

# Validation of Free Energy Methods in AMBER

Hsu-Chun Tsai,<sup>§</sup> Yujun Tao,<sup>§</sup> Tai-Sung Lee, Kenneth M. Merz, Jr., and Darrin M. York\*



Cite This: *J. Chem. Inf. Model.* 2020, 60, 5296–5300



Read Online

ACCESS |



Metrics & More

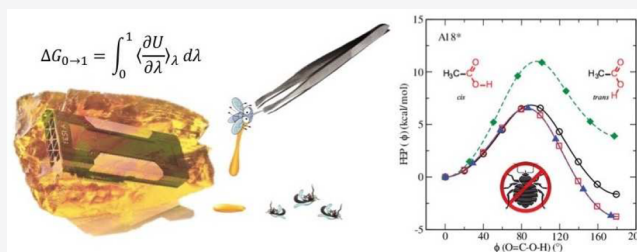


Article Recommendations



Supporting Information

**ABSTRACT:** Herein we provide high-precision validation tests of the latest GPU-accelerated free energy code in AMBER. We demonstrate that consistent free energy results are obtained in both the gas phase and in solution. We first show, in the context of thermodynamic integration (TI), that the results are invariant with respect to “split” (e.g., stepwise decharge–vdW–recharge) versus “unified” protocols. This brought to light a subtle inconsistency in previous versions of AMBER that was traced to the improper treatment of 1–4 vdW and electrostatic interactions involving atoms across the softcore boundary. We illustrate that under the assumption that the ensembles produced by different legs of the alchemical transformation between molecules A and B in the gas phase and aqueous phase are very small, the inconsistency in the relative hydration free energy  $\Delta\Delta G_{\text{hydr}}[A \rightarrow B] = \Delta G_{\text{aq}}[A \rightarrow B] - \Delta G_{\text{gas}}[A \rightarrow B]$  is minimal. However, for general cases where the ensembles are shown to be substantially different, as expected in ligand–protein binding applications, these errors can be large. Finally, we demonstrate that results for relative hydration free energy simulations are independent of TI or multistate Bennett’s acceptance ratio (MBAR) analysis, invariant to the specific choice of the softcore region, and in agreement with results derived from absolute hydration free energy values.



Alchemical free energy simulations are widely used for many computational biophysics and drug discovery applications.<sup>1–3</sup> The recent development of GPU-accelerated free energy methods<sup>4–7</sup> has enabled free energy simulations to be performed with sufficient precision for rigorous stress testing of implementations for consistency, reproducibility, and reliability. This, along with the development of rich data sets of solvation,<sup>8</sup> absolute,<sup>9</sup> and relative ligand binding free energies<sup>10–12</sup> for drug discovery,<sup>13,14</sup> is vitally important to ensure the predictive capability of these methods, particularly for computational lead optimization, where the relative binding affinities of candidate ligands may differ by less than 0.5 kcal/mol.

Recently, a study by Loeffler and co-workers<sup>15</sup> compared the reproducibilities of free energy calculations across different widely used molecular simulation software packages. In that work, it was brought to light that some calculations using AMBER16 did not produce internally consistent results. Herein we establish rigorous validation of free energy methods in the updated version of AMBER using the same data set of molecules and transformations (chosen in part to avoid complex sampling and allow high precision to be achieved) and extending the scope of the analysis. We illustrate the source of the reported inconsistent behavior in AMBER16 and demonstrate consistency of the results to less than 0.1 kcal/mol with AMBER18 (and also the most recent AMBER20 release<sup>16</sup>). It is the hope that the benchmarks and protocols reported here will be instrumental to a broad community for

the validation and practical application of other free energy methods and simulation software.

We first examine the test set of molecules illustrated by Loeffler et al.<sup>15</sup> and demonstrate using their procedures that inconsistencies in the “split” versus “unified” protocols observed with versions of AMBER prior to AMBER18 (designated pA18) no longer exist in AMBER18 with patch updates (designated A18\*) and the most recent AMBER20 release.<sup>16</sup> For the unified protocol, both electrostatic (Ele) and van der Waals (vdW) interactions are scaled simultaneously using softcore potentials from real atoms that are transformed into dummy atoms (or vice versa). The unified protocol is also sometimes called a “single-step” or “one-step” transformation. Different from the unified protocol are so-called “split” (or alternatively “multistep”, “stepwise”, or “decoupled”) procedures.<sup>17</sup> In these procedures, all or parts of the Ele and vdW transformations are decoupled and performed as separate steps. The split protocol described by Loeffler et al.<sup>15</sup> is a two-step procedure that is also used in the present work to provide a direct comparison. Later in the paper, we adopt the following three-step “decharge–vdW–recharge” split protocol: the atoms in the softcore region (i.e., the atoms that will be

**Special Issue:** Novel Directions in Free Energy Methods and Applications

**Received:** March 23, 2020

**Published:** June 18, 2020

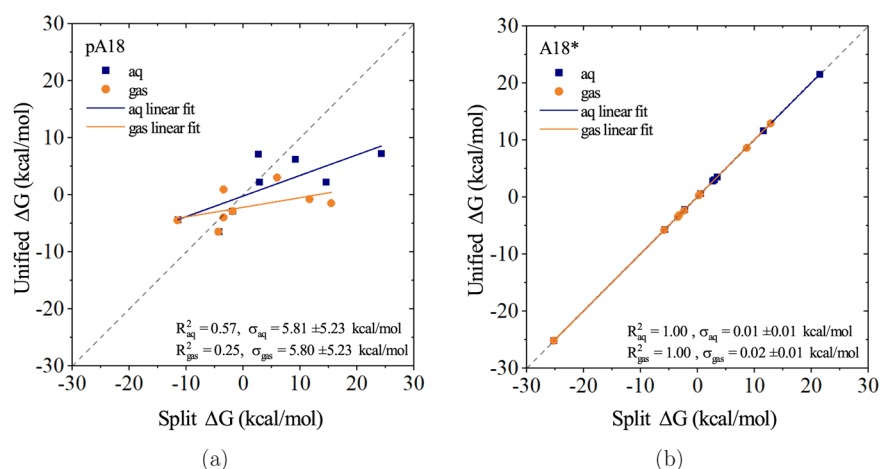


ACS Publications

© 2020 American Chemical Society

5296

<https://dx.doi.org/10.1021/acs.jcim.0c00285>  
*J. Chem. Inf. Model.* 2020, 60, 5296–5300



**Figure 1.** Comparison of  $\Delta G_{aq}$  and  $\Delta G_{gas}$  values (designated generically as  $\Delta G$  on the axis labels) using the unified and split protocols for nine transformations from simulations using (a) pre-AMBER18 (pA18) (values listed in Table S3) and (b) current updated AMBER18 (A18\*) (values listed in Table S4).

transformed into dummy atoms) are first decharged; next, these decharged atoms undergo a vdW transformation using softcore potentials; finally, the atoms are recharged to the final state. Herein, we use the terms “unified” and “split” in order to be consistent with those used in the work by Loeffler et al.,<sup>15</sup> although in subsequent work we prefer the terms “concerted” and “stepwise”.

In the discussion that follows, we separate the atoms involved in the alchemical transformation into two regions: the softcore region and the common core region. The common core atoms are those that are transformed from a real atom in the initial state to another real atom in the final state and in intermediate  $\lambda$  states interact with other (non-softcore) atoms via normal vdW and Ele interactions. The softcore atoms, on the other hand, are those selected to interact with other atoms (including the common core atoms) via a softcore potential<sup>18</sup> in intermediate  $\lambda$  states. Often the atoms of the softcore region are transformed from real atoms in the pure state to dummy atoms in the final state. The requirement that the dummy atoms reproduce the ensemble and potential of mean force of the real state has been discussed extensively by Boresch and Karplus<sup>19,20</sup> and Roux and co-workers.<sup>21,22</sup>

All of the calculations presented here were performed with the latest update version of the AMBER18 GPU code, PMEMD.CUDA (simulation details are provided in the Supporting Information). This latest updated version has been modified from the original AMBER18 release<sup>6</sup> by us to fix several problems we uncovered, including the treatment of 1–4 vdW and Ele interactions involving atoms in the softcore region. Flags have been added that enable reproduction of free energy simulation results from previous GPU and CPU versions of PMEMD. The main difference between pA18 and A18\* is in the treatment of interactions between atoms of the softcore and common core regions, as summarized in Table S1. Of particular note is the fact that in pA18, the 1–4 vdW and Ele terms across the softcore boundary are present at full strength (i.e., not scaled by  $\lambda$  and thus wrong), whereas in A18\* those interactions are correctly scaled by  $\lambda$ . This difference affects the sampling, thermodynamic derivatives, and end states.

In the analysis below, we examine the absolute hydration free energy,  $\Delta G_{hydr}$ , of a molecule A, defined as

$$\Delta G_{hydr}[A] = G_{aq}[A] - G_{gas}[A] \quad (1)$$

and the relative hydration free energy between molecules A and B, given by

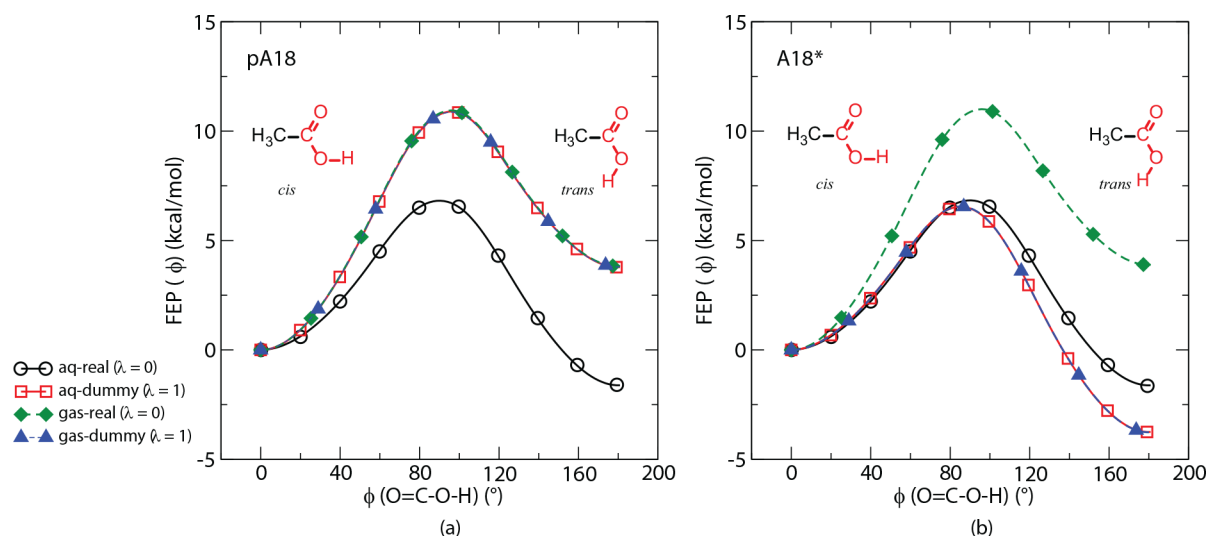
$$\begin{aligned} \Delta\Delta G_{hydr}[A \rightarrow B] &= \Delta G_{hydr}[B] - \Delta G_{hydr}[A] \\ &= \Delta G_{aq}[A \rightarrow B] - \Delta G_{gas}[A \rightarrow B] \end{aligned} \quad (2)$$

where  $\Delta G_{aq}[A \rightarrow B] = G_{aq}[B] - G_{aq}[A]$  and  $\Delta G_{gas}[A \rightarrow B] = G_{gas}[B] - G_{gas}[A]$ . In the discussion that follows, we will compute absolute and relative free energy values using a TI formulation<sup>23</sup> as well as a thermodynamic perturbation (TP) formulation<sup>24</sup> with Bennett’s acceptance ratio (BAR)<sup>25</sup> analysis and its multistate generalization (MBAR).<sup>26</sup> In order to facilitate comparison of the relative hydration free energy values obtained from direct alchemical transformations to differences in absolute hydration free energy values computed independently, we will generate benchmark data for the latter using a split protocol with analysis using BAR<sup>25</sup> (see Table S2), as we find this to be generally the most robust protocol for the data set considered here.

## ■ COMPARISON OF PAST AND CURRENT VERSIONS OF AMBER FOR HYDRATION FREE ENERGIES

Following the procedures described by Loeffler et al.,<sup>15</sup> we examine a set of nine alchemical free energy transformations to obtain relative hydration free energy values and analyze the degree to which  $\Delta G_{aq}$  and  $\Delta G_{gas}$  values obtained using the unified and split protocols agree. The results are summarized in Figure 1 (details are provided in Tables S3 and S4). Whereas the pA18 results show very poor correlation ( $R^2 = 0.3$ – $0.6$ ) and large variation ( $\sigma \approx 5.8$  kcal/mol), the A18\* results are in very close agreement ( $R^2 = 1.0$ ,  $\sigma \approx 0.02$  kcal/mol).

While the values for  $\Delta G_{aq}$  and  $\Delta G_{gas}$  obtained with pA18 clearly are inconsistent and in error, the overall  $\Delta\Delta G_{hydr}$  values are in much closer agreement (Tables S3 and S4). This is due to the fact that for the small and conformationally restricted molecules considered here and by Loeffler et al.,<sup>15</sup> the ensembles generated in the gas phase and in solution are quite similar. As a consequence, the contributions from the 1–4 vdW and Ele terms between the softcore and common core



**Figure 2.** Free energy profiles for acetic acid along the O=C-O-H torsion angle ( $\phi$ ) in the gas phase and in solution for both real ( $\lambda = 0$ ) and dummy ( $\lambda = 1$ ) states computed using (a) pA18 and (b) A18\*. The torsion angle values  $\phi = 0^\circ$  and  $\phi = 180^\circ$  represent the *cis* and *trans* proton configurations, respectively.

**Table 1. Comparison of Relative Hydration Free Energy Values (in kcal/mol) for Methanol  $\rightarrow$  Ethane and Acetic Acid  $\rightarrow$  Acetaldehyde Transformations Calculated Using the Split and Unified Protocols in Pre-AMBER18 (pA18) and Updated AMBER18 (A18\*)<sup>a</sup>**

transformation	AMBER version	$\Delta G_{\text{aq}}$		$\Delta G_{\text{gas}}$		$\Delta \Delta G_{\text{hydr}}$		$\Delta_{\text{abs}}^b$
		unified	split	unified	split	unified	split	
methanol $\rightarrow$ ethane	pA18	2.21(04)	2.85(04)	-3.99(03)	-3.35(03)	6.20(04)	6.20(04)	6.23(03)
	A18*	3.04(04)	3.03(04)	-3.16(03)	-3.16(03)	6.20(04)	6.19(04)	6.23(03)
acetic acid $\rightarrow$ acetaldehyde	pA18	-9.15(04)	32.83(04)	-15.64(04)	26.41(04)	6.49(05)	6.42(05)	4.80(06)
	A18*	38.65(03)	38.63(03)	33.83(03)	33.83(03)	4.82(04)	4.80(04)	4.80(06)

<sup>a</sup>The hydration free energy values were obtained by unified and split protocols using softcore region 2 and analyzed using the TI method. Standard errors are shown in parentheses (multiplied by  $10^2$ ). Relative hydration free energy values were obtained by differences of absolute hydration free energies between the real end state molecules. <sup>b</sup>The absolute hydration free energies were obtained with the split protocol and analyzed by BAR.

atoms in the aqueous and gas phases largely cancel one another.

As a specific example, consider the relative hydration free energy between acetic acid and acetaldehyde. Acetic acid is well-known to have a different orientation of the titratable proton in the gas and aqueous phases.<sup>27</sup> In the gas phase, the proton makes an internal 1–4 hydrogen bond with the opposite carboxylic acid oxygen (a roughly  $0^\circ$  *cis* configuration about the O=C-O-H torsion angle). In solution, this proton prefers to occupy a roughly  $180^\circ$  *trans* configuration, which has an enhanced dipole moment (here note that our use of the terms *cis* and *trans* is with reference to the O=C-O-H torsion angle and not the R-C-O-H torsion angle).

Figure 2 shows the free energy profiles (computed using umbrella sampling simulations; see the Supporting Information for details) along the O=C-O-H torsion angle coordinate for pA18 and A18\* in both the “real states” ( $\lambda = 0$ ) and the “dummy states” ( $\lambda = 1$ ). The pA18 and A18\* profiles for the real states ( $\lambda = 0$ ) are identical, with the *cis* configuration being favored by 3.8 kcal/mol in the gas phase and the *trans* configuration being favored by 1.6 kcal/mol in aqueous solution. In both cases, there is a significant barrier ( $>5$  kcal/mol) separating the *cis* and *trans* configurations. Unlike those for the real states, the pA18 and A18\* free energy profiles for the dummy states ( $\lambda = 1$ ) are markedly different. We expect the dummy states in the gas phase and in solution

to be identical, as there is no interaction of the acetic acid with the surroundings for  $\lambda = 1$ . We further expect the free energy profile for  $\lambda = 1$  to differ from that for the real state ( $\lambda = 0$ ) in the gas phase, as the internal energy should have 1–4 vdW and Ele terms between the softcore and common core regions that are  $\lambda$ -dependent (i.e., scaled by  $\lambda$ ). Although this is indeed the case for A18\*, it is not the case for pA18. In the case of pA18, the profile of the dummy state exactly matches that of the real state in the gas phase. This derives from the fact that pA18 does not scale the 1–4 vdW and electrostatic interactions between the softcore and common core regions (Table S1) and thus gives the same energy and forces as for the real state in the gas phase.

Sampling the proper O=C-O-H torsion angle distributions in either the real state or the dummy state is challenging.<sup>27</sup> Here we adopt a procedure that rigorously takes into account the appropriate predicted distributions as determined by the computed free energy profiles in Figure 2b (see the Supporting Information for details). When sampling of these distributions is taken into account, it is clear that pA18 produces qualitatively incorrect  $\Delta \Delta G_{\text{hydr}}$  values for both the unified and split protocols, whereas A18\* gives  $\Delta \Delta G_{\text{hydr}}$  values with either protocol that are in agreement with one another and with the reference values derived from absolute hydration free energy calculations (Table 1). While this benchmark demonstration is for the relative hydration free energies of two

simple molecules, the issue is even more relevant for the calculation of relative binding free energies of more complex ligands when the conformational ensemble of the ligand in solution changes upon binding to the protein.<sup>13</sup>

## ■ COMPARISON OF RELATIVE AND ABSOLUTE HYDRATION FREE ENERGIES WITH TI AND MBAR

As the free energy is a state function, free energy changes are formally independent of the pathway chosen to connect states, which would be determined by the specific transformation protocol and choice of softcore region, and also independent of the thermodynamic integration or perturbation formalism used for analysis. In practice, however, these choices often have a profound effect on the amount of sampling required to achieve a target degree of statistical precision. To show consistency of the free energy methods, we compare precise benchmark results derived from absolute hydration free energy calculations with those for relative hydration free energy values using both unified and split protocols, two different softcore regions, and either a TI<sup>23</sup> or TP<sup>24</sup>+MBAR<sup>25,26</sup> formalism. In the previous sections, results were shown for transformations where the softcore region was determined in accord with the procedures used by Loeffler et al.,<sup>15</sup> with the strategy to minimize the number of unpaired atoms in the softcore region and maximize the common substructure (i.e., the number of common core atoms). Alternately, we consider a second choice of softcore atoms based on grouping of the softcore atoms into chemical functional groups. We designate these choices of the softcore region as “softcore region 1” and “softcore region 2”, respectively.

Detailed comparison of results for different softcore regions with both TI and TP+MBAR are provided in Figure S2 and Tables S6 and S7. Overall, all of the relative hydration free energy values agree closely with the reference values obtained from the absolute hydration free energies. It should be noted that formally care should be taken that the bonded terms connecting the softcore atoms to the common core region obey certain constraints and conditions<sup>19–22</sup> to ensure that the ensembles generated in the state that contains dummy atoms reproduce the same potential of mean force on the real atoms as the real system without the dummy atoms. We considered these terms in an explicit correction step by eliminating all but a subset of bonded terms obeying the theoretical constraints. In all cases, the magnitude of this contribution was found to be well below the sampling precision (typically less than 0.1 kcal/mol), and hence, the corrections are not statistically significant.

Comparison of the results using different softcore regions suggests that softcore region 2 leads to slightly smaller average estimated errors (0.02–0.03 kcal/mol) relative to softcore region 1 (0.04–0.11 kcal/mol). The latter range is dominated by the methanol → ethane transformation, which has two of the H atoms on ethane as softcore region 1. During the transformation, the negative charge of the methanol O atom causes H atoms of the TIP3P water molecules, which lack Lennard-Jones repulsive terms, to closely approach the softcore H atoms of the ethane molecule. This results in a sharp spike in the thermodynamic derivative that leads to numerical integration errors (see Figure S3). The problem persists with use of the split protocol since the ethane H atoms are already almost decharged (0.031e), making the unified and split procedures almost identical. The same transformation using softcore region 2 has the –CH<sub>3</sub> group in ethane and the –OH group in methanol as the softcore regions. With this

functional group approach, there are no softcore transformations involving H atoms that do not also consider at the same time the heavy atoms to which they are covalently attached.

Excluding the methanol → ethane transformation (softcore region 1) outlier above, overall the statistical precision for softcore region 2 is still higher (smaller statistical errors) than for softcore region 1 using either the unified or split protocol (Tables S6 and S7). This arises from smaller fluctuations of the quantities being averaged to obtain the thermodynamic estimates using the functional group softcore approach with the present data set. The results derived from TI and TP+MBAR are quite similar (typically differing by less than 0.05 kcal/mol), with the greatest deviation occurring for the outlier mentioned above.

## ■ CONCLUSION

Herein we provide a comparison of the consistency of alchemical transformations of relative and absolute hydration free energies in updated AMBER18 (and recently released AMBER20). We demonstrate that the free energy results are internally consistent to typically 0.1 kcal/mol or better independent of the use of a unified or split protocol, the choice of softcore region, and TI versus TP+MBAR analysis. We further illustrate the need for proper treatment of the  $\lambda$  dependence of 1–4 van der Waals and electrostatic interactions between the softcore and common core atoms for general cases. The benchmark data provided herein will be valuable for the testing of new methods and implementations of tools for conducting and analyzing alchemical free energy simulations.

## ■ ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.jcim.0c00285>.

Simulation protocols, softcore region definition, simulation setup, comparison of past and current versions of AMBER for hydration free energies, and an extended discussion of the comparison of relative and absolute hydration free energies with TI and MBAR (PDF)

## ■ AUTHOR INFORMATION

### Corresponding Author

Darrin M. York – Laboratory for Biomolecular Simulation Research, Center for Integrative Proteomics Research, and Department of Chemistry and Chemical Biology, Rutgers University, Piscataway, New Jersey 08854, United States; [orcid.org/0000-0002-9193-7055](https://orcid.org/0000-0002-9193-7055); Email: [Darrin.York@rutgers.edu](mailto:Darrin.York@rutgers.edu)

### Authors

Hsu-Chun Tsai – Laboratory for Biomolecular Simulation Research, Center for Integrative Proteomics Research, and Department of Chemistry and Chemical Biology, Rutgers University, Piscataway, New Jersey 08854, United States; [orcid.org/0000-0001-7027-5649](https://orcid.org/0000-0001-7027-5649)

Yujun Tao – Laboratory for Biomolecular Simulation Research, Center for Integrative Proteomics Research, and Department of Chemistry and Chemical Biology, Rutgers University, Piscataway, New Jersey 08854, United States; [orcid.org/0000-0002-4520-941X](https://orcid.org/0000-0002-4520-941X)



**Tai-Sung Lee** – Laboratory for Biomolecular Simulation Research, Center for Integrative Proteomics Research, and Department of Chemistry and Chemical Biology, Rutgers University, Piscataway, New Jersey 08854, United States;  
orcid.org/0000-0003-2110-2279

**Kenneth M. Merz, Jr.** – Department of Chemistry and the Department of Biochemistry and Molecular Biology, Michigan State University, East Lansing, Michigan 48824, United States;  
orcid.org/0000-0001-9139-5893

Complete contact information is available at:  
<https://pubs.acs.org/10.1021/acs.jcim.0c00285>

### Author Contributions

<sup>§</sup>H.-C.T. and Y.T. contributed equally to this work.

### Notes

The authors declare no competing financial interest.

## ACKNOWLEDGMENTS

The authors thank Benoît Roux and Stefan Boresch for useful discussions. The authors are grateful for financial support provided by the National Institutes of Health (GM107485 to D.M.Y. and GM130641 to K.M.M.). Computational resources were provided by the MSU HPC, the Office of Advanced Research Computing (OARC) at Rutgers, The State University of New Jersey, and by the Extreme Science and Engineering Discovery Environment (XSEDE), which is supported by National Science Foundation Grant OCI-1053575 (Project TG-MCB110101). We gratefully acknowledge the support of nVidia Corporation through the donation of several Pascal, Volta, and Turing GPUs and GPU time on a GPU cluster where the reported benchmark results were performed.

## REFERENCES

- (1) Gumbart, J. C.; Roux, B.; Chipot, C. Standard Binding Free Energies from Computer Simulations: What Is the Best Strategy? *J. Chem. Theory Comput.* **2013**, *9*, 794–802.
- (2) Chipot, C. Frontiers in free-energy calculations of biological systems. *Wiley Interdisciplinary Reviews: Computational Molecular Science* **2014**, *4*, 71–89.
- (3) Duarte Ramos Matos, G.; Kyu, D. Y.; Loeffler, H. H.; Chodera, J. D.; Shirts, M. R.; Mobley, D. L. Approaches for calculating solvation free energies and enthalpies demonstrated with an update of the FreeSolv database. *J. Chem. Eng. Data* **2017**, *62*, 1559–1569.
- (4) Lee, T.-S.; Hu, Y.; Sherborne, B.; Guo, Z.; York, D. M. Toward Fast and Accurate Binding Affinity Prediction with pmemdGTI: An Efficient Implementation of GPU-Accelerated Thermodynamic Integration. *J. Chem. Theory Comput.* **2017**, *13*, 3077–3084.
- (5) Giese, T. J.; York, D. M. A GPU-Accelerated Parameter Interpolation Thermodynamic Integration Free Energy Method. *J. Chem. Theory Comput.* **2018**, *14*, 1564–1582.
- (6) Lee, T.-S.; Cerutti, D. S.; Mermelstein, D.; Lin, C.; LeGrand, S.; Giese, T. J.; Roitberg, A.; Case, D. A.; Walker, R. C.; York, D. M. GPU-Accelerated Molecular Dynamics and Free Energy Methods in Amber18: Performance Enhancements and New Features. *J. Chem. Inf. Model.* **2018**, *58*, 2043–2050.
- (7) Eastman, P.; Swails, J.; Chodera, J. D.; McGibbon, R. T.; Zhao, Y.; Beauchamp, K. A.; Wang, L.-P.; Simmonett, A. C.; Harrigan, M. P.; Stern, C. D.; Wiewiora, R. P.; Brooks, B. R.; Pande, V. S. OpenMM 7: Rapid development of high performance algorithms for molecular dynamics. *PLoS Comput. Biol.* **2017**, *13*, e1005659.
- (8) Gosink, L. J.; Overall, C. C.; Reehl, S. M.; Whitney, P. D.; Mobley, D. L.; Baker, N. A. Bayesian Model Averaging for Ensemble-Based Estimates of Solvation-Free Energies. *J. Phys. Chem. B* **2017**, *121*, 3458–3472.
- (9) Mobley, D. L.; Guthrie, J. P. FreeSolv: a database of experimental and calculated hydration free energies, with input files. *J. Comput.-Aided Mol. Des.* **2014**, *28*, 711–720.
- (10) Gathiaka, S.; Liu, S.; Chiu, M.; Yang, H.; Stuckey, J. A.; Kang, Y. N.; Delproposto, J.; Kubish, G.; Dunbar, J. B.; Carlson, H. A.; Burley, S. K.; Walters, W. P.; Amaro, R. E.; Feher, V. A.; Gilson, M. K. D3R grand challenge 2015: Evaluation of protein-ligand pose and affinity predictions. *J. Comput.-Aided Mol. Des.* **2016**, *30*, 651–668.
- (11) Wang, L.; et al. Accurate and reliable prediction of relative ligand binding potency in prospective drug discovery by way of a modern free-energy calculation protocol and force field. *J. Am. Chem. Soc.* **2015**, *137*, 2695–2703.
- (12) Shirts, M. R.; Klein, C.; Swails, J. M.; Yin, J.; Gilson, M. K.; Mobley, D. L.; Case, D. A.; Zhong, E. D. Lessons learned from comparing molecular dynamics engines on the SAMPL5 dataset. *J. Comput.-Aided Mol. Des.* **2017**, *31*, 147–161.
- (13) Cournia, Z.; Allen, B.; Sherman, W. Relative Binding Free Energy Calculations in Drug Discovery: Recent Advances and Practical Considerations. *J. Chem. Inf. Model.* **2017**, *57*, 2911–2937.
- (14) Schindler, C.; et al. Large-Scale Assessment of Binding Free Energy Calculations in Active Drug Discovery Projects. *ChemRxiv* **2020**, DOI: 10.26434/chemrxiv.11364884.v2.
- (15) Loeffler, H. H.; Bosisio, S.; Duarte Ramos Matos, G.; Suh, D.; Roux, B.; Mobley, D. L.; Michel, J. Reproducibility of Free Energy Calculations across Different Molecular Simulation Software Packages. *J. Chem. Theory Comput.* **2018**, *14*, 5567–5582.
- (16) Case, D. et al. AMBER 20; University of California, San Francisco: San Francisco, CA, 2020.
- (17) Song, L. F.; Lee, T.-S.; Zhu, C.; York, D. M.; Merz, K. M. Using AMBER18 for Relative Free Energy Calculations. *J. Chem. Inf. Model.* **2019**, *59*, 3128–3135.
- (18) Steinbrecher, T.; Joung, I.; Case, D. A. Soft-Core Potentials in Thermodynamic Integration: Comparing One- and Two-Step Transformations. *J. Comput. Chem.* **2011**, *32*, 3253–3263.
- (19) Boresch, S.; Karplus, M. The Role of Bonded Terms in Free Energy Simulations: 2. Calculation of Their Influence on Free Energy Differences of Solvation. *J. Phys. Chem. A* **1999**, *103*, 119–136.
- (20) Boresch, S.; Karplus, M. The Role of Bonded Terms in Free Energy Simulations: 1. Theoretical Analysis. *J. Phys. Chem. A* **1999**, *103*, 103–118.
- (21) S, S.; Roux, B.; Andersen, O. S. Free Energy Simulations: Thermodynamic Reversibility and Variability. *J. Phys. Chem. B* **2000**, *104*, 5179–5190.
- (22) Jiang, W.; Chipot, C.; Roux, B. Computing Relative Binding Affinity of Ligands to Receptor: An Effective Hybrid Single-Dual-Topology Free-Energy Perturbation Approach in NAMD. *J. Chem. Inf. Model.* **2019**, *59*, 3794–3802.
- (23) Kirkwood, J. G. Statistical mechanics of fluid mixtures. *J. Chem. Phys.* **1935**, *3*, 300–313.
- (24) Zwanzig, R. W. High-temperature equation of state by a perturbation method. I. Nonpolar gases. *J. Chem. Phys.* **1954**, *22*, 1420–1426.
- (25) Bennett, C. H. Efficient estimation of free energy differences from Monte Carlo data. *J. Comput. Phys.* **1976**, *22*, 245–268.
- (26) Shirts, M. R.; Chodera, J. D. Statistically optimal analysis of samples from multiple equilibrium states. *J. Chem. Phys.* **2008**, *129*, 124105.
- (27) Lim, V. T.; Bayly, C. I.; Fusti-Molnar, L.; Mobley, D. L. Assessing the Conformational Equilibrium of Carboxylic Acid via Quantum Mechanical and Molecular Dynamics Studies on Acetic Acid. *J. Chem. Inf. Model.* **2019**, *59*, 1957–1964.