Computational study of product release in LinB haloalkane dehalogenase

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Motivation:

The following document is a writeup of the initial simulation setup to be used in the project conducted by Lada under DAAD postdoctoral fellowship [1]. The project aims at better understanding the substrate entrance and product exit through tunnels in enzymes with buried active sites.

Methods:

The project setup has been designed based on literature search as well as discussions with Rebecca Wade, Jiří Damborský and MCM and PEG group members.

Results:

Systems to be studied:

The LinB haloalkane dehalogenase has been chosen for its robustness, broad substrate specificity and suitability for kinetic experiments. LinB enzyme and its L177 mutants (L177 being positioned at the opening of largest - "lower" - LinB tunnel and at the edge of the "upper" tunnel) are currently subject of intensive experimental investigation in PEG group in Brno. In addition to the L177 mutants of LinB (17 well folded mutants, 15 of which active, [2] several other mutants of LinB

have been constructed [3], but these involve mutations in the active site and therefore are not suitable for the present study.

The ligands selected are cyclohexanol and 2-bromoethane-1-ol. The corresponding substrates are converted by a range of other haloalkane dehalogenases and are well suited for transient kinetics experiments, therefore lot of experimental data is/will be available.

Kinetic parameters of the selected systems:

The rate limiting step in LinBwt for its good substrates (including 1,2-dibromoethane and bromocyclohexane) is the alkyl-enzyme intermediate (E-R.X^-) hydrolysis step, as shown by pre-steady state kinetics [4], solvent kinetic isotopic effect [5] and MD simulations [6]. Product exit is fast and alcohol and halide leave in random order [4]. For a poor substrate, 1,2-dichloroethane, the SN2 step has been reported as rate limiting by experiment [5,7] and simulation [6].

There is currently no data on the rate limiting step in the L177 mutants. Transition kinetic experiments in these systems are pending. However, it can be expected that the substitution for large amino acid (Trp), which was shown to decrease enzyme activity, has no direct effect on the hydrolytic step due to the distance from the active site (12,5 Å C_α-C_α distance to catalytic Asp108), but presumably slows down the product release. Due to the comparable rate of all enzymatic steps, this affects the overall catalytic efficiency.

On the other hand, the effect of substitution for other residues is more difficult to rationalize. The overall most active mutants are L177A, L177F, L177M and L177G. The least active are L177R, L177D and 177H. Hypothetically, the mutations which increase the activity could do so by increasing the speed of hydration of the active site cavity after the formation of alkyl-enzyme and halide. Although the simulations of Negri et al. [6] assumed the active site hydration to take place *after* the

hydrolytic step, it is also possible that the waters enter as soon as the charged product (halide) is formed and that their presence is required for the proper positioning of the attacking OH- ion in the AdN step, which could probably be elucidated by classical MD.

For the comparison of experimental data with RAMD simulations of ligand exit, we actually do not need mutants with product release rate limiting, since we can compare the simulated probability of exit with the kinetic constant of the product release (k_4). The important thing is that the mutants differ in the speed of this step, as expected for LinBwt, LinBL177W and L177G. Also, since at the current stage we try to understand the underlying mechanisms rather than optimize the enzyme for a given substrate, we do not care whether the mutants are more active or less active.

As far as the DbjA enzyme (another possible candidate for simulations) is concerned, here the rate limiting step is also hydrolysis [8]. The order in which halide and alcohol leave the active site is not known yet. Generally, this enzyme has been less studied so far.

Experiments planned:

In addition to the detailed kinetic and stability characterization of the L177 mutants, including transient kinetics, there are some unique experiments scheduled for LinB:

LinB and its L177 mutants will be studied in the group of prof. Bertil Halle, Lund University, using a unique NMR method able to reveal H2O in the active site of the free enzyme. Measurements with ligand bound are also possible. These experiments can be directly compared with MD simulations. We have the opportunity to suggest model systems for these experiments.

Preliminary results with L177G and L177W show that both proteins posses improved

thermostability (up to 5°C of T_m). Change in thermostability by modification of the tunnel was set as important criterion to move from DhaA to LinB. Following mutants will be purified and studied for thermostability in the next step: L177A, L177D, L177R and L177S. Based on the results, the set will be extended further or (1) steady-state and (2) transient kinetic analysis will be performed.

Solvent relaxation experiments are on-going with the mutants L177A and L177S. Also this set will be extended as necessary.

Multiple mutants focused in the tunnel mouth of LinB will be constructed in two different ways: (1) rationally in an attempt to block the tunnel as much as possible; (2) using CASTing (saturated mutagenesis) in the hot spots identified by modeling. For this purpose, the identification of tunnel residues using RAMD simulations will be useful. The effect of mutations on stability, activity and individual reaction steps will be studied and the results will be compared to the findings obtained by RAMD and US. These experiments are to be started in about 1 month (as a new PhD project).

Planned simulations:

We will start with the RAMD simulations of LinBwt and LinBL177W with the products cyclohexanol and 2-bromoethane-1-ol. Simulations should be performed both with and without halide bound in active site, due to its random sequential exit with respect to the alcohol (see above). The effect of mutations on the permeability of the tunnels for different substrates could be studied using Umbrella Sampling similar to Hub and de Groot, [9]. This will enable us to derive not only qualitative, but also quantitative information on the barriers, which is essential for the comparison with the experiments. The residues revealed as barriers for ligand passage will be subject of mutagenesis. It is also possible to suggest other mutations (e.g. closing the tunnels).

In case of the large product, cyclohexanol, the release will need to be simulated using RAMD (especially in case of LinBL177W). Although the exit of the smaller product, 1-bromoethane-1-ol, has been observed using classical MD [6] on a time scale of ~5 ns, it has to be simulated using RAMD for consistency. It will be interesting to see whether the alcohol really leaves through one pathway only, as postulated by Negri et al. [6] based on a single trajectory, or whether it can use other pathways as well.

The conformational changes induced in the protein (especially in the tunnel residues, but also the whole cap domain) by the egress of ligands of different size could be compared with the dynamical behavior of the free enzyme, as revealed by normal mode analysis (NMA). If we could confirm on other systems the observation of Negri et al. [6] that LinB low frequency modes correspond to tunnel(s) opening, than the Caver tool could be enhanced by the possibility to search for tunnels in conformational ensemble generated by NMA.

The Caver tool should also be supplemented by the analysis of water molecules in the trajectories. However, the precise methodology how to do that (simple list of H2O in the tunnel in each snapshot, analysis of water passage, analysis of interaction with individual tunnel residues/ ligands?) needs to be devised.

Technical details:

The general procedure in the Klvana et al. manuscript [10] will be followed. The mutant modeling will be done using PyMol, docking of the ligands using Autodock. The system will be heated and equilibrated in the presence of the two products (alcohol and Br-) in the active site. During equilibration, following properties will be monitored: rmsd, energy, rmsf, rgyr, hbonds, ligands, water (using ptraj). Then a snapshot will be selected for RAMD. It might be beneficial to use a newer version of the forcefield (ff99sb or ff93) but the halogen parameters need to be consistent. Amber10 will be used for the initial set up and equilibration, while the production

runs will be done with NAMD. TIP3P water model should be OK for the purposes of these simulations. Initially, similar parameter values to the those used by Klvana et al. [10] will be used and than adjusted. For NMA using an ANM or ENM on the crystal structure and modeled mutants we will use a method including the side-chain atoms (not just C_α), e.g. NOMAD-Ref. [11,12]

Conclusions:

Enzymes to be studied: LinBwt, LinB-L177W, LinB-L177G, (L177R, L177D)
Ligands: cyclohexanol, 1-bromoethane-1-ol
Simulations: RAMD and US of product egress, MD of hydration process of the active site, NMA of free enzymes
Expected results: Higher free energy barriers and lower probability of product release in case of ligands of larger size, and in case of mutations to bulky residues (Trp). Difference in the speed of active site hydration for different mutants (hypothetical). Protein conformational changes during ligand exit comparable to low frequency normal modes.

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