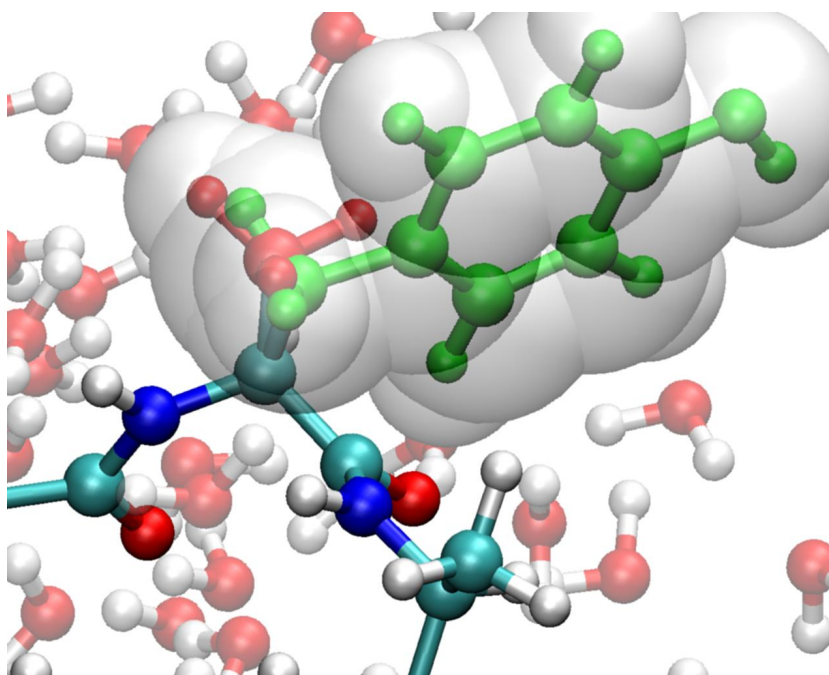


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## ***In silico* alchemy: A tutorial for alchemical free-energy perturbation calculations with NAMD**

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## Abstract

This tutorial explains how NAMD and related tools can be used to setup and perform alchemical free-energy simulations within the free-energy perturbation (FEP) theory. The force-field independent, “zero-sum” transformation of ethane into ethane is used as an introductory, prototypical example. FEP is then used to compute the free energy of charging a naked Lennard-Jones particle into a sodium ion. Next, the variation in solvation free energy upon mutation of a tyrosine residue into alanine is examined in the Ala–Tyr–Ala tripeptide. Last, the concept of standard binding free energy is illustrated in the simple case of a potassium ion binding a ionophore, 18–crown–6. Prior knowledge of NAMD and standard molecular dynamics simulations is assumed.

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## Introduction

The goal of this tutorial is to guide the user in setting up free energy calculations of alchemical transformations [1] within NAMD. [2, 3] We will first perform the rather simple, “zero-sum” transformation of ethane into ethane in water. In a second case example, we calculate the free energy of charging a naked Lennard-Jones sphere into a sodium ion, recovering in a computer simulation a result predicted over eighty years ago by the Born model. [4] Last, as a more practical example, we will compute the difference in hydration free energy resulting from the mutation of tyrosine into alanine in the Ala–Tyr–Ala tripeptide. As has been commented on amply, such *in silico* experiments have not reached yet the maturity to be viewed as black-box, routine jobs, [5, 6, 7] which implies that both the sampling strategy and the analysis of the results should be considered with great care.

NAMD makes use of the so-called *dual topology* approach, [8, 9] in which both the initial state, *viz.*  $\lambda = 0$ , and the final state, *viz.*  $\lambda = 1$ , are defined concurrently. As the molecular dynamics (MD) simulation progresses, the potential energy function characteristic of  $\lambda = 0$  is scaled into that representative of  $\lambda = 1$ . Whereas the initial and the final states do interact with the environment, we typically do not allow them to see each other in the course of the transformation. For versions of NAMD prior to 2.7b2, achieving these conditions requires that a list of excluded atoms be defined in the PSF topology file. Since the `psfgen` software supplied with NAMD does not offer a way of building such a list, we provide the `alchemify` program. `alchemify` processes PSF files written by `psfgen` or CHARMM and makes them suitable for simulating alchemical transformations. However, in NAMD 2.7b2, the appropriate exclusions are generated automatically at the start of the simulations.

The reader of this tutorial is assumed to be familiar with the use of NAMD to perform “standard” calculations, including energy minimization and MD simulations. General documentation, tutorials and templates of NAMD configuration files are available from the Documentation section of the NAMD web page. The simulation times in this tutorial are very short in the interest of expediency; however, one should be aware that they are generally insufficient for most applications. Additionally, although not always used in the tutorial, equilibration of a system prior to running FEP simulations is highly recommended.

Completion of this tutorial requires:

- various files contained in the archive `FEP_tutorial.zip`, provided with this document;

- NAMD 2.7b2 [3] or later (<http://ks.uiuc.edu/Research/namd>);
- VMD 1.8.4 [10] or later (<http://ks.uiuc.edu/Research/vmd>);
- `alchemify` (only needed for versions of NAMD before 2.7b2 <http://www.edam.uhp-nancy.fr/Alchemify>).

## 1. Ethane-to-ethane “zero-sum” transformation

Perhaps the simplest alchemical transformation one could imagine, the result of which is completely independent of the potential energy function utilized, is the *zero-sum* ethane  $\rightarrow$  ethane mutation, [11, 9] wherein a methyl group vanishes at one end of the molecule, while another one appears at the other end. The accuracy of the computed free energy only depends on the sampling strategy adopted, regardless of the force field employed.

### 1.1. System setup

To set up a system, you will need a structure file (PSF) and a coordinate file (PDB). We have provided you with the coordinate files for both ethane and water, as well as the topology files needed to build the structure files. In addition, FEP calculations in NAMD require the `alchFile` which will be introduced to you in greater detail later.

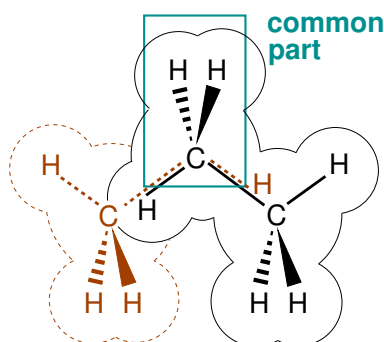


Figure 1: Dual-topology hybrid molecule used for the *zero-sum* ethane  $\rightarrow$  ethane alchemical transformation. The initial state, *viz.*  $\lambda = 0$  (black), and the final state *viz.*  $\lambda = 1$  (brown), are defined concurrently. The central  $-\text{CH}_2-$  moiety is common to both topologies.

Using dual topology means that we will need a topology file that describes the structure of the hybrid

molecule. Although such a file, `zero.top`, has been provided for you in this case, the hybrid topology file is in general created by the user.

- 1 Open the CHARMM topology file `zero.top` with a text editor. You will see the hybrid structure described as follows:

```
* Topology for ethane-to-ethane transformation
27 1 ! pretend we are CHARMM27_1

RESI ZERO      0.00      ! ethane -> ethane
GROUP          !
ATOM CI  CT3  -0.27      !
ATOM HI1 HA   0.09      !
ATOM HI2 HA   0.09      !
ATOM HI3 HA   0.09      !
GROUP          !   HI1      HM1  HF2  HF3
ATOM CM  CT3  -0.27      !   \      |      |  /
ATOM HM1 HA   0.09      !   \HF     |      |  /
ATOM HM2 HA   0.09      !   CI-----CM-----CF
ATOM HI  HA   0.09      !   /  |      |      HI\
ATOM HF  HA   0.09      !   /  |      |      \
GROUP          !   HI2  HI3  HM2      HF1
ATOM CF  CT3  -0.27      !
ATOM HF1 HA   0.09      !
ATOM HF2 HA   0.09      !
ATOM HF3 HA   0.09      !
BOND  CI  HI1      CI  HI2      CI  HI3      ! ethane 1
BOND  CF  HF1      CF  HF2      CF  HF3      ! ethane 2
BOND  CI  CM      CF  CM      ! common
BOND  CM  HM1      CM  HM2      ! common
BOND  CM  HI      ! ethane 1
BOND  CM  HF      ! ethane 2

! No patching
PATCHING FIRST NONE LAST NONE
END
```

In this transformation, the methyl group `CI-HI1-HI2-HI3` is replaced by hydrogen atom `HF`, while the methyl group `CF-HF1-HF2-HF3` replaces the hydrogen atom `HI`.

- 2 Now, write a `psfgen` script to build the PSF. Create a file named `setup.pgn` containing the following script using any text editor:

```
package require psfgen
topology ../common/top_all22_prot.inp
topology zero.top

segment ZERO { residue 1 ZERO }
segment WAT { auto none; pdb water.pdb }

coordpdb ethane.pdb ZERO
coordpdb water.pdb WAT
```

```
writepsf setup.psf
writepdb setup.pdb
```

The script you have entered tells `psfgen` to generate a system consisting of the hybrid molecule and water (PDB provided), using structural information from the named topology files.

- 3 In the VMD TkConsole, run the script using `source setup.pgn`. You have now created a PDB and a PSF file, `setup.pdb` and `setup.psf`. An example output has been provided in the `example-output` folder.

In the next step, you will create the `alchFile`. Recall that the system you have created contains a) atoms in the initial state that will gradually vanish, b) atoms that will gradually appear towards the final state, and c) atoms that are present throughout the whole transformation. NAMD distinguishes between these three classes of atoms based on information in the `alchFile`. The `alchFile` is essentially the PDB file of your system, except that every atom has been tagged according to the class to which it belongs.

- 4 Open `setup.pdb` in a text editor. Change the B column so that vanishing and appearing atoms are tagged with a -1.00 and 1.00 flag respectively, while unchanging atoms are tagged with 0.00. The required changes are shown below:

```
ATOM      1  CI  ZERO  1      -1.167  0.224  0.034  1.00 -1.00  ZERO
ATOM      2  HI1 ZERO  1      -2.133 -0.414  0.000  1.00 -1.00  ZERO
ATOM      3  HI2 ZERO  1      -1.260  0.824  0.876  1.00 -1.00  ZERO
ATOM      4  HI3 ZERO  1      -1.258  0.825 -0.874  1.00 -1.00  ZERO
ATOM      5  CM  ZERO  1      0.001 -0.652 -0.002  1.00  0.00  ZERO
ATOM      6  HM1 ZERO  1      0.000 -1.313 -0.890  1.00  0.00  ZERO
ATOM      7  HM2 ZERO  1      0.005 -1.308  0.889  1.00  0.00  ZERO
ATOM      8  HI  ZERO  1      1.234  0.192  0.000  1.00 -1.00  ZERO
ATOM      9  HF  ZERO  1      -1.237  0.190  0.000  1.00  1.00  ZERO
ATOM     10  CF  ZERO  1      1.289  0.150 -0.078  1.00  1.00  ZERO
ATOM     11  HF1 ZERO  1      2.149 -0.425 -0.001  1.00  1.00  ZERO
ATOM     12  HF2 ZERO  1      1.256  0.837 -0.893  1.00  1.00  ZERO
ATOM     13  HF3 ZERO  1      1.131  0.871  0.940  1.00  1.00  ZERO
ATOM     14  OH2 TIP3  1      -5.574 -5.971 -9.203  1.00  0.00  WAT
ATOM     15  H1  TIP3  1      -5.545 -5.020 -9.301  1.00  0.00  WAT
...
```

- 5 Once you have made the necessary changes, save the file as `zero.fep`.

You may find it helpful at this stage to visualize the system with its initial and final groups. Doing so makes it easy to spot errors made in the previous steps.

- 6 Run VMD with the following command: `vmd setup.psf -pdb zero.fep`.
- 7 In the Graphics/Representations menu, set the selection text to “not water” and select the coloring method `Beta`. Appearing atoms are colored blue and vanishing atoms are colored red, while the unperturbed part of the molecule appears in green. Compare the result with Figure 1. Can you see clearly which atoms will vanish and which will appear?
- 8 If you are using NAMD 2.7b2 or later, you can skip this step. Call `alchemify` using the following command line:  

```
alchemify setup.psf zero.psf zero.fep
```

In the system we have defined, appearing and vanishing atoms interact in two ways: through non-bonded forces, and because some of the angle and dihedral parameters automatically generated by `psfgen` couple the two end-points of the transformation. For instance, the `CI-CM-CF` angle should not be assigned a force field term by NAMD. To prevent unwanted non-bonded interactions, `alchemify` processes PSF files and creates the appropriate non-bonded exclusion list. At the same time, irrelevant bonded terms that involve appearing and vanishing atoms are removed. `alchemify` retrieves the necessary data about the transformation from `alchFile`. These unwanted terms are automatically ignored in NAMD versions 2.7b2 and later.

If you have called `alchemify` the resulting file `zero.psf` will be used when performing the FEP calculations.

## 1.2. Running the free energy calculation

- 1 Open with a text editor the provided configuration files for the forward and backward transformations, `forward-noshift.namd` and `backward-noshift.namd` respectively.

You can tell from the configuration files that we will be doing an MD run at a constant temperature of 300 K and pressure of 1 bar, using particle-mesh Ewald (PME) electrostatics. Furthermore, we have set the `rigidBonds` option to `all` and the time step to 2 fs. To impose isotropic fluctuations of the periodic box dimensions, we set the `flexibleCell` variable to `no`. We define the initial periodic box

to be a cube with an edge length of 21.8 Å. The simulation takes as its input the output files (provided) of an equilibration run, so there is no need to run an equilibration in this example.

Take a look at the FEP section of the configuration file. This section defines the sampling strategy of the calculation. This section calls a TCL script `fep.tcl` that defines three commands to simplify the syntax of FEP scripts:

- `runFEP` runs a series of FEP windows between equally-spaced  $\lambda$ -points, whereas
- `runFEPList` uses an arbitrary, user-supplied list of  $\lambda$  values.
- `runFEPmin` is identical to `runFEP` but adds an initial minimization step as well (use this only for equilibration runs).

The FEP section of the configuration file for the forward transformation reads:

```
# FEP PARAMETERS

source                ../tools/fep.tcl

alch                  on
alchType              FEP
alchFile              zero.fep
alchCol               B
alchOutFile           forward-noshift.fepout
alchOutFreq           10

alchVdwLambdaEnd     1.0
alchElecLambdaStart  1.0
alchVdWShiftCoeff    0.0
alchDecouple         no

alchEquilSteps       100
set numSteps         500

runFEP 0.0 1.0 0.0625 $numSteps
```

In the above example, the potential energy function of the system is scaled from  $\lambda = 0$  to  $\lambda = 1$  by increments  $\delta\lambda = 0.0625$ , *i.e.* 16 intermediate  $\lambda$ -states or “windows”. [7] In each window, the system is equilibrated over `alchEquilSteps` MD steps, here 100 steps, prior to `$numSteps - alchEquilSteps = 400` steps of data collection, from which the ensemble average is evaluated. As you can see from the script, there are other FEP variables defined in this section that we have not mentioned. These variables correspond to the soft-core potential and will be introduced to you later.

- Run the forward and backward simulations by entering the commands
 

```
namd2 forward-noshift.namd > forward-noshift.log
namd2 backward-noshift.namd > backward-noshift.log
```

 in a terminal window. Each simulation should require only a few minutes.

### 1.3. Results

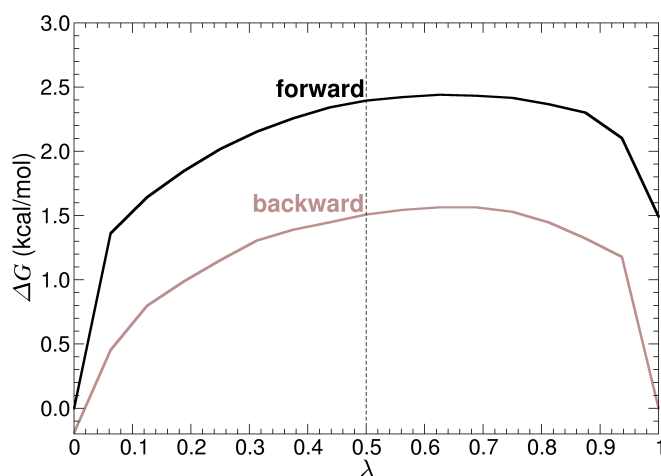


Figure 2: Free-energy change for the ethane  $\rightarrow$  ethane *zero-sum* mutation, measured in the forward and in the backward direction. This simulation was performed in the absence of a soft-core potential. [12, 13] Singularities are manifested at the end points, when  $\lambda = 0$  or 1.

The `alchOutFile` contains all data resulting from the FEP calculation. In order to obtain the  $\Delta G$  profile shown above, we will have to extract only the relevant portions and parse it into a format amenable to graph plotting.

- If you are on a UNIX or Macintosh system, extract the  $\Delta G(\lambda)$  profile using this command line:

```
grep change forward-noshift.fepout | awk '{print $9, $19}' >
forward-noshift.dat
```

Alternatively, you can use the provided script `deltaA.awk` in the `tools` directory, i.e.,

```
../tools/deltaA.awk forward-noshift.fepout > forward-noshift.dat
```

This creates a two-column data file that can be read by most plotting programs, *e.g.* `xmgrace`, `gnuplot`, or

any spreadsheet application.

An example of the expected result is plotted in Figure 2. One of the notorious shortcomings of the dual-topology paradigm can be observed in the  $\Delta G(\lambda)$  profile when  $\lambda$  approaches 0 or 1. In these regions, interaction of the reference or the target topology with its environment is minute, yet strictly nonzero. Molecules of the surroundings can in turn clash against incoming or outgoing chemical moieties, which can lead to numerical instabilities in the trajectory, manifested in large fluctuations in the average potential energy. The result is slow convergence. [7]

There are two ways we can go about handling these so-called “end-point catastrophes”. One is to employ a better-adapted sampling strategy. The other is to use a soft-core potential, [12, 13, 14] which effectively eliminates the singularities at  $\lambda = 0$  or 1, by progressively decoupling interactions of outgoing atoms and coupling interactions of incoming atoms. This feature is available by default in NAMD. For pedagogical purposes, however, the parameters that control the soft-core potential in the example you have just done — *viz.* `alchVdwLambdaEnd`, `alchElecLambdaStart`, `alchVdWShiftCoeff` and `alchDecouple`, were not set to apply a correction.

Having seen the deleterious effects of possible end-point singularities, we will now use a soft-core potential to prevent such singularities from occurring.

#### 1.4. Why a soft-core potential should always be included

We will now run the FEP calculation again, but using this time a soft-core potential, hence avoiding the need for narrower windows as  $\lambda$  gets closer to 0 or 1.

1 Open `forward-shift.namd` in a text editor.

Note that the configuration script differs from `forward-noshift.namd` in only the FEP section, which is as follows:

```
# FEP PARAMETERS
source          ../tools/fep.tcl
```

```
alch                on
alchType            FEP
alchFile            zero.fep
alchCol             B
alchOutFile         forward-shift.fepout
alchOutFreq         10

alchVdwLambdaEnd    1.0
alchElecLambdaStart 0.5
alchVdWShiftCoeff   6.0
alchDecouple        yes

alchEquilSteps      100
set numSteps         500

runFEP 0.0 1.0 0.0625 $numSteps
```

The parameters utilized for the soft-core potential are rather detailed and can be somewhat confusing, so be sure to read the following carefully. Outgoing atoms will see their electrostatic interactions with the environment decoupled during  $\lambda = 0$  to  $1 - \text{alchElecLambdaStart} = 0.5$ , while the interactions involving incoming atoms are progressively coupled during  $\lambda = 1 - \text{alchElecLambdaStart}$  to  $1$ .

At the same time, van der Waals interactions involving vanishing atoms are progressively decoupled during  $\lambda = 1 - \text{alchVdwLambdaEnd}$ , *i.e.* 0 to 1, while the interactions of appearing atoms with the environment become coupled during  $\lambda = 1$  to  $1 - \text{alchVdwLambdaEnd}$ , *i.e.* 0.

## 2 Run the new forward and backward simulations using the commands

```
namd2 forward-shift.namd > forward-shift.log
namd2 backward-shift.namd > backward-shift.log
```

in a terminal window and analyze the resulting output.

Figure 3 shows a typical result for this new simulation. The effect of the soft-core potential is apparent in the total free energy change, now close to zero, and the symmetry of the profile with respect to  $\lambda = 0.5$ , which altogether suggests that the calculation has converged. However, greater sampling will improve convergence further. If desired, try running the simulation `forward-shift-long.namd`, which multiplies the length by a factor of 10, to see if it improves the result (NOTE: this simulation may take 30 minutes or more!).

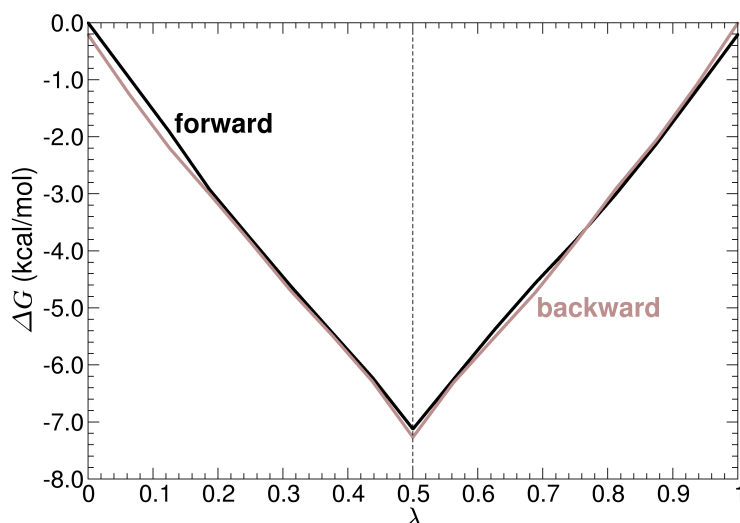


Figure 3: Result of an ethane  $\rightarrow$  ethane *zero-sum* mutation including a soft-core potential correction to circumvent the “end-point catastrophes” highlighted in Figure 2. Using an equally modest sampling strategy, the expected zero free-energy change is recovered. Note the overlapping profiles obtained from the forward and the backward transformations.

### Probing the convergence properties of the simulation



We can assess convergence of the free energy calculation by monitoring the time-evolution of  $\Delta G(\lambda)$  for every individual  $\lambda$ -state and the overlap of configurational ensembles embodied in their density of states,  $P_0[\Delta U(\mathbf{x})]$  and  $P_1[\Delta U(\mathbf{x})]$ , where  $\Delta U(\mathbf{x}) = U_1(\mathbf{x}) - U_0(\mathbf{x})$  denotes the difference in the potential energy between the target and the reference states. Since a soft-core potential [12, 13] has been introduced to avoid the so-called “end-point catastrophes”, the potential energy no longer varies linearly with the coupling parameter  $\lambda$ . Therefore, it is necessary that the reverse transformation be carried out explicitly to access  $\Delta G_{\lambda+\delta\lambda \rightarrow \lambda}$ , as is proposed in the above NAMD scripts.

## 2. Charging a spherical ion

In the second example of this tutorial, we will charge a naked Lennard-Jones particle into a sodium ion in an aqueous environment.

### 2.1. System setup

Our system consists of a sodium ion immersed in a bath of water molecules. Using the dual-topology paradigm, we could charge a Lennard-Jones particle by shrinking a naked spherical particle, while growing concomitantly the ion. However, in this particular example, the single-topology approach would have the benefit of perturbing only the electrostatic component of the nonbonded potential, thus avoiding perturbing the Lennard-Jones terms and improving convergence. We can emulate such a single-topology paradigm within the dual-topology approach implemented in NAMD, simply by choosing appropriate parameters for the soft-core potential and the coupling of the particle with its environment.

- 1 Generate the PSF and PDB files for the system, which is a sodium ion SOD at the center of a water box. You can do this by creating a file named `setup.pgn` containing the script below. Run the script you have written by going to the VMD TkConsole and entering `source setup.pgn`.

```
topology ../common/top_all122_prot.inp

segment SOD {
  residue 1 SOD
}

writepsf setup.psf
writepdb setup.pdb
```

- 2 Next, you will surround the ion with a water box using the `Solvate` plugin of VMD. Call the `Solvate` plugin in the TkConsole with the following: `solvate setup.psf setup.pdb -t 15 -o solvate`

We want the primary cell be large enough to minimize the self-interaction of the cation between adjacent boxes. For this purpose, a box length of 30 Å will do. The distance separating the cation in the primary

and the adjacent cells being equal to 30 Å, the interaction energy reduces to  $q^2/4\pi\epsilon_0\epsilon_1r = 0.1$  kcal/mol with an ideal macroscopic permittivity of 78.4 for bulk water.

- 3 Unlike the previous example, the system has not been pre-equilibrated for you. Conduct an equilibration run using the provided configuration file `equilibration.namd` by entering the command

```
namd2 equilibration.namd > equilibration.log
```

in a terminal window.

- 4 You are now ready to run the FEP calculation. As before, you must build the `alchFile` by editing the B column of the PDB file to indicate which atoms are appearing and disappearing. Open with a text editor the `solvate.pdb` file generated by the `Solvate` plugin.

- 5 Edit the relevant line to reflect the growing sodium ion, then save the file as `solvate.fep`.

```
ATOM      1  SOD  SOD      1      0.000  0.000  0.000  1.00  1.00      SOD
...
```

- 6 In the present system, since SOD does not coexist with any other perturbed particle, you do not have to use `alchemify` to remove extra terms in the PSF file.

- 7 As a safety check, you should consider both forward, *i.e.* charge creation, and backward, *i.e.* charge annihilation, transformations. Repeat the procedure described in this section for the backward calculation.

## 2.2. Running the free energy calculation

The NAMD implementation of the soft-core potential [12] allows us to decouple at a different pace the van der Waals and the electrostatic interactions of the perturbed system with its environment, as depicted in Figure 4. In our current case, we can independently modify each of the forces so that the Lennard-Jones coupling remains the same throughout (the neutral particle never disappears) while electrostatic interactions are scaled up or down.

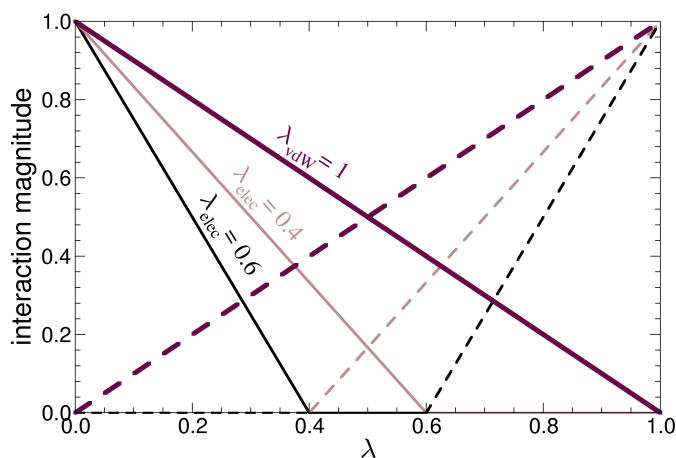


Figure 4: Decoupling of electrostatic and van der Waals interactions within the NAMM implementation of the soft-core potential. Solid lines represent outgoing atoms and dashed lines represent incoming atoms. Two variables define how the perturbed system is coupled or decoupled from its environment, *viz.*  $\lambda_{\text{elec}}$  (`alchElecLambdaStart`) and  $\lambda_{\text{vdW}}$  (`alchVdWLambdaEnd`). In the present scenario,  $\lambda_{\text{vdW}} = 1.0$ , meaning that the van der Waals interactions for outgoing and incoming particles will be, respectively, scaled down starting from  $\lambda = 1.0 - \lambda_{\text{vdW}} = 0.0$  to  $\lambda = 1.0$ , and scaled up starting from  $\lambda = 0.0$  to  $\lambda = \lambda_{\text{vdW}}$ . If  $\lambda_{\text{elec}} = 0.4$ , electrostatic interactions for outgoing and incoming particles are, respectively, scaled down from 0.0 to  $1.0 - \lambda_{\text{elec}} = 0.6$ , and scaled up from  $\lambda_{\text{elec}}$  to 1.0.

By setting both `alchElecLambdaStart` and `alchVdWLambdaEnd` to 0.0, we begin scaling down electrostatic interactions of the vanishing atoms at  $\lambda = 0.0$ , until the interactions completely disappear at  $\lambda = 1.0$ , and for appearing atoms, scaling up from  $\lambda = 0.0$  to  $\lambda = 1.0$ , while van der Waals interactions remain unchanged.

You can set the above parameters in the FEP section of the NAMM configuration file. Take a look, for example, at `forward.namd`:

```
# FEP PARAMETERS

source                ../tools/fep.tcl

alch                  on
alchType              FEP
alchFile              solvate.fep
alchCol               B
alchOutFile           forward.fepout
alchOutFreq           10

alchVdWLambdaEnd     0.0
alchElecLambdaStart  0.0
alchVdWShiftCoeff    5.0
alchDecouple         on
```

```
alchEquilSteps      100
set numSteps        500

runFEP 0.0 1.0 0.0625 $numSteps
```

The sampling strategy we will use involves 16 equally spaced windows with  $\delta\lambda = 0.0625$ . Each window features 500 steps of MD sampling, among which 100 steps are equilibration. Assuming a time step of 2 fs for integrating the equations of motion, the total simulation time amounts to 16 ps. We will find that this time scale is appropriate for reproducing the expected charging free energy.

In the traditional MD section, we use standard NAMD configuration parameters. Langevin dynamics is employed to maintain constant temperature at 300 K. The Langevin piston enforces a constant pressure of 1 bar. Long-range electrostatic forces are handled by means of the PME algorithms. All chemical bonds are frozen to their equilibrium value and a time step of 2 fs is used. To impose isotropic fluctuations of the periodic box dimensions, `flexibleCell` has been set to `no`. The initial periodic cell, prior to equilibration, is defined as a cube with an edge length of 30.0 Å.

- 1 Before performing the FEP calculations, you will first need to equilibrate the system. Run an equilibration using `namd2 equilibration.namd > equilibration.log`.

- 2 Run both the forward and backward simulations using the commands

```
namd2 forward.namd > forward.log
namd2 backward.namd > backward.log
```

in a terminal window.

### 2.3. Results

The free energy profile delineating the alchemical transformation is shown in Figure 5.

- 1 Obtain this profile by using again the `grep` command on the `alchOutFile`, as you did in the first example of this tutorial.

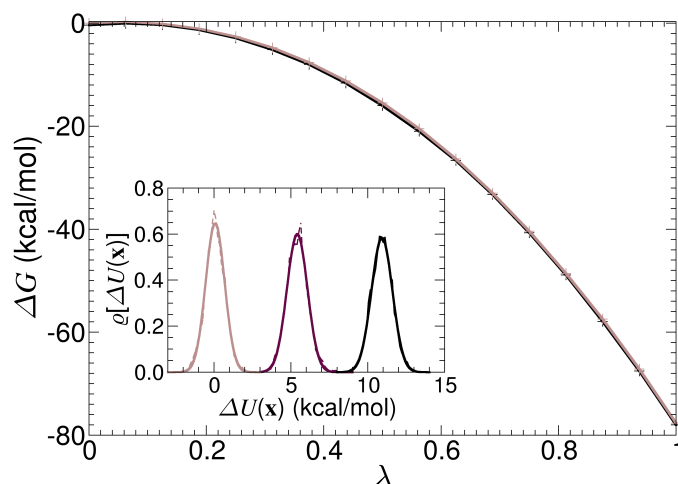


Figure 5: Free energy change for the charging of a naked Lennard-Jones particle into a sodium ion. Inset: Distribution of  $\Delta U(x)$  at different values of  $\lambda$  (indicated by arrows on the free energy curve), with Gaussian fits.

Figure 5 reveals that the free energy of charging the spherical particle in water is considerable, amounting to  $-77.6$  kcal/mol. As is, this estimate is incomplete, because it ignores the size-dependence of the system — *i.e.*  $\Delta G$  is expected to vary with the size of the primary cell. [15] In the case of an Ewald summation-like simulation, with a cubic periodic simulation box, the size-dependence correction is equal to  $+1/2 \xi_{\text{Ewald}} (q_1^2 - q_0^2)$ , where  $q_0$  and  $q_1$  are the charges for the reference and the target states — *i.e.*  $+1$  and  $0$ , and  $\xi_{\text{Ewald}} = -2.837/L$ , where  $L$  is the length of the cell.

A periodic cell length of  $L = 29.1$  Å yields a size-dependence correction of  $-16.2$  kcal/mol. Added to the raw charging free energy, the corrected estimate amounts to  $-93.8$  kcal/mol, in reasonable agreement with the value of  $-96.8$  kcal/mol obtained by Hummer *et al.*, [15] using a different set of Lennard-Jones parameters for sodium — *viz.*  $R_{ii}^* = 1.425$  Å and  $\varepsilon_{ii} = 0.04793$  kcal/mol, and with a box of 256 SPC water molecules. As a basis of comparison, the complete free energy of ionic hydration measured experimentally by Marcus is equal to  $-87.2$  kcal/mol. [16] At the theoretical level, Straatsma and Berendsen, [17] using a simulation box containing 216 water molecules, estimated this free energy to  $-121.4$  kcal/mol. It is apparent from these different results that the charging free energy is very sensitive to the force field parameters utilized.

The density of states for a selection of intermediate  $\lambda$ -states are depicted in the inset of Figure 5. From the onset, it is apparent that these distributions obey a normal law, thereby suggesting that second-order perturbation theory is sufficient to describe with a reasonable accuracy the charging process. [7]

**Improving the agreement with experiment**

Replacing the standard CHARMM Lennard-Jones parameters for sodium by those chosen by Hummer *et al.*, [15] verify that the charging free energy coincides with the estimate reached by these authors.

**Recovering predictions from the Born model**

Granted that water is a homogeneous dipolar environment, the charging free energy writes  $\Delta G = -\beta/2 q^2 \langle V^2 \rangle_0$ , where  $V$  is the electrostatic potential created by the solvent on the charge,  $q$ . Increasing  $q$  to  $+2$ , show that the charging free energy varies quadratically with the charge, as predicted by the Born model — *i.e.*  $\Delta G = (\epsilon - 1)/\epsilon \times q^2/2a$ , where  $a$  is the radius of the spherical particle and  $\epsilon$ , the macroscopic permittivity of the environment.

### 3. Mutation of tyrosine into alanine

In this section, we will calculate the free energy change involved in the point mutation of the N- and C-terminally blocked Ala–Tyr–Ala tripeptide. Our calculation will be based on the thermodynamic cycle of Figure 6. [18] We will calculate the quantities  $\Delta G_{\text{alch.}}^1$  and  $\Delta G_{\text{alch.}}^2$  by running two FEP simulations of the mutation, one *in vacuo* and the other in bulk water. This case is a computationally affordable example of how point mutations in more complex protein systems may be studied.

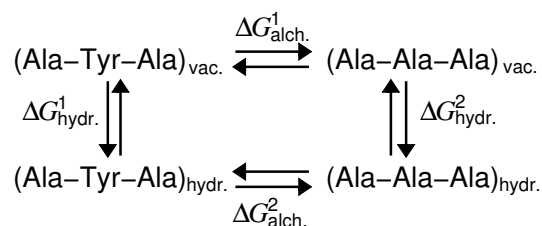


Figure 6: Thermodynamic cycle used in the Ala–Tyr–Ala  $\rightarrow$  (Ala)<sub>3</sub> alchemical transformation. The vertical arrows correspond to the hydration of the wild-type tripeptide and its mutant. The horizontal arrows correspond to the point mutation in bulk water and *in vacuo*, so that:  $\Delta G_{\text{alch.}}^2 - \Delta G_{\text{alch.}}^1 = \Delta G_{\text{hydr.}}^2 - \Delta G_{\text{hydr.}}^1$ .

#### 3.1. System setup

We will prepare two systems: the isolated tripeptide, and the same solvated in explicit water. You can build the latter by using the `solvate` plugin of VMD on the former. The files required to get started with this setup can be found in the `03.Mutating-tyrosine-into-alanine` subdirectory of the archive.

As in the ethane-ethane transformation example, you will need a topology file describing the hybrid molecule, which is depicted in Figure 7. The provided topology file `tyr2ala.top` is based on the standard CHARMM topologies for alanine and tyrosine. You can also obtain a topology file containing hybrid amino acids for any point mutation (except those involving proline) with the VMD plugin Mutator, available with VMD 1.8.5 and later. Recent releases of VMD include a database of hybrid topologies compatible with the CMAP corrections [19] of the CHARMM force field. The Mutator plugin may be used to prepare hybrid protein topologies and coordinates suitable for alchemical FEP. The manual procedure that we follow here, however, is much more flexible, should one want to include specific patches in the structure.

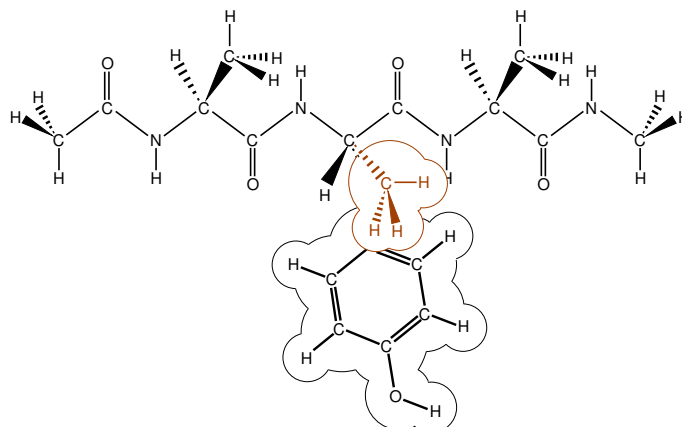


Figure 7: Dual topology hybrid molecule used for the Ala-Tyr-Ala  $\rightarrow$  (Ala)<sub>3</sub> alchemical transformation. The initial state, *viz.*  $\lambda = 0$  (black), and the final state *viz.*  $\lambda = 1$  (brown), are defined concurrently. Apart from the two side chains, the chemical groups of the tripeptide are common to the two topologies.

\* Topology for tyrosine-to-alanine transformation  
27 1 ! Version: pretend we are CHARMM27b1

```
RESI Y2A      0.00
GROUP
ATOM N      NH1  -0.47
ATOM HN     H    0.31
ATOM CA     CT1  0.07
ATOM HA     HB   0.09
GROUP
ATOM CBA    CT2  -0.18
ATOM HB1A   HA   0.09
ATOM HB2A   HA   0.09
GROUP
ATOM CGA    CA   0.00
GROUP
ATOM CD1A   CA   -0.115
ATOM HD1A   HP   0.115
GROUP
ATOM CE1A   CA   -0.115
ATOM HE1A   HP   0.115
GROUP
ATOM CZA    CA   0.11
ATOM OHA    OH1  -0.54
ATOM HHA    H    0.43
GROUP
ATOM CD2A   CA   -0.115
ATOM HD2A   HP   0.115
GROUP
ATOM CE2A   CA   -0.115
ATOM HE2A   HP   0.115
GROUP
ATOM CBB    CT3  -0.27
ATOM HB1B   HA   0.09
ATOM HB2B   HA   0.09
ATOM HB3B   HA   0.09
GROUP
ATOM C      C    0.51
ATOM O      O    -0.51
BOND N      HN
BOND N      CA
BOND C      CA
BOND C      +N
BOND CA     HA
BOND O      C
BOND CBA    CA
BOND CGA    CBA
```

```

BOND CD2A CGA
BOND CE1A CD1A
BOND CZA CE2A
BOND OHA CZA
BOND CBA HB1A
BOND CBA HB2A
BOND CD1A HD1A
BOND CD2A HD2A
BOND CE1A HE1A
BOND CE2A HE2A
BOND OHA HHA
BOND CD1A CGA
BOND CE1A CZA
BOND CE2A CD2A
BOND CBB CA
BOND CBB HB1B
BOND CBB HB2B
BOND CBB HB3B
IMPR N -C CA HN
IMPR C CA +N O
END

```

- 1 Create the PSF file by making the file `setup.pgn` containing the script below and then running it in the VMD TkConsole using `source setup.pgn`. Note that the first and third residues are standard alanine residues, so you have to use both the topology file from CHARMM27 and the custom hybrid topology.

```

package require psfgen
topology ../common/top_all122_prot.inp
topology tyr2ala.top

# Build the topology of both segments
segment Y2A {
  pdb tyr2ala.pdb
  first ACE
  last CT3
}
# The sequence of this segment is Ala-Y2A-Ala

# Read coordinates from pdb files
coordpdb tyr2ala.pdb Y2A

writepsf y2a.psf
writepdb y2a.pdb

```

- 2 Create the `alchFile` by opening `y2a.pdb` in a text editor and editing the B column to reflect which atoms vanish and appear. Save the file as `y2a.fep`. The modified part should read:

```

...
ATOM 17 N Y2A 2 5.841 -1.926 -3.336 1.00 0.00 YTOA N
ATOM 18 HN Y2A 2 5.362 -2.371 -4.106 1.00 0.00 YTOA H
ATOM 19 CA Y2A 2 7.291 -1.926 -3.336 1.00 0.00 YTOA C
ATOM 20 HA Y2A 2 7.655 -0.898 -3.336 1.00 0.00 YTOA H
ATOM 21 CBA Y2A 2 7.842 -2.640 -2.100 1.00 -1.00 YTOA C

```

|      |    |      |     |   |        |        |        |      |       |      |   |
|------|----|------|-----|---|--------|--------|--------|------|-------|------|---|
| ATOM | 22 | HB1A | Y2A | 2 | 7.014  | -2.994 | -1.485 | 1.00 | -1.00 | YTOA | H |
| ATOM | 23 | HB2A | Y2A | 2 | 8.452  | -3.487 | -2.411 | 1.00 | -1.00 | YTOA | H |
| ATOM | 24 | CGA  | Y2A | 2 | 8.687  | -1.679 | -1.298 | 1.00 | -1.00 | YTOA | C |
| ATOM | 25 | CD1A | Y2A | 2 | 8.856  | -0.360 | -1.739 | 1.00 | -1.00 | YTOA | C |
| ATOM | 26 | HD1A | Y2A | 2 | 8.377  | -0.028 | -2.660 | 1.00 | -1.00 | YTOA | H |
| ATOM | 27 | CE1A | Y2A | 2 | 9.640  | 0.531  | -0.996 | 1.00 | -1.00 | YTOA | C |
| ATOM | 28 | HE1A | Y2A | 2 | 9.771  | 1.557  | -1.339 | 1.00 | -1.00 | YTOA | H |
| ATOM | 29 | CZA  | Y2A | 2 | 10.254 | 0.104  | 0.187  | 1.00 | -1.00 | YTOA | C |
| ATOM | 30 | OHA  | Y2A | 2 | 11.016 | 0.969  | 0.909  | 1.00 | -1.00 | YTOA | O |
| ATOM | 31 | HHA  | Y2A | 2 | 11.063 | 1.844  | 0.516  | 1.00 | -1.00 | YTOA | H |
| ATOM | 32 | CD2A | Y2A | 2 | 9.302  | -2.106 | -0.115 | 1.00 | -1.00 | YTOA | C |
| ATOM | 33 | HD2A | Y2A | 2 | 9.170  | -3.132 | 0.227  | 1.00 | -1.00 | YTOA | H |
| ATOM | 34 | CE2A | Y2A | 2 | 10.086 | -1.215 | 0.627  | 1.00 | -1.00 | YTOA | C |
| ATOM | 35 | HE2A | Y2A | 2 | 10.564 | -1.547 | 1.548  | 1.00 | -1.00 | YTOA | H |
| ATOM | 36 | CBB  | Y2A | 2 | 7.842  | -2.640 | -2.100 | 1.00 | 1.00  | YTOA | C |
| ATOM | 37 | HB1B | Y2A | 2 | 7.014  | -2.994 | -1.485 | 1.00 | 1.00  | YTOA | H |
| ATOM | 38 | HB2B | Y2A | 2 | 8.452  | -3.487 | -2.411 | 1.00 | 1.00  | YTOA | H |
| ATOM | 39 | HB3B | Y2A | 2 | 8.687  | -1.679 | -1.298 | 1.00 | 1.00  | YTOA | C |
| ATOM | 40 | C    | Y2A | 2 | 7.842  | -2.640 | -4.572 | 1.00 | 0.00  | YTOA | C |
| ATOM | 41 | O    | Y2A | 2 | 7.078  | -3.122 | -5.407 | 1.00 | 0.00  | YTOA | O |
| ...  |    |      |     |   |        |        |        |      |       |      |   |

**3** Visualize the system containing the hybrid amino-acid. Run VMD with the following command:

```
vmd y2a.psf -pdb y2a.fep.
```

**4** In the Graphics/Representations menu, set the coloring method to Beta. The appearing alanine side chain should be colored blue and the tyrosine side chain should be red, while the backbone and the two unperturbed alanine residues should be green. Compare the result with Figure 7.

**5** (Optional if using NAMD 2.7b2 or later) Remove spurious bonded force-field terms and add a non-bonded exclusion list using `alchemify` in the command prompt:

```
alchemify y2a.psf y2a-alch.psf y2a.fep
```

Use the resulting file `y2a-alch.psf` when performing the *in vacuo* FEP calculations.

**6** You are now ready to prepare the hydrated system. Load the isolated tripeptide in VMD: `vmd y2a.psf y2a.pdb`.

**7** Open the Solvate interface (Extensions/Modeling/Add Solvation Box).

**8** Define a cubic,  $30 \times 30 \times 30 \text{ \AA}^3$  water box: Uncheck the “Use Molecule Dimensions” box and the “Waterbox Only”, set the minimum value of  $x$ ,  $y$  and  $z$  to  $-13$  and their maximum to  $+13$ . Run the plugin to create the files `solvate.psf` and `solvate.pdb`.

- 9 Prepare a new `alchFile` for the solvated structure, by opening `solvate.pdb` in a text editor and editing the B column to reflect the same information you entered in `y2a.fep` for the unsolvated structure. Save the new `alchFile` as `solvate.fep`.
  
- 10 When running `solvate`, information about non-bonded exclusions is lost, so if you are using a version of NAMD earlier than 2.7b2, `solvate.psf` should be treated with `alchemify` again:  

```
alchemify solvate.psf tyr2ala.hydrated.psf solvate.fep
```

### 3.2. Running the free energy calculations

You can find the configuration files for the isolated and solvated systems in the `In-vacuo` and `In-aqua` folders respectively.

Traditional MD is configured to run at a constant temperature of 300 K, with cutoff electrostatics and no particular boundary conditions. The FEP sections are configured based on several considerations.

The *in vacuo* transformation requires relatively long sampling times, because there are no solvent fluctuations that could couple to conformational fluctuations of the peptide (see Results). Fortunately, for such a small system, a 0.5-nanosecond trajectory can be generated very quickly on a single processor. Here, we will use 20 contiguous windows, involving 25 ps of MD sampling — interspersed with 2 ps of equilibration (carried out in steps 40 to 42):

```
# FEP PARAMETERS

source          ../../tools/fep.tcl

alch            on
alchType        FEP
alchFile        y2a.fep
alchCol         B
alchOutFile     forward.fepout
alchOutFreq     10

alchVdwLambdaEnd 1.0
alchElecLambdaStart 0.5
alchVdWShiftCoeff 4.0
alchDecouple     off

alchEquilSteps  4000
set numSteps    50000
```

```
runFEP 0.0 1.0 0.05 $numSteps
```

We will use a similar strategy for the solvated system, albeit with a somewhat larger time step. In each window, the system is equilibrated over `alchEquilSteps` MD steps, *viz.* here 100 steps, prior to 400 steps of data collection, making a total of 0.5 ps of MD sampling. Altogether, the alchemical transformation is carried out over 10 ps, and the backward transformation over the same time. (carried out in steps 43 to 45)

```
# FEP PARAMETERS

source                ../../tools/fep.tcl

alch                  on
alchType              FEP
alchFile              solvate.fep
alchCol               B
alchOutFile           forward-off.fepout
alchOutFreq           10

alchVdwLambdaEnd     1.0
alchElecLambdaStart  0.5
alchVdWShiftCoeff    4.0
alchDecouple         off

alchEquilSteps       100
set numSteps         500

runFEP 0.0 1.0 0.05 $numSteps
```

**1** Navigate to the In-vacuo folder.

**2** First, run `equilibration.namd` in the command prompt:

```
namd2 equilibration.namd > equilibration.log
```

**3** Next, repeat the above command for `forward.namd` and `backward.namd` to generate the required data files for analysis.

**4** Now navigate to the In-aqua folder. Note that the difference between the files suffixed by `-on` and those by `-off` lies in the `alchDecouple` switch. For now you will work with the files with suffix `off`.

The `alchDecouple` switch determines whether or not the vanishing and appearing moieties interact with each other. Switching decoupling off in this example causes the perturbed moieties to interact not only with the environment but also with each other. We discuss the use of this option at greater length in the Results section.

5 Perform the equilibration run.

6 Perform the forward and backward simulations.

### 3.3. Results

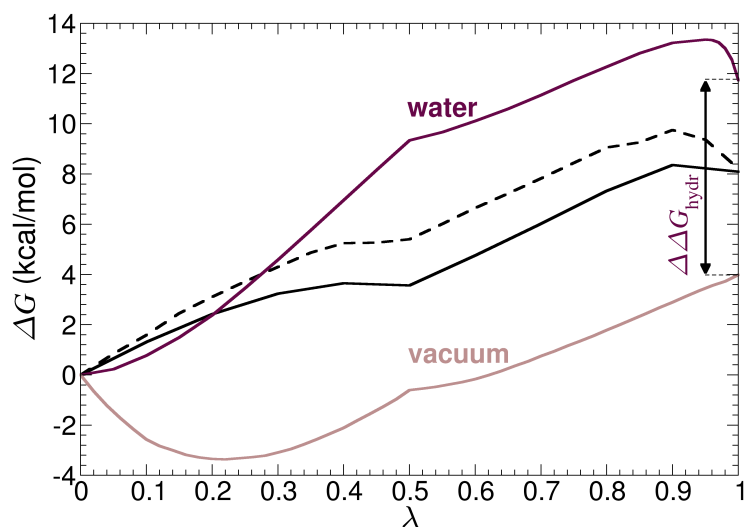


Figure 8: Results for the Tyr  $\rightarrow$  Ala mutations in water and in vacuum, in the Ala–Tyr–Ala blocked tripeptide. The difference between the corresponding free-energy changes yields the relative hydration free energy of Ala–Tyr–Ala with respect to (Ala)<sub>3</sub>. The transformation in vacuum accounts for intramolecular interactions between the perturbed moieties, *i.e.* `alchDecouple` off. As a basis of comparison and a consistency check, the results of decoupling-recoupling simulations, *i.e.* forward (dark solid line) and backward (dark dashed line) transformations with `decouple` on, in water are shown. Because this option ignores intra-perturbed interactions, it also obviates the need for separate gas-phase simulations.

1 Use `grep` to extract the free energy profiles from the `alchOutFiles` generated by your forward and backward simulations.

The  $\Delta G(\lambda)$  curves for both simulations, as well as an alternate method, are shown in Figure 8. Using an adapted protocol for each of the two mutations, the free energy difference for the hydrated state is

+11.7 kcal/mol, and +4.0 kcal/mol for the isolated state; your values may be slightly different due to the very short simulation times used here. Using the thermodynamic cycle of Figure 6, one may write:

$$\Delta\Delta G = \Delta G_{\text{alch.}}^2 - \Delta G_{\text{alch.}}^1 = \Delta G_{\text{hydr.}}^2 - \Delta G_{\text{hydr.}}^1.$$

The net solvation free energy change  $\Delta\Delta G$  for the Ala-Tyr-Ala  $\rightarrow$  (Ala)<sub>3</sub> transformation is found to be +7.7 kcal/mol. Alternately, a single decoupling/recoupling simulation indicates a hydration free energy difference of +8.1 kcal/mol, in agreement with the double annihilation scheme. This result may be related to the differential hydration free energy of side-chain analogues, *i.e.* the difference in the hydration free energy of methane and *p*-cresol, that is, respectively, 1.9 + 6.1 = +8.0 kcal/mol [20, 21]. Interestingly enough, Scheraga and coworkers have estimated the side-chain contribution for this mutation to be equal to +8.5 kcal/mol [22].

This very close agreement with experimental determinations based on side-chain analogues, as well as other computational estimates, may be in part coincidental or due to fortuitous cancellation of errors. Indeed, some deviation could be expected due to environment effects — *viz.* the mutation of a residue embedded in a small peptide chain *versus* that of an isolated, prototypical organic molecule[23] — and, to a lesser extent, the limited accuracy of empirical force fields. The first explanation may be related to the concept of “self solvation” of the side chain. Here, the tyrosyl fragment is not only solvated predominantly by the aqueous environment, but also, to a certain degree, by the peptide chain, which, under certain circumstances, can form hydrogen bonds with the hydroxyl group.

Moreover, it should be noted that even for a small and quickly relaxing system such as the hybrid tripeptide, convergence of the FEP equation requires a significant time. In some cases, very short runs may give better results than moderately longer ones, because the former provide a local sampling around the starting configuration, while the latter start exploring nearby conformations, yet are not long enough to fully sample them.

In general, whether or not intramolecular interactions ought to be perturbed — *i.e.* `alchDecouple` set to `off` or `on`, respectively — requires careful attention. As has been seen here, ignoring perturbed intramolecular interactions is computationally advantageous in the sense that it obviates the need for the gas-phase simulation depicted in Figure 7. This choice is fully justified in the case of rigid, or sufficiently small molecules. If, however, the system of interest can assume multiple conformations, setting `alchDecouple` to `on` may no longer be appropriate. This is due to the fact that on account

of the environment, specifically its permittivity, the conformational space explored in the low-pressure gaseous phase is likely to be different from that in an aqueous medium.

In all cases, visualizing MD trajectories is strongly advisable if one wishes to understand the behavior of the system and to solve possible sampling issues. Looking at the present tyrosine-to-alanine trajectories, it appears that the main conformational degree of freedom that has to be sampled is the rotation of the tyrosine hydroxyl group. Convergence is actually faster for the solvated system than for the tripeptide in vacuum, because fluctuations of the solvent help the tyrosine side chain pass the rotational barriers, which does not happen frequently in vacuum.

#### 4. Binding of a potassium ion to 18-crown-6

For this final example, we will go through the theoretical framework for measuring standard binding free energies, using the simple example of a potassium ion associated to a crown ether, namely 18-crown-6. 18-crown-6:K<sup>+</sup> has been investigated previously by means of molecular simulations. In their pioneering work, Dang and Kollman evaluated the potential of mean force delineating the reversible separation of the ion from the ionophore [24, 25]. Alchemical FEP, however, has proven over the years [7] to constitute a method of choice for measuring *in silico* host:guest binding free energies.

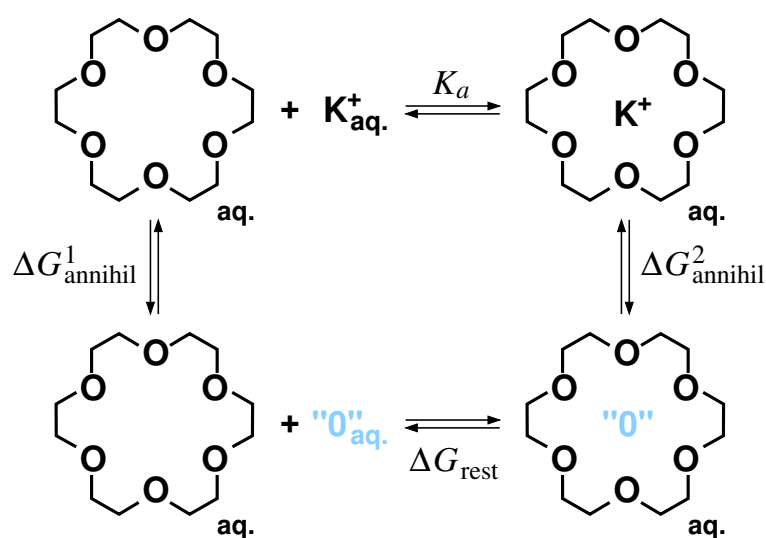


Figure 9: Thermodynamic cycle delineating the reversible association of a potassium ion to the 18-crown-6 crown ether in an aqueous environment. Binding of the ion to the ionophore is characterized by the association constant  $K_a$  and can be measured directly by means of a potential of mean force-like calculation. Alternatively, FEP can be employed to annihilate, or create the potassium ion both in the free and in the bound states. It follows from the latter that  $\Delta G_{\text{binding}} = -1/\beta \ln K_a = \Delta G^1_{\text{annihil}} - \Delta G^2_{\text{annihil}}$ . To prevent the cation from moving astray when it is only weakly coupled to the crown ether, *i.e.* at the very end of the annihilation transformation, or at the beginning of the creation transformation, positional restraints ought to be enforced, contributing  $\Delta G_{\text{rest}}$  to the overall binding free energy.

Here, the standard binding affinity of a potassium ion towards 18-crown-6 will be determined using this approach and following the thermodynamic cycle of Figure 9, wherein the alkaline ion undergoes a double annihilation [26], in its free and bound states.

## 4.1. System setup

Double annihilation refers to the ion being annihilated both in the free and in the bound state. In other words, we have to build two different molecular systems, *viz.* . a potassium ion in a water bath and the complex 18-crown-6:K<sup>+</sup>, also in a water bath.

The topology of 18-crown-6 is supplied in `18crown6.top`. In addition, the initial coordinates of the ionophore are provided `18crown6.pdb`. We will use these files to set up the host-guest complex. The first step of the setup is to build the PSF file for the host.

- 1 In the VMD TkConsole, navigate to the folder `04.Binding-of-a-potassium-ion-to-18-crown-6` and use the following script:

```
package require psfgen
topology 18crown6.top
segment 18C6 { pdb 18C6.pdb }
coordpdb 18crown6.pdb
writepsf 18crown6.psf
```

- 2 Now you will build the PDB and PSF files for the guest. Without exiting VMD, enter the following script in the TkConsole:

```
resetpsf
topology top_all122_prot.inp
segment POT { residue 0 POT }
writepdb potassium.pdb
writepsf potassium.psf
```

- 3 Merge the 18-crown-6 and potassium ion into a single PSF and PDB file by issuing the following commands in the VMD TKConsole:

```
resetpsf
readpsf 18crown6.psf
coordpdb 18crown6.pdb
readpsf potassium.psf
coordpdb potassium.pdb
writepsf complex.psf
writepdb complex.pdb
```

Now, we will center the ion with respect to the center of mass of 18-crown-6.

- 4 Open in VMD the files `complex.psf` and `complex.pdb`. Measure the center of mass of 18-crown-6, then move the potassium ion to the center, using

```
set sel [atomselect top "segname 18C6"]
set vec [measure center $sel]
set sel2 [atomselect top "segname POT"]
$sel2 moveby $vec
```

- 5 Write a new pdb file for the system: `writpdb complex_new.pdb`

Before you immerse the complex in water, recall that the free energy of the system is dependent on the size of the hydration box. The size-dependence correction imposed by the long-range nature of charge-dipole interactions is expected to cancel out if the dimensions of the simulation cell are identical for the two vertical legs of the thermodynamic cycle of Figure 9. In both cases, a box of dimension  $28 \times 28 \times 28 \text{ \AA}^3$  appears to constitute a reasonable compromise in terms of cost-effectiveness.

- 6 We now proceed to preparing the hydrated system. Load `complex.psf` and `complex_new.pdb` into VMD. Use the `solvate` plugin to create a water box of the recommended dimensions. Uncheck "Use Molecule Dimensions" and "Waterbox Only" before running. You will obtain `solvate.psf` and `solvate.pdb`. Move these files to the folder labeled `complex`.
- 7 You must solvate also the lone potassium ion. Start a new session of VMD. Load `POT.psf` and `POT.pdb` into VMD. Repeat the solvation step described previously to `solvate.psf` and `solvate.pdb`. Move these files to the folder labeled `potassium`.
- 8 Using a text editor, make an `alchFile` for each of the two systems you have created by editing their respective PDB files. Give the vanishing potassium ion a value of -1 in the B column, and then save the new files as `solvate.fep` in their respective folders.

## 4.2. Running the free energy calculations

We will minimize each system and then carry out equilibration, as a preamble to the free-energy calculations. In the case of the hydrated 18-crown-6:K<sup>+</sup> complex, this thermalization step will be utilized to appreciate how strongly bound is the guest in its dedicated binding site.

Equilibrium MD will be run for both systems at a constant temperature of 300 K with a damping coefficient of 1 ps<sup>-1</sup>, and pressure of 1 bar, using PME electrostatics. The `rigidBonds` option will be set to `all` to increase the integration time step of 2 fs. To impose isotropic fluctuations of the periodic box dimensions, the `flexibleCell` variable will be set to `no`. The trajectory will be saved in a DCD file with a frequency of *e.g.* 100 time steps.

- 1 At the command prompt, navigate to the folder `complex`. Run the minimization by entering  
`namd2 minimization.namd > minimization.log.`
- 2 Run the equilibration by entering  
`namd2 equilibration.namd > equilibration.log.`
- 3 Repeat the minimization and equilibration steps for the unbound potassium ion in the `potassium` folder.

To measure how the position of alkaline ion fluctuates in the ionophore, the trajectory of the 18-crown-6:K<sup>+</sup> complex can be loaded and the coordinates of the system aligned with respect to the heavy atoms of the crown ether.

- 4 Start a session of VMD and load `solvate.psf` and `solvate.pdb` in the `complex` folder. Then load into the molecule the trajectory `solvate.dcd`.
- 5 The alignment and calculation of the distance separating the center of mass of 18-crown-6 from the ion every frame can be performed using the following simple TCL script:

```
set outfile [open COM-ion.dat w]
```

```

set nf [molinfo top get numframes]

set all [atomselect top "all"]

set crown [atomselect top "resname 18C6 and noh"]
set crown0 [atomselect top "resname 18C6 and noh" frame 0]

set ion [atomselect top "segname POT"]

for { set i 1 } { $i <= $nf } { incr i } {
  $all frame $i
  $crown frame $i
  $ion frame $i
  $all move [measure fit $crown $crown0]
  $crown update
  $ion update
  set COM [measure center $ion weight mass]
  set dist [expr sqrt(pow([lindex $COM 0],2)+pow([lindex $COM 1],2)+pow([lindex $COM 2],2))]
  puts $outfile [format "%8d %8f" $i $dist]
}

close $outfile

```

The output file lists two columns of numbers, the first being the frame number and the second being the distance of the ion from the center of mass of 18–crown–6. You can use any plotting software to plot a graph of the distance against frame number. The maximum fluctuation in the distance between the center of mass of 18–crown–6 and the potassium ion will serve as the basis for a positional restraint introduced in the subsequent free-energy calculation, in which the ion is annihilated in its bound state.

- Now we move on to the free energy calculations. Navigate to the `complex` folder and inspect the `forward.namd` configuration file.

Look at the FEP section. An identical sampling strategy will be used for the annihilation of the potassium ion in the free and in the bound state. Here, use will be made of 32 equally spaced intermediate states:

```

# FEP PARAMETERS

source          ../../tools/fep.tcl

alch            on
alchType       FEP
alchFile       solvate.fep
alchCol        B
alchOutFreq    10

```

```
alchOutFile          forward.fepout

alchElecLambdaStart  0.1
alchVdwLambdaEnd     1.0
alchVdwShiftCoeff    5.0
alchdecouple         on

alchEquilSteps       50
set numSteps         200

set dLambda          0.03125

runFEP 0.0 1.0 $dLambda $numSteps
```

We will employ the COLVARS module of NAMD to enforce a positional restraint by means of an isotropic harmonic potential — *i.e.* an external potential that confines the ion within a sphere of given radius, centered about the center of mass of the ionophore. To do so, the following two lines have been added to the NAMD configuration file:

```
colvars              on
colvarsConfig        COMCOM.in
```

In addition, a separate, dedicated `colvarsConfig` file, `COMCOM.in`, has been written to instruct COLVARS of the positional restraint to be enforced:

```
colvarsTrajFrequency 100
colvarsRestartFrequency 100

colvar {
  name COMDistance

  width 0.1

  lowerboundary 0.0
  upperboundary 0.5

  lowerWallConstant 100.0
  upperWallConstant 100.0

  distance {
    group1 {
      atomnumbers { 43 }
    }
    group2 {
      atomnumbers { 1  4  5  8  11  12
                   15 18  19  22  25  26
                   29 32  33  36  39  40 }
    }
  }
}
```

```

    }
  }
}

```

Here, a confinement potential will be imposed on the ion to remain within a sphere of 1 Å diameter. This positional restraint results in a loss of translational entropy equal to  $-1/\beta \ln(c_0 \Delta v)$ , where  $\Delta v$  is the effective volume sampled by the guest and  $c_0$  is the usual standard concentration.

**7** In the command prompt, run `namd2 forward.namd > forward.log`. After the forward run has ended, run also the backward transformation.

**8** Run the forward and backward transformations in the folder `potassium`.

### 4.3. Results

The results of the two independent free-energy calculations are shown in Figure 10. To probe micro-reversibility of the transformation, both forward, annihilation, and backward, creation simulations were run. The marginal hysteresis between either pair of free-energy profiles is suggestive of a low finite-length systematic error. Still, a closer examination of the associated probability distributions,  $P_0[\Delta U(\mathbf{x})]$  and  $P_1[\Delta U(\mathbf{x})]$ , is necessary to ascertain that this is indeed the case [27, 7].

The difference between the net free-energy changes for the ion in its free and bound states yields the binding free energy, to which the contribution due to the positional restraint ought to be added. Confining the ion in a spherical volume enclosed in the ionophore to prevent it from escaping as host:guest coupling fades out is tantamount to a loss of translational entropy, which can be evaluated analytically. This entropic term corresponds to a free-energy contribution equal to  $-1/\beta \ln(c_0 \Delta v)$ , which in the case of a spherical volume element of  $0.52 \text{ \AA}^3$ , amounts to about 4.8 kcal/mol.

Put together, the theoretical estimate for the binding free energy of a potassium ion associated to 18-crown-6 is equal to  $-2.6$  kcal/mol, which appears to be in good agreement with the available experimental measurement of  $-2.91$  kcal/mol [28]. The accuracy is clearly improved compared to estimates from the early 1990s by Dang and Kollman, based on potential of mean force calculations with the limited sampling times then feasible: their first calculations [24] yielded  $-3.5$  kcal/mol, whereas the second publication [25] reports  $-2.0 \pm 0.3$  kcal/mol.

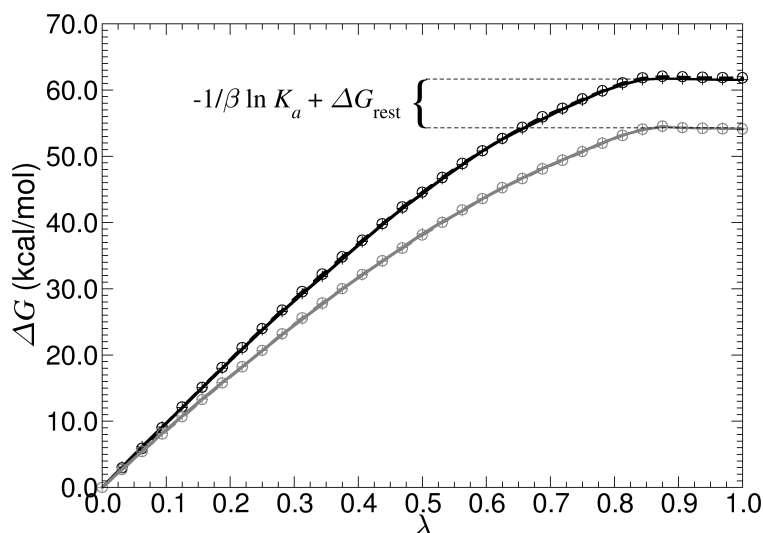


Figure 10: Free-energy change for the double annihilation of a potassium ion in its bound state, associated to 18-crown-6 (black solid line and pluses for the forward transformation, black dashed line and circles for the backward transformation), and in its free state, in a bulk aqueous environment (light solid line and pluses for the forward transformation, red dashed line and circles for the backward transformation). The difference at  $\lambda = 1$ , between the net free-energy changes,  $\Delta G_{\text{annihil}}^1 - \Delta G_{\text{annihil}}^2$ , viz.  $54.1 - 61.5 = -7.4$  kcal/mol, corresponds to the binding free energy, *i.e.*  $-1/\beta \ln K_a$ , to which the contribution due to the confinement of the ion in the crown ether, viz. 4.8 kcal/mol, ought to be added. The resulting standard binding free energy of  $-2.6$  kcal/mol is in good agreement with the experimental measurement of  $-2.91$  kcal/mol of Michaux and Reisse [28].

### Role of confinement potentials



Introduction of a positional restraint by means of the COLVARS module can be substituted by the addition of pseudo bonds between the ionophore and the alkaline cation. These bonds, aimed at tethering the ion as its coupling to the crown ether vanishes, are declared in the NAMD configuration file by:

```
extraBonds      yes
extraBondsFile  restraints.txt
```

The reader is invited to refer to the NAMD user's guide for the syntax employed for defining pseudo bonds in the external `extraBondsFile` file, and verify that following this route and subsequently measuring the free-energy cost incurred for fading out these pseudo bonds — *i.e.* by zeroing out reversibly the associated force constants, the aforementioned contribution due to the loss of translational entropy can be recovered.



### Finite-length bias

For each individual  $\lambda$ -intermediate state, monitor the probability distribution functions  $P_0[\Delta U(\mathbf{x})]$  and  $P_1[\Delta U(\mathbf{x})]$ , and verify that the systematic error due to the finite length of the free-energy calculation [7] is appreciably small.

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