FEW: A Workflow Tool for Free Energy Calculations of Ligand Binding

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In the later stages of drug design projects, accurately predicting relative binding affinities of chemically similar compounds to a biomolecular target is of utmost importance for making decisions based on the ranking of such compounds. So far, the extensive application of binding free energy approaches has been hampered by the complex and time-consuming setup of such calculations. We introduce the free energy workflow (FEW) tool that facilitates setup and execution of binding free energy calculations with the AMBER suite for multiple ligands. FEW allows performing free energy calculations according to the implicit solvent molecular mechanics (MM-PB(GB)SA), the linear interaction energy, and the thermodynamic integration approaches. We describe the tool's architecture and functionality and demonstrate in a show case study on Factor Xa inhibitors that the time needed for the preparation and analysis of free energy calculations is considerably reduced with FEW compared to a fully manual procedure. © 2013 Wiley Periodicals, Inc.

Introduction

Drug discovery projects can greatly benefit from computational methods that are able to accurately predict the affinity of drug candidates to a biomacromolecular target. In the lead optimization phase, correctly predicting the *relative* binding affinities of structurally similar drug candidates are of utmost importance for making decisions based on the ranking of such compounds. Accordingly, much effort has been undertaken in the last two decades to develop methods for computing free energies of ligand binding.[1] The developed approaches range from scoring functions describing the properties of ligand and target molecules by simplified energy models, via implicit solvent molecular mechanics and linear interaction energy (LIE) methods (so-called end-point free energy methods) to rigorous, but computationally demanding thermodynamic integration (TI) and free-energy perturbation (FEP) calculations.[2,3] Fast scoring approaches are routinely used today in high-throughput screens in the early phase of drug discovery. In contrast, the end-point free energy methods and rigorous statistical thermodynamics methods are still sparsely applied in later stages of a drug design project, despite them having proven successful in structure-based lead optimization.[4] Besides their computational demand, which is expected to become less of a problem with increasing computational power, a main obstacle for these methods being more widely applied is that setting up such computations usually requires many separate steps, which becomes cumbersome for even medium-size ligand sets. Accordingly, first efforts to facilitate a setup resulted in the FEP module of Desmond,[5] the MCPRO+ program,[6] the CHARMM-GUI[7] module Ligand Binder, the BEAR program,[8] and the AMBER plugin for Pymol;[9] these implementations allow setting up a single type of free energy calculation according to a mostly predetermined protocol. In contrast, workflow tools for a fast, easy, and user-tunable setup of binding free energy calculations for medium-size ligand sets are largely missing. Furthermore, such tools should allow choosing between different free energy methods depending on the desired accuracy of the predictions and the computing time available for a given project. To the best of our knowledge, a tool providing these features has not yet been developed.

The end-point free energy methods molecular mechanics Poisson–Boltzmann surface area (MM-PBSA) and molecular mechanics generalized Born surface area (MM-GBSA)[10] are less computationally expensive than TI or FEP calculations because they evaluate only the bound and unbound states of ligand and target. In MM-PB(GB)SA, the free energy of a molecule is computed as the sum of the molecular mechanics energy in the gas phase, the solvation free energy, and the configurational entropy. Conformational effects are usually taken into account by averaging over a conformational ensemble generated by molecular dynamics (MD) simulations. From the free energies of a ligand, a receptor, and a complex, the binding free energy is computed by subtracting the first two from the latter one. The conformational ensembles for these calculations can either be obtained from separate simulations for complex, receptor, and ligand (3-trajectory approach) or from a single simulation of the complex (1-trajectory approach). As the MM-PB(GB)SA method does not take into account water and ions explicitly but models their effects by a continuum approach, the application of the method is limited to non-highly charged systems, where screening by water and

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ions is not that essential. Furthermore, entropic contributions due to the release or trapping of water and/or ions are neglected in this approach. Despite these and other known limitations to the accuracy of binding free energy predictions by MM-PB(GB)SA, especially for structurally diverse ligands, the method can yield good binding affinity rankings for medium-size ligand sets.[11,12]

The LIE method[13] also belongs to the class of end-point free energy methods. Here, the binding free energy is estimated from the changes in the electrostatic (elec) and van der Waals (vdW) interaction energies of the ligand with its surroundings upon transfer from aqueous solution to the target binding site. Sometimes, changes in the ligand's solvent accessible surface area (SASA) upon binding are considered, too.[14,15] The energetic contributions are determined as averages from conformational ensembles generated by MD or Monte Carlo simulations of the complex and of the free ligand in solution and scaled individually. While standard scaling factors can be applied for weighting the energetic components,[16,17] this does not always result in an optimal prediction.[18] Consequently, experimental binding affinity data of a training set of known ligands are often used for fitting the scaling factors. Despite this shortcoming the LIE approach has proven valuable for obtaining good binding affinity predictions for diverse ligand sets at relatively low-computational costs.[17]

TI and FEP methods belong to the class of pathway approaches that determine the binding free energy by a gradual transformation of one physical state of interest into another one; detailed descriptions of the methods have been presented elsewhere.[4,17,19] Both methods have been shown to give very accurate predictions of ligand binding free energies: in an ideal case, the prediction error can be <1 kcal mol^{-1}, which is close to the experimental uncertainty.[2,19] However, the high-computational demand of these methods has led to these methods being preferentially applied to a small number of structurally similar compounds only.[4] Even with today's computing resources, which allow an evaluation of the relative binding affinities of medium-size ligand sets by rigorous approaches, it might be worthy to restrict TI or FEP analyses to especially interesting compounds while using MM-PB(GB)SA or LIE approaches for a prescreening.

Here, we describe the development of a Workflow tool for Free Energy calculations of ligand binding, referred to as FEW. FEW has been designed (i) to facilitate the setup of MM-PB(GB)SA, LIE, and TI calculations for multiple ligands, (ii) to allow for an online analysis of TI calculations to adjust these calculations with respect to the desired level of precision and the available amount of computing time, (iii) to be used in a heterogeneous hardware environment including high-performance compute clusters, (iv) to require only minimal input information, and (v) to be highly adaptable to a user's needs. Free energy calculations setup with FEW can be run for ligand sets of 10-30 compounds, typical for the later stages of drug design, within a time span of 3 days by MM-PBSA, 3$1/2$ days by LIE, and 1 month by TI on a state-of-the-art compute cluster with 40 cores per ligand (if the target has a size of $\sim$300 amino acids). FEW functions as a front-end to the AMBER suite of programs,[20] which provides functionalities for MM-PB(GB)SA and TI calculations and from which the interaction energies required for LIE analyses can be derived. As a show case example, FEW is applied to a set of inhibitors of Factor Xa.

Architecture and Functionality of the Workflow Tool

FEW has been written in Perl and consists of three modules for the setup of free energy calculations: workflow for automated MM-PBSA and MM-GBSA (WAMM) for MM-PBSA and MM-GBSA calculations, linear interaction energy workflow (LIEW) for LIE analyses, and thermodynamic integration workflow (TIW) for TI calculations (Fig. 1). The modules prepare the
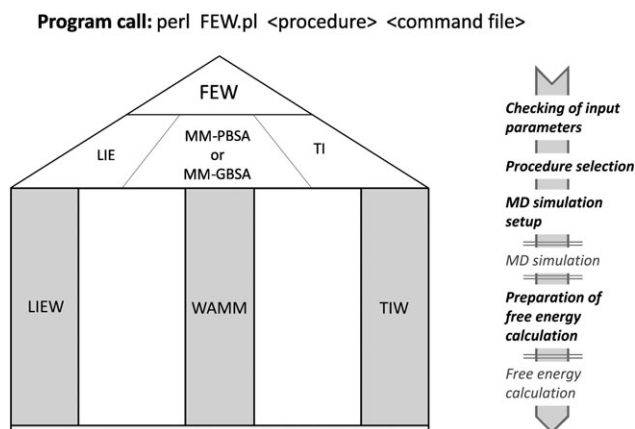

Figure 1. General design (left) and task workflow (right) of the FEW program. The main subroutine FEW.pl is called by the user. After checking the input parameters the specific module for the setup of free energy calculations is selected based on the user input. Within each module (column), the files needed for MD simulations and free energy calculations are prepared. The actual calculations (marked by gray letters) are invoked externally, for example, by using batch scripts prepared by FEW for the submission of jobs via a queuing system.

files needed for the respective free energy analyses using tools of the AMBER suite of programs.[20] For running FEW and for carrying out the free energy calculations an installation of AMBER[21] and AmberTools[22] is required. FEW was optimized for usage with Version 12 of AMBER[21] and will be provided in the next Amber release (http://ambermd.org).

FEW is invoked via the command line. The command consists of three components (Fig. 1): a call of the main Perl routine FEW.pl, the name of the free energy calculation procedure to be used, and the path and name of a command file containing the required parameters. Within the main routine, the correctness of the general user settings is checked before the subroutine for the requested free energy calculation is invoked. This hierarchical design guarantees a straightforward expandability of FEW and ensures an easy usage of the program via a central routine that handles general tasks and invokes specific modules.

The command file (Supporting Information Fig. S1), in which the parameters for the setup of the free energy calculations are specified, consists of a list of keywords. Different

functionalities can be selected by setting the flag of the respective keyword to 'on', either be changing its status from '0' to '1' or by providing the name of a file from which additional information is read. The user can modify most of the parameters according to his needs; additionally, he can adapt the provided template files. As such, experienced users can use their own parameter files for MD simulations and free energy calculations by specifying their locations in the command file. For inexperienced users, we recommend to use the provided template files and to modify only specifications related to the computing environment. Overall, this design makes FEW a "gray box," where all parameter settings can still be changed if required.

The setup of free energy calculations by FEW can be requested for one or multiple ligands. In the latter case, all tasks conducted within one setup step are carried out sequentially for all ligands, that is, when a setup step is finished for one compound, the same step will be performed again for the next ligand, and so on. Input files for the calculations are then prepared such that calculations can be started for all ligands simultaneously and run in parallel.

MD simulations

MM-PB(GB)SA and LIE calculations are carried out based on structural ensembles and therefore require MD simulations to be performed first. TI calculations can either be started from provided ligand and complex structures or can be conducted based on structures that have already been pre-equilibrated by energy minimizations and MD simulations.

The functionality for the setup of MD simulations of FEW (Fig. 2) can be accessed from each of the three modules for

Figure 2. Schematic representation of the procedure for the preparation of MD simulations. If multistructure files are provided, the ligand structures are first stored in separate files. Subsequently, charges and structural parameters for the ligands are determined by standard procedures using external programs. These are then used to setup coordinate and topology files of the systems according to the 1- or 3-trajectory approach. Finally, input files for the equilibration and production phase of the MD simulations are prepared based on template files with simulation parameters.

free energy calculations. Module-internal rules ensure that the setup of the simulations is consistent with the requested free energy calculation method. As input structures for the MD setup procedure, a receptor structure in PDB format, and one or multiple ligand structures in mol2 format are expected. Ligands can also be provided in a multistructure file, which is then split into separate ligand files before the MD preparation is started.

For conducting MD simulations, force-field parameters and atomic charges are needed for each ligand. In the FEW workflow, the parameterization of the ligands is automated as far as possible, requiring only minimal user intervention. First, the atomic charges are determined. They can either be calculated using the restrained electrostatic potential (RESP)[23] or the Austin model 1 - bond charge corrections (AM1-BCC)[24,25] procedure. AM1-BCC charges are computed with the AMBER tools Antechamber and SQM. If a RESP charge calculation is requested, input files for geometry optimization and electrostatic potential (ESP) calculations with Gaussian[26] are prepared. The generated batch- and shell-scripts allow a fast submission of the Gaussian jobs via a queuing system. Gaussian output files are checked by FEW for completeness. XYZ-files with the coordinates of the optimized structures are created that can be inspected by the user with common visualization programs. The presence of intramolecular hydrogen bonds in structures used for RESP fitting can have a significant influence on the derived charges and should therefore be avoided. FEW checks the geometry optimized structures for the presence of A⋯H—D hydrogen bonds (where A and D are oxygen or nitrogen) using an A⋯H distance <2.5 Å and an A⋯H—D angle >150° as cutoff criteria. If a hydrogen bond is found, a warning is issued. When all checks are passed, RESP charges are computed with Antechamber based on the determined ESP. If requested, FEW can assign identical charges to chemically equivalent atoms of enantiomers by averaging the charges calculated by the RESP or the AM1-BCC procedure for the respective atoms. The finally determined charges and the geometry optimized structures are used to generate a library ("lib") file in an Amber-specific format for the ligand; this file is required to provide topological, atom type, and charge information for nonstandard residues within AMBER. Force-field parameters of the ligand are drawn from the general Amber force field[27] (gaff) developed for organic molecules with a special focus on drug-like compounds. Force-field parameters missing in gaff are determined with the parmchk tool of AMBER. Parameters that cannot be provided by parmchk are finally reported to the user.

Next, parameter/topology and coordinate files of the solvated complex, receptor, and/or ligand are created, which serve as input files for the MD simulations. The molecular systems are neutralized by the addition of sodium or chloride ions, and then solvated in a water box extending at least 11 Å in each direction from the solute. While the ligands are described by the parameters derived in the previous setup step, the protein is modeled by the ff99SB force field,[28,29] which has been developed for biomacromolecules. If the receptor contains nonstandard residues, for example, a phosphorylated amino acid, additional library and force-field files can
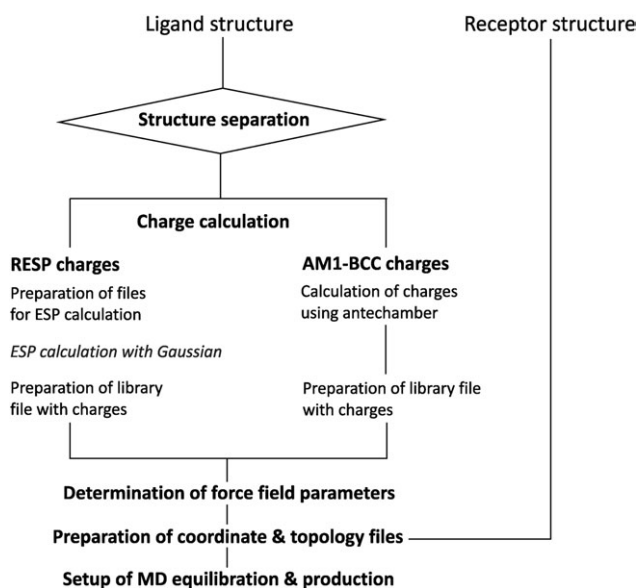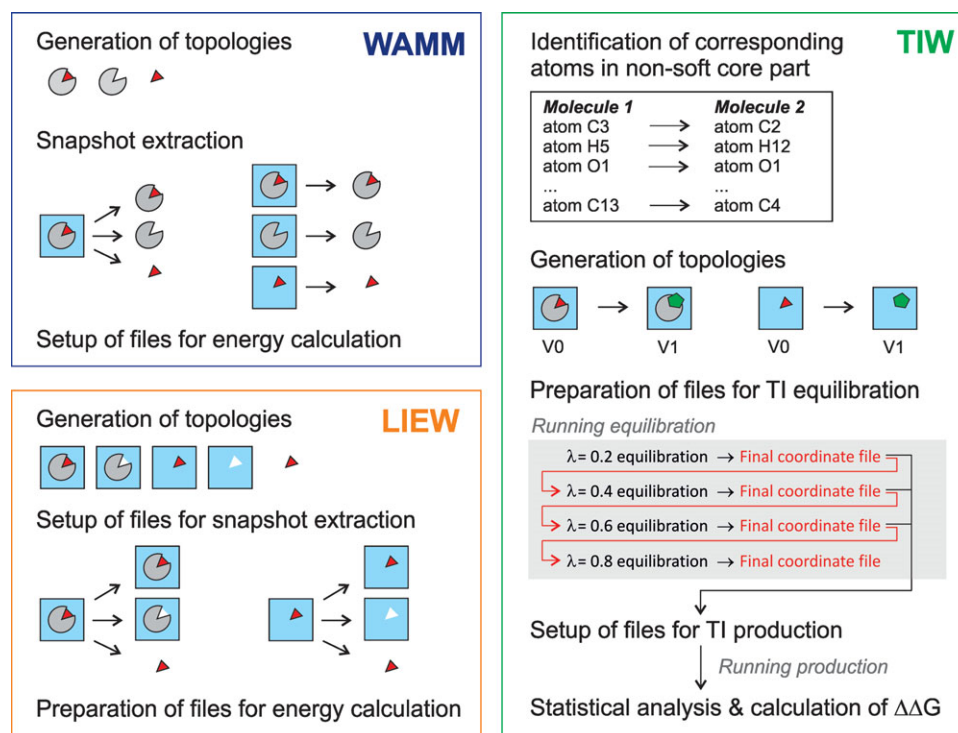
Figure 3. Schematic overview of the steps conducted by FEW in the setup of the three types of free energy calculations. The preparation of implicit solvent, linear interaction energy, and thermodynamic integration analyses are shown in blue, orange, and green boxes, respectively.

be provided by the user. Furthermore, crystal water molecules present in the receptor structure can be carried over into the simulation setup.

In the last step of the setup process files with input parameters for the equilibration and production phase of the simulations are prepared based on template files as well as the requested simulation time and procedure. Batch- and shell-scripts for job submission via a queuing system, generated based on templates with user-defined computing environment-specific settings, then allow initiating the MD jobs comfortably.

MM-PBSA and MM-GBSA module

The WAMM module prepares implicit solvent molecular mechanics analyses that allow determining the effective binding energy of a ligand. The effective binding energy is the sum of the changes in the gas phase and solvation free energy upon complex formation, whereas contributions due to changes in the configurational entropy of the solutes are neglected.[30] The latter is in line with reports according to which taking into account entropic contributions calculated by normal mode analysis or quasiharmonic analysis introduces a considerable uncertainty in the predicted binding affinities.[30–33] Furthermore, these entropic contributions are often similar for a set of structurally similar compounds so that they cancel when it comes to computing relative binding affinities.

Currently, seven alternatives of implicit solvent free energy calculation methods are available in FEW. These include the

well-established MM-GBSA methods of Tsui et al.[34] and Onufriev et al.,[35] conventional MM-PBSA methods in which the nonpolar solvation free energy is considered proportional to the SASA[10] and novel MM-PBSA methods as the one developed by Tan et al.[36] in which the nonpolar part of the solvation free energy is determined from separate repulsive and attractive components, or the hybrid model of Metz.[37] Furthermore, an MM-PB(GB)SA decomposition option enabling the setup of residue- and pairwise interaction analyses is implemented in WAMM. A table listing all available implicit solvent methods is provided in Supporting Information (Supporting Information Table S1).

The setup of the MM-PB(GB)SA calculations (Fig. 3) starts with creating parameter/topology files stripped off water molecules and ions, using the parameters recommended for the requested analysis method. Subsequently, conformational ensembles of complex, receptor, and ligand are generated by automated extraction of snapshots from a user-specified interval of the MD simulation. Snapshots can either be created according to the 1-trajectory approach by extracting them from the trajectory of the complex, or by using the 3-trajectory approach in which snapshots are taken from three separate trajectories of complex, receptor, and ligand. In the final step of the setup process, the files needed for carrying out the energy calculations with the mm_pbsa.pl script of AMBER are created. The calculations can then be started using provided batch- and shell-scripts on any computing system were AMBER[21] is available.

LIE module

The LIEW module sets up calculations according to the LIE method of Åqvist et al.[13] This module generates all files needed for determining the interaction energies between the ligand and its environments, that is, the bound ligand and the free ligand in water. Interaction energies E_{inter} for both states are determined by eq. (1) based on single-point calculations of the elec and vdW energy for each snapshot using the program Sander.

$$E_{inter}^t = E_{total}^t - E_{ligand}^t - E_{rest}^t \quad \text{with } t = \text{elec|vdW} \quad (1)$$

The energy contribution of the ligand (E_{ligand}) and of all other components (E_{rest}), for example, water, receptor, and ions, are

subtracted from the total energy of the system (E_{total}). Mean interaction energies are obtained by averaging over the interaction energies of all n snapshots recorded during a user-specified interval in the MD trajectories [eq. (2)].

$$\left\langle E^t_{\text{inter,state}} \right\rangle = \frac{1}{n} \sum_{i=1}^{n} E^t_{\text{inter,state}} \quad \text{with } t = \text{elec|vdW} \quad (2)$$

From these, the differences in the elec and vdW interaction energies between bound and free states are calculated and reported [eq. (3)].

$$\left\langle \Delta E^t_{\text{inter}} \right\rangle = \left\langle E^t_{\text{inter,bound}} \right\rangle - \left\langle E^t_{\text{inter,free}} \right\rangle \quad \text{with } t = \text{elec|vdW} \quad (3)$$

Estimates of the binding free energy ΔG_{bind} [eq. (4)] are then determined by FEW using default coefficients of $\alpha = 0.16$, $\beta = 0.5$, and $\gamma = 0$.[13,17] Since using dataset or ligand-type specific weighting factors may yield better results in some cases,[18,38] we recommend to the user in the results file to thoroughly investigate whether other coefficients are more suitable. For this, the user can recalculate the binding free energy based on the reported $\left\langle \Delta E^t_{\text{inter}} \right\rangle$ values with a statistical program such as R[39] using other coefficients for α and β reported in the literature (e.g. $\alpha = 0.18$ and $\beta = 0.33$–0.5[16,17]) or using coefficients obtained by a linear fit of ΔG_{bind} to experimental binding affinities of a training set of ligands.

$$\Delta G_{\text{bind}} = \alpha \left\langle \Delta E^{\text{vdW}}_{\text{inter}} \right\rangle + \beta \left\langle \Delta E^{\text{elec}}_{\text{inter}} \right\rangle + \gamma \quad (4)$$

In addition to the interaction energies, the SASA of the ligand is calculated in the bound and in the free state using the ICOSA method available in Sander. The difference in SASA is provided together with the interaction energies in the output file to allow the user to consider a cavity term in the binding free energy calculations according to the approach by Jorgensen and coworkers[14,15,40] [eq. (5)].

$$\Delta G_{\text{bind}} = \alpha \left\langle \Delta E^{\text{vdW}}_{\text{inter}} \right\rangle + \beta \left\langle \Delta E^{\text{elec}}_{\text{inter}} \right\rangle + \gamma \left\langle \Delta \text{SASA} \right\rangle \quad (5)$$

Analogous to setting up MM-PB(GB)SA calculations, first, topology files are created for the components of the system for which interaction energies need to be determined (Fig. 3). Corresponding snapshots are then extracted from the specified intervals of MD trajectories of the complex and of the ligand in solution. The elec and vdW interaction energy differences are calculated by a Perl program provided with FEW. For reasons of efficiency, both the snapshot extraction and energy calculation jobs can be started via batch scripts in the case of LIE analyses.

TI module

The TIW module conducts the setup of TI simulations according to the dual topology, one-step, soft-core approach[4,41] and allows to determine the difference in the free energy of binding $\Delta\Delta G_{\text{bind}}$ between two structurally similar ligands. If $\Delta\Delta G_{\text{bind}}$ energies shall be determined for a set of ligand

compounds, we recommend using the algorithm of Kruskal[42] to identify those ligand pairs for the transformation simulations that require overall the smallest structural changes. This identification is based on a user-provided matrix of similarity scores; as a score, for example, the TanimotoCombo score of ROCS[43] can be applied. To conduct this analysis, a Perl script for use in conjunction with the Graph::Kruskal package available at CPAN (http://www.cpan.org) is provided with FEW.

For the preparation of the calculations, the user needs to specify the atoms of the compounds that shall be part of the soft-core in the TI simulations. All atoms that are not located within the soft-core are treated as being equivalent in both molecules during the simulations. To identify such atoms, the TIW module uses a connectivity-based search algorithm distinguishing atoms by atom types or element information. Special care is taken for mapping equivalent atoms connected to a prochiral center.

For setting up the calculations (Fig. 3), initially coordinate and topology files are created for complex and ligand. In doing so, the information about corresponding atoms is used to build the end state of the transformation from structural information of the start state such that all corresponding atoms get identical atomic names and coordinates. Force-field parameters for both systems are assigned according to the procedure described for the MD setup module of FEW above. Next, files with input parameters for the equilibration phase of the transformation simulations at user-defined λ-values are created. To keep the total equilibration time small, the equilibrations are setup such that simulations at different λ-values are conducted in sequential order from small to large λ's, with each simulation starting from the final structure of the equilibration of the next smaller λ-value. This sequential procedure can currently only be run for equidistant λ-values, that is, $\Delta\lambda$ needs to be of the same size for all user-defined λ-steps. The submission of the equilibration jobs, run with the program Sander,[21] is automated by way of a batch script provided by FEW. After the equilibration phase, input files for the production phase of the transformation simulations can be prepared with FEW. Before the files for production are setup, the completion of the equilibrating phase is analyzed using a Shapiro–Wilk test-based procedure as suggested by Yang et al.[44] For maximal throughput, production simulations are setup such that they can be run in parallel for different λ-values. As the total simulation time needed to obtain average $dV/d\lambda$ values with a desired precision can vary considerably, the convergence of the $dV/d\lambda$ values is repeatedly checked in user-specified intervals. The user can select one of two options for this: (i) the difference in the standard error of the mean $dV/d\lambda$ values (SEM)[41] in two successive checking steps is evaluated; (ii) the deviation of the true mean of $dV/d\lambda$ from the sample mean that can be expected is determined by using the Student's distribution and a confidence limit of 95%. If the value of the convergence measures from (i) or (ii) is below a user-defined threshold, the simulation at the given λ step is terminated. That way, the simulations are ended when no significant additional information is to be expected from further sampling, allowing to balance with minimal user intervention

the desired precision with the computational resources available.

After termination of all transformation simulations, FEW calculates the difference in the free energy ΔG between the two compounds according to eq. (6) for the complex and ligand transformation, respectively.

$$\Delta G = \int_0^1 \left\langle \frac{\delta V(\lambda)}{\delta \lambda} \right\rangle_\lambda d\lambda \qquad (6)$$

The relative binding free energy then results from taking the difference of these values [eq. (7)]:

$$\Delta\Delta G = \Delta G_{complex} - \Delta G_{ligand} \qquad (7)$$

By default, the numerical integration in eq. (6) is performed by the trapezoidal rule. As recommended for the soft-core approach in AMBER, simulations at the physical end points ($\lambda = 0$ and $\lambda = 1$) are not conducted; hence, a linear extrapolation of $dV/d\lambda$ to $\lambda = 0$ and $\lambda = 1$ is required, which can result in large errors if the size of the λ steps is large. Two reasons account for this: First, the uncertainty in the extrapolation increases with increasing $\Delta\lambda$ for nonlinear $dV/d\lambda$ curves. Second, in the one-step soft-core approach kinks in the $dV/d\lambda$ curves can occur[41] that might not be visible in the case of large $\Delta\lambda$. If such kinks are present, this can result in erroneous $dV/d\lambda$ estimates for the end-points. Accordingly, assuming that for large $\Delta\lambda$ steps the error introduced by neglecting the end-points of a transformation is smaller than the one introduced by the extrapolation, an additional option was implemented in FEW that allows conducting the numerical integration over the central part of the $dV/d\lambda$ versus λ curve only [eq. (8)]

$$\Delta G = \sum_{l=1}^n \left\langle \frac{dV}{d\lambda} \right\rangle_{\lambda_1} \times \Delta\lambda_l \qquad (8)$$

with n being the number of sampled λ states. Along these lines, we found better agreement with experimentally determined binding affinity changes for two data sets from industrial lead optimization projects when the end-states were neglected, in contrast to when they were considered (Homeyer and Gohlke, unpublished results).

Show Case Example: Relative Binding Free Energies of Factor Xa Inhibitors

The potential of FEW is demonstrated in the following by computing relative binding free energies for a show case example of 10 inhibitors binding to Factor Xa, a serine protease involved in the blood coagulation cascade. Inhibitors of Factor Xa are promising agents for the therapy of thromboembolic diseases and have, therefore, been widely studied in drug design projects during the last decades. In general, free energy calculation approaches based on MD simulations are limited to receptor systems that experience only small conformational changes upon binding because such changes need

to be sampled. Factor Xa does not undergo large conformational changes upon inhibitor binding and thus can well be studied by these approaches.

The inhibitors were taken from Ref. [45] and modeled based on the crystal complex structure of 7-fluoroindazole bound to Factor Xa (PDB[46] code: 2RA0). The inhibitors (Supporting Information Table S2) were selected with a focus on covering a wide range of binding affinities while keeping the structural modifications among them small. Structural modifications were introduced using the programs SYBYL[47] and MAESTRO,[48] and the positions of the modeled moieties were optimized with MOLOC.[49] The protein from the complex structure was taken as the receptor structure. All water molecules present in the structure were kept and saved together with the receptor structure. Details about the preparation of the systems are given in Supporting Information.

The receptor and ligand structures were used as input structures for FEW. To generate conformational ensembles for MM-PBSA and LIE analyses, MD simulations of the complex and ligand were prepared using the AM1-BCC charge option, and the MD template files provided with FEW (the protocol used for the MD simulations is described in detail in Supporting Information). The preparation of the 10 complex and 10 ligand simulations including the charge calculations took 4 h with only ~20 min of user intervention (Table 1). Equilibration and 10 ns MD production runs were completed on a compute cluster using 8 cores per ligand within $4\frac{1}{2}$ days.

MM-PBSA calculations were conducted for 200 snapshots of the complex simulations recorded after 6 ns of MD production because energetic analyses had suggested that the systems are largely equilibrated in the 6–10 ns time interval. The preparation of the MM-PBSA calculations for all 10 ligands by the WAMM module of FEW, including snapshot extraction, took ~$\frac{1}{2}$ h with only 15 min of user intervention. In comparison, this task can take several hours if done fully manually because parameter/topology files for all systems stripped of water and counter ions need to be created, and the snapshot extraction requires system-specific input files. Once snapshots have been created, setting up another type of MM-PB(GB)SA calculation (cf. Supporting Information Table S1) with FEW can be conducted within 5 min. This is a significant speed up compared to when this preparation is performed manually because switching between different alternatives of implicit solvent models usually requires generating parameter/topology files with appropriate radii sets for all systems. Conventional MM-PBSA calculations[10] with Parse radii[50] for the 10 ligands yielded $\Delta G_{effective}$ values (Supporting Information Table S2) that showed a fair correlation ($r^2 = 0.64$, Supporting Information Fig. S2A) with experimental pK_i data from Ref. [45].

LIE analyses with the LIEW module of FEW were performed based on 200 snapshots recorded during the time interval 6–10 ns in the simulations of the complexes and the free ligands in solution. The preparation of the analyses took ~$\frac{1}{2}$ h. After snapshot extraction, the energetic calculations were run with a parallel installation of Sander on 8 cores for each ligand. The whole calculation took ~5 h (Table 1). Binding energies (Supporting Information Table S2) were estimated

Table 1. Time needed for setup and execution of free energy calculations for the sample data set of Factor Xa inhibitors[a].

Task	No. of ligands[b]	Time with FEW[c]	Time without FEW[d]
Setup of command files for preparation of MD simulations	[e]	20 min[f]	[h]
Charge calculation	10	3.5 h[g]	4 h[f]
Determination of parameters and setup of coordinate and parameter/topology files	10	5 min[g]	2 h[f]
Generation of MD input files for complex and ligand (20 simulations)[f]	10	5 min[g]	4 h[f]
Minimization and MD equilibration (1 node per ligand)	10	7 h[g]	7 h[g]
10 ns MD production (1 node per ligand)	10	4 days[g]	4 days[g]
Generation of command files for the setup of MM-PBSA calculations	[e]	15 min[f]	[h]
Setup of MM-PBSA calculations including snapshot extraction	10	10 min[g]	4 h[f]
Running MM-PBSA calculations for 200 snapshots (1 node per ligand)	10	35 min[g]	35 min[g]
Preparation of command files for setup of LIE analyses	[e]	25 min[f]	[h]
Setup for LIE analyses	10	5 min[g]	10 h[f]
Running LIE analyses	10	5 h[g]	[i]
Preparation of TI simulations for first transformation	1	30 min[f]	2 days[f]
Setup of TI simulations for further transformation(s)	1	10 min[f]	1 day[f]
Running equilibration TI simulations (9 λ steps; 5 nodes per complex; 1 node per ligand)	1	8.5 days[g]	8.5 days[g]
Generation of input files for TI production	1	5 min[f]	3 h[f]
Running TI production (1 node per ligand)	1	10.5 days[g]	10.5 days[j]
Calculation of $\Delta\Delta G$	1	5 min[f]	3 h[f]

[a] The setup was conducted on a desktop computer, and the calculations were run on a multi-node compute cluster with two quadcore Intel Xeon Nehalem 2.4 GHz processors per node. [b] Number of ligands / transformations considered. [c] Approximate time needed for the task or calculation using FEW. Times rounded up to days, hours, or 5 minutes, respectively. [d] Estimate of the time that is needed for the task by a user familiar with the free energy calculation methods performing a purely manual setup, that is, without using FEW. [e] The time needed for this task is independent of the number of ligands. [f] Task requires user intervention; the command file preparation includes the creation of all specified template files. [g] No user intervention required apart from starting the setup procedures / calculations. [h] Not necessary. [i] Impossible without an automation by a shell-script or program because a single-point energy calculation needs to be invoked for every snapshot. [j] Requires manual checking of convergence.

by a linear fit of the computed differences in elec and vdW interaction energies to experimental binding free energies derived from K_i values.[45] A fair correlation ($r^2 = 0.68$, Supporting Information Fig. S2B) with the experimental data was found when the coefficient β was set to 0.5 in the fit, leaving two coefficients to determine [eq. (4)]. This correlation was only slightly better than the correlation obtained with the default coefficients of $\alpha = 0.16$, $\beta = 0.5$, and $\lambda = 0$ used in FEW ($r^2 = 0.66$), which shows that the established coefficients yield fair relative binding affinity estimates for the 10 ligands. Detailed information about the LIE binding free energy calculations is available in Supporting Information.

TI analyses, prepared with the TIW module of FEW, were conducted for nine transformations identified as optimal by Kruskal's algorithm based on similarity scores derived from the TanimotoCombo score of ROCS[43] (Table 2). Due to the automated identification of corresponding atoms of start and end state and the automated setup of parameter/topology files and input files for the calculations, the preparation of these calculations with FEW consumed only 2 h of user intervention. Simulations were run at 9 λ values with $\Delta\lambda = 0.1$. As termination criterion a difference in the SEM of 0.01 kcal mol^{-1} was used. A detailed description of the TI procedure is provided in Supporting Information.

The conducted sample calculations show that it is possible to perform TI calculations within $\sim$2$^{1}/_{2}$ weeks per transformation for medium-size protein systems on a state-of-the-art computer cluster using 72 cores (Table 1). For the sample transformations, the root mean-square difference between computed and experimental relative binding free energies is 2.2 kcal mol^{-1} [1.9 kcal mol^{-1} when $\Delta\Delta G$ is estimated

according to eq. (8)]. The largest differences between computed and experimentally determined $\Delta\Delta G$ were primarily found for transformations involving the exchange of ring moieties and mobile side chains, as given for L51a → L51b, L51g → L51a, and L51h → L51c (cf. Table 2 and Supporting Information Table S2). If only the five smallest transformations identified in the similarity analysis with Kruskal's algorithm are considered, the root mean-square error reduces to 0.9 kcal mol^{-1},

Table 2. Differences in binding free energies calculated according to the TI approach for nine sample transformations[a].

Simulated transformation	$\Delta\Delta G_{method1}$[b]	$\Delta\Delta G_{method2}$[b]	$\Delta\Delta G_{experiment}$[c]
L51c → L51d	3.48 ± 0.03	2.66 ± 0.03	3.36
L51c → L51g	−1.28 ± 0.04	−1.05 ± 0.04	−0.36
L51i → L51k	−1.71 ± 0.05	−1.57 ± 0.05	−2.95
L51e → L51f	−2.93 ± 0.05	−2.07 ± 0.04	−2.32
L51k → L51h	−1.21 ± 0.08	−1.12 ± 0.07	−0.11
L51c → L51f	−2.60 ± 0.04	−2.36 ± 0.04	−0.59
L51a → L51b	4.18 ± 0.09	3.12 ± 0.09	−1.45
L51g → L51a	−0.83 ± 0.10	−0.53 ± 0.10	0.77
L51h → L51c	1.70 ± 0.09	1.60 ± 0.09	0.41

[a] Calculations were conducted with (method 1) and without [(method 2, eq. (8)] linear extrapolation to $\lambda = 0$ and $\lambda = 1$. Computed binding free energies are listed with corresponding error estimates, ordered by decreasing similarity between the end states as determined based on TanimotoCombo scores using Kruskal's algorithm. [b] In kcal/mol. Error in ΔG estimated according to reference,[41] and error in $\Delta\Delta G$ calculated by $\sigma_{\Delta\Delta G} = \sqrt{(\sigma_{\Delta G,lig}{}^2 + \sigma_{\Delta G,com}{}^2)}$. [c] In kcal/mol. Computed based on experimental K_i values[45] by $\Delta\Delta G = -RT \ln (K_i^{V0} / K_i^{V1})$ with $T = 300$ K.

which shows that "chemical accuracy"[51] is reached in these cases. This shows that the TI method can be valuable for reliably determining binding affinity differences of chemically similar compounds.

Conclusions

We have introduced the workflow tool FEW that facilitates setup and execution of ligand binding free energy calculations with AMBER for multiple ligands. FEW allows setting up three types of free energy calculations that operate at different levels of rigor and computational demand: MM-PB(GB)SA, LIE, and TI calculations. The hierarchical and template-based design makes that FEW requires minimal input information but, at the same time, remains highly adaptable. Thus, the program constitutes a "gray box" and should be of interest to both beginners in the field of free energy calculations and expert users.

In a case study, we demonstrated that the time needed for the preparation and analysis of free energy calculations for medium-size ligand sets was considerably reduced with FEW compared to a fully manual procedure. Furthermore, the required total calculation time and the results of the sample analyses demonstrated that free energy calculations can be valuable to estimate relative binding affinities and thus guide drug design projects in the later stages. FEW will render such calculations more feasible by reducing the effort and complexity of the preparation procedure, which has provided a major obstacle for the widespread application of these methods so far. Overall, we believe that FEW will help foster computational binding affinity studies in the lead optimization phase of drug design projects with the AMBER package.

Keywords: lead optimization · linear interaction energy · molecular mechanics Poisson–Boltzmann surface area · molecular mechanics generalized Born surface area · thermodynamic integration

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