding free energy, ΔG_{bind}^0 , were obtained by combining the estimates of W_{pull} and $-W_{push}$ with the work of initial spring attachment and final spring detachment, according to Eq. 1, using a standard concentration of 1 M; note that these $\pm$ ranges are not a reflection of statistical noise but of the inequality expressed in Eq. 6.

	Pulling Scheme	W_{pull} (kcal/mol)	$-W_{push}$ (kcal/mol)	ΔG_{bind}^0
Steered MD	N ₁ -C ₁	27.3	9.5	-18.7 $\pm$ 8.8
	COM _g -COM _h	27.5	16.0	-20.7 $\pm$ 6.1
	FGP	26.1	25.1	-24.8 $\pm$ 0.5
US-BAR	COM _g -COM _h	26.4	16.4	-20.7 $\pm$ 5.0
	FGP	27.3	25.5	-25.7 $\pm$ 0.9