Initial RAMD simulations of LinBwt and LinBL177W

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Motivation:

- Find out appropriate RAMD parameters for the simulation of LinBwt and LinBL177W in complex with cyclohexanol and 2-bromoethanol ligands.
- Get first insight into the ligand exit pathways in these systems.
- Identify critical residues for product egress, i.e. those interacting with the ligands on the way from the active site, for directed evolution experiments.
- Test the performance of the in-house RAMD implementation for NAMD.

Methods:

Preparation of structures:

Crystal structure of LinBwt (PDB code 1MJ5 [1], resolution 0.95Å) was taken as a starting point for modeling. The terminal His-tag residues were removed. Atoms with alternate locations were modeled in position A. The LinBL177W mutant was modeled based on this structure using the mutagenesis wizard of PyMol 1.0r2 [2], adopting the rotamer with least clashes.

Ligand docking:

Docking of the cyclohexanol and 2-bromoethanol ligands into the LinBwt structure was performed using the AutoDock 4.0 program suite [3]. All crystal water molecules in the LinBwt protein structure were removed, as well as all ions except the active site Cl^-. All hydrogens in the protein structure were removed and polar hydrogens were added using AutoDockTools (ADT) [4] graphical user interface of AutoDock 4.0 [3].

Structures of cyclohexanol and 2-bromoethanol were taken from the PEG in-house database. The structures were obtained previously according to the procedure described in Kmunicek et al. [5] (full minimization at MP2/6-31G* level). Auto-merge of non-polar hydrogens was turned off for the ligands (modeling them with both polar as well as non-polar hydrogens). The rotatable bonds and Gasteiger charges were assigned using ADT. The charge of Cl^- was assigned to -1.0. Grid box of 81x81x81 points in x, y and z dimensions was used with grid spacing of 0.25 Å. The grid was positioned so as to cover the whole active site cavity and the tunnels in the cap domain. Electrostatic map and atomic interaction maps for all atom types of the ligands, i.e. carbon, oxygen, bromine and hydrogen, were calculated by AutoGrid.

Ten independent docking calculations were performed for both cyclohexanol and 2-bromoethanol by AutoDock module of AutoDock suite using Lamarckian genetic algorithm for global and Solis&Wets algorithm for local search with initial population size of 150 and default AutoDock 4.0 [3] settings for elitism and cross-over. Maximum of 27 000 generations or 250 000 energy evaluations were performed. Resulting conformations were clustered with tolerance of 2.0 Å. For both cyclohexanol and 2-bromoethanol the lowest energy docking pose in the highest populated cluster resulting from docking to LinBwt was selected as a starting configuration for subsequent molecular dynamics simulations of the LinBwt complex as well as the LinBL177W mutant complex.

Preparation of molecular dynamics (MD) simulations:

The polar as well as non-polar hydrogens of LinBwt and LinBL177W protein structures were added using WHAT IF v5.2 [6]. Gln152 side-chain was flipped, His272 was modeled as double-protonated, based on the calculation of WHAT IF v5.2 [6] and in accordance with the MD simulations and analysis of LinB crystal structure [1], which showed the His272 to be at least partially double-protonated in the enzyme's ligand-free form. In other words, we assume here that the alcohol product leaves first, before the H+ and the halide. All crystallographic water molecules not overlapping with the docked ligands were added to the complexes. The

active site Cl^- was converted to Br^-. The system was neutralized by the addition of 12 Na^+ cations using Leap module of Amber 9 [7]. Finally, the complexes were immersed in a rectangular box of TIP3P [8] water molecules with a minimal distance of 10.0 Å between the boundaries of the box and the nearest protein atoms. About 12760 water molecules were added to solvate each system.

System equilibration:

The energy minimization and equilibration was performed using Sander module of Amber 9 [7] using ff99SB force field [9] Parameters for halogenated substrates (hal01.dat) were adopted from ref. [5]. Parameters for Br^- were adopted from Ref.[10]. The equilibration protocol consisted of the following steps: (i) 500 steps of steepest descent minimization and 500 steps of conjugate gradient minimization of water molecules and ions, with the rest of the system restrained by 500 kcal.mol^{-1}.Å^{-2}; (ii) 1000 steps of steepest descent minimization and 1500 steps of conjugate gradient minimization of the whole system; (iii) gradual heating of the whole system from 0 to 300 K in 20 ps, maintaining the temperature with Langevin thermostat and temperature coupling constant of 1.0 ps. Positions of protein and ligand atoms were fixed by weak restraints of 10.0 kcal.mol^{-1}.Å^{-2}. Constant volume periodic box conditions were used. The time step was 2 fs and all bonds involving hydrogen atoms were constrained by using the SHAKE algorithm. The Particle Mesh Ewald (PME) method was used for the long-range electrostatic interactions with non-bonded cutoff of 10 Å; (iv) 300 ps of unrestrained MD simulation at 300K in NPT periodic box conditions, using same parameters as in previous equilibration step and a constant pressure of 1.0 atm.

Production MD simulations:

The production runs were performed with NAMD version 2.7b1 simulation package [11], using the same Amber force field parameters as in the equilibration phase. Time step was 2 fs and all bonds involving hydrogen were constrained. PME method was used to calculate Coulomb interactions. Cutoff of 10 Å was applied for non-bonded interactions, switching functions were turned on. The simulation was propagated for 1ns (will be more), snapshots were gathered every 2 ps.

RAMD simulations:

RAMD simulations [12] of the complexes of LinBwt and LinBL177W with cyclohexanol and

2-bromoethanol were performed in NAMD version 2.6 [11]. The starting snapshots of the systems for RAMD simulations were extracted from the production runs after 1 ns. (More snapshots will be used in future.) Maximum duration of RAMD simulation was set to 1 ns; when ligand exit event was detected, i.e. distance between ligand center of mass (COM) and protein COM exceeded 30 Å, the simulation was halted.

First, proper setting of RAMD parameters was tested on the LinBL177W complexed with cyclohexanol. The force constant was varied using the following values: 20.0, 15.0, 10.0, 7.0, 5.0, 3.0 and 1.0 kcal.mol^{-1}.Å^{-2}. Force direction was reevaluated every 10 steps with a threshold on the distance traveled by ligand being 0.002 Å. In the next round of simulations, the force constant was kept at 5.0 kcal.mol^{-1}.Å^{-2} and the threshold distance was varied between 0.001 and 0.004 Å with a step of 0.001 Å. These settings were tested on all systems, i.e. complexes of each LinBwt and LinBL177W with either cyclohexanol or 2-bromoethanol.

Analysis of MD and RAMD simulations:

Stability of the MD trajectories was assessed by plotting total energy, RMSD, and radius of gyration, calculated using Ptraj module of AMBER 9 [7], against time. Per residue B-factors were also measured by Ptraj. *(More analyses will be performed, especially monitoring of internal H-bonds and of water dynamics.)* Trajectories were visually inspected in VMD [13].

Results:

Ligand docking:

Docking cyclohexanol to LinBwt resulted in only 1 cluster of docked ligand poses, see Table 1. In all runs, the ligand was placed in the active site above the ring plane of residue His272, with the hydroxyl group of the ligand pointing toward Asp108 and forming H-bond with atom OD2. The lowest energy pose (run 5) was selected. Docking of 2-bromoethanol to LinBwt resulted in 3 clusters. The lowest energy clusters 1 and 2 correspond to a position of the ligand not in active site, but in the “lower” tunnel. The most populated cluster 3, however, the ligand is placed in the active site, and similarly to the case of cyclohexanol, with the hydroxyl group forming H-bond to Asp 108. The lowest energy pose (run 4) in this cluster has been selected for further simulations.

Table 1. Docking results for cyclohexanol and 2-bromoethanol. Selected docking pose shown in bold.

Cyclohexanol			**2-bromoethanol**		
Docking run #	**Cluster #**	**Binding free E [kcal/mol]**	**Docking run #**	**Cluster #**	**Binding free E [kcal/mol]**
1	1	-3,85	*1*	*2*	*-2,65*
2	1	-3,85	2	3	-2,50
3	1	-3,85	3	3	-2,51
4	1	-3,83	**4**	**3**	**-2,51**
5	**1**	**-3,86**	5	3	-2,50
6	1	-3,85	*6*	*2*	*-2,67*
7	1	-3,84	7	3	-2,51
8	1	-3,85	*8*	*1*	*-2,72*
9	1	-3,85	9	3	-2,49
10	1	-3,85	10	3	-2,51

Equilibration MD:

The four complexes (LinBwt/cyclohexanol, LinBwt/2-bromoethanol, LinBL177W/ cyclohexanol and LinBL177W/2-bromoethanol) were equilibrated for 320 ps according to the protocol described in Methods. Monitoring of energy, per residue rmsd and radius of gyration of the system confirmed stable trajectory and achievement of equilibrium.

Production MD:

Each system has been simulated for 1ns. Visual inspection in VMD showed stable trajectory. *(Longer simulation and more detailed analysis will be performed in near future.)*

RAMD simulations:

Initial setting of RAMD parameters to be used in the NAMD RAMD implementation has been performed on the LinBL177W/cyclohexanol complex and confirmed on the remaining three systems (LinBwt/cyclohexanol, LinBwt/2-bromoethanol, LinBL177W/2-bromoethanol). The exit time of the ligand was found to be sensitive to the force applied. On the other hand, no direct relationship between threshold distance and exit time has been observed in the tested value range (see Table 2).

Table 2. Ligand exit time in LinBL177W/cyclohexanol complex for various RAMD parameter values.

Force Constant [kcal.mol^{-1}.Å^{-2}]	Distance threshold [Å]	Exit time [ps]	Exit route
20.0	0,002	10,6	lower t.
15.0	0,002	17,5	lower t.
10.0	0,002	53,5	lower t.
7.0	0,002	161,3	below a4
5.0	0,002	N/A	N/A
3.0	0,002	N/A	N/A
1.0	0,002	N/A	N/A
5.0	0,001	896,2	lower t.
5.0	0,002	N/A	N/A
5.0	0,003	725,2	lower t.
5.0	0,004	N/A	N/A

For the selected value of force constant of 5.0 kcal.mol^{-1}.Å^{-2} and the distance threshold varied between 0.001 and 0.004 Å with a step of 0.001 Å, the ligand exit event has been observed in two out of four simulations for all systems, except the least hindered one, i.e. LinBwt/2-bromoethanol, in which case ligand exit has been observed in all simulations, see Table 3. At the same time, the simulations in all cases spanned at least 150 ps, suggesting reasonably balanced RAMD parameters.

Table 3. Ligand exit time in all four studied systems with varying value of distance threshold. Force constant kept constant at 5.0 kcal.mol^{-1}.Å^{-2}

System	Distance threshold [Å]	Exit time [ps]	Exit route
LinBwt / Cyclohexanol	0,001	328,8	lower t.
	0,002	1000,0	N/A
	0,003	1000,0	N/A
	0,004	566,1	lower t.
LinBwt / 2-bromoethanol	0,001	352,2	lower t.
	0,002	378,5	upper t.
	0,003	356,6	upper t.
	0,004	164,4	upper t.
LinBL177W / Cyclohexanol	0,001	896,2	lower t.
	0,002	1000,0	N/A
	0,003	725,2	lower t.
	0,004	1000,0	N/A
LinBL177W / 2-bromoethanol	0,001	1000,0	N/A
	0,002	1000,0	N/A
	0,003	287,902	slot
	0,004	362,6	lower t.

The most frequent alcohol exit pathway is through the “lower” tunnel. *(This is just a preliminary result based on visual inspection, more rigorous analysis is needed.)* For the cyclohexanol, it is the only pathway observed. The smaller ligand, 2-bromoethanol, uses also the “upper” tunnel and the slot. In contradiction to the conclusion of Negri et al. [14] that the alcohol exit in LinBwt happens through the “slot” only, (based on single 8 ns trajectory of 2-bromoethanol in LinBwt), we show here that the “slot” is not the only pathway available to the alcohol.

No bromide exit event has been observed neither in the 1ns production MD simulations, nor in any of the RAMD simulations (spanning up to 1ns). This is consistent with the simulation of Negri et al. [14], where the progressive hydration of the cavity started at about 4 ns time, 2-bromoethanol exit started to leave the cavity at about 5 ns, and the bromide exit was observed only after about 7 ns. This shows that the spontaneous exit of the halide ion is probably slower than the exit of alcohol and a relatively long simulation times are therefore needed if it is to be observed.

In the ~2 ns MD simulations of Klvana et al. [15] of DhaAwt and its mutants with 2,3-dichloropropane-1-ol, the Cl^- exit event through pathway p1 was observed, but only rarely. It occurred in 1 of 2 trajectories of both DhaAwt and DhaA15 (I135F+C176Y), but not in any other of the remaining 7 mutants. The RAMD simulations were performed without Cl^- ion in active site.

Regarding the RAMD implementation for NAMD, it seems to work fine with NAMD 2.6, but problems were encountered with the new version 2.7 during the initialization of the program, probably due to clashes with a newly introduced module of TI for alchemical transformations. *(Vlad is now trying to solve this matter together with NAMD developers.)*

Conclusions:

- Suitable RAMD parameters for the simulation of LinBwt and LinBL177W in complex with cyclohexanol and 2-bromoethanol ligands have been identified.
- First insight into the ligand exit pathways in these systems has been obtained, although much more rigorous analysis of the trajectories is needed. Especially, the identification of residues forming exit pathways (importance for directed evolution experiments) and monitoring of water dynamics inside the tunnels are planned. Besides that, the changes in the tunnel properties will be analyzed using the new version of Caver. The conformational changes induced in the protein by the egress of ligand will be compared with the normal modes of a free enzyme.
- The in-house RAMD implementation for NAMD 2.6 has been successfully tested. Problems encountered with the new NAMD version 2.7 have been promised to be solved by the NAMD developers.

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