First insights into the ligand exit pathways in LinBwt and LinBL177W using RAMD simulations

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Motivation:

- Validate the RAMD parameters derived for the simulation of cyclohexanol and 2-bromoethanol exit from LinBwt and LinBL177W as described in previous report [1].
- Complete the RAMD data of the previous report by a second set of simulations; draw first conclusions regarding 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:

System set-up, equilibration and production MD

The methods used for modeling, docking, equilibration and production are described in the previous report [1]. Briefly, the crystal structure with PDB code 1MJ5 was used for LinB wild type, the LinBL177W mutant was modeled based on this structure using PyMol [2]. The cyclohexanol and 2-bromoethanol ligands were docked into the LinBwt structure (water and ions removed, except active site Cl-) using AutoDock 4.0 [3]. The polar as well as non-polar hydrogens of LinBwt and LinBL177W protein structures were added using WHAT IF v5.2 [4]. The His272 residue was modeled as double-protonated. The active site Cl^- was converted

to Br^- and all non-overlapping crystallographic water molecules were added. Systems were neutralized by addition of Na+ cations and immersed in a rectangular box of TIP3P [5] water molecules with a 10.0 Å minimum wall thickness.

The systems were equilibrated using Amber 9 with ff99SB force field. Additional parameters were used for halogenated substrates and for Br- anion. The production runs were performed with NAMD [6], using the same Amber force field parameters as in the equilibration phase. Time step was 2 fs and all bonds involving hydrogen were constrained. The simulations were propagated for 2 ns, gathering snapshots every 2 ps. For more details on system set-up, equilibration and production, please refer to the July report [1].

RAMD simulations

RAMD simulations [7] of the complexes of LinBwt and LinBL177W with cyclohexanol and 2-bromoethanol were performed in NAMD version 2.6 and 2.7 [6]. The RAMD simulation was performed twice for each system and each RAMD parameter combination, taking the starting snapshot after 1 or 2 ns of production MD, respectively. The maximum duration of RAMD simulation was set to 1 ns; when a ligand exit event was detected, i.e. distance between ligand center of mass (COM) and protein COM exceeded 30 Å, the simulation was halted.

The RAMD parameters found according to the procedure described in the previous report [1] were used. Briefly, the system of LinBL177W with cyclohexanol was used for parameter set up. The force constant was varied first, decreasing its value from 20.0 $kcal.mol^{-1}.Å^{-1}$ to 1.0 $kcal.mol^{-1}.Å^{-1}$, with a threshold on the distance traveled by ligand being kept at 0.002 Å. Next, the force constant was kept at 5.0 $kcal.mol^{-1}.Å^{-1}$ while 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. In all simulations, force direction was reevaluated every 10 steps.

Analysis of RAMD simulations

The RAMD trajectories were visually inspected in VMD [8]. The evolution of RMSD against time was analyzed for each trajectory. The residues in contact with the ligand during its exit were calculated as those closer than 5 Å.

Results:

Parameter validation:

In addition to the results described in previous report [1] (RAMD simulations starting from snapshot taken after 1ns of production MD), another simulation was performed for each system and parameter setting, starting from a snapshot after 2 ns of production MD. The additional set of simulation confirms the observations made in previous report, namely that the ligand exit time is sensitive to the value of force constant (as already mentioned in previous report [1]), see Table 1 and Figure 1, but rather insensitive to the value of distance threshold, see Table 2 and Figure 2. The dependence of the number of new force vectors (directions) applied during the simulation on the value of the distance threshold is shown in Figure 3.

Table 1. Ligand exit times and pathways in LinBL177W/cyclohexanol complex for varying force constant values. Distance threshold kept at 0,002 Å.

Force Constant [kcal.mol^{-1}.Å^{-1}]	**Exit time [ps]**	**Exit route**
20.0	10,6	lower t.
15.0	17,5	lower t.
10.0	53,5	lower t.
7.0	161,3	**below a4** [1)]
5.0	N/A	N/A
3.0	N/A	N/A
1.0	N/A	N/A

1) Unorthodox pathway U1 described below.

Table 2. Ligand exit time in all four studied systems, starting from two different snapshots of PMD, with varying value of distance threshold. Force constant kept constant at 5.0 kcal.mol^{-1}.Å^{-1}

System	snapshot [ns of PMD]	Distance threshold [Å]	Exit time [ps]	Exit route
LinBwt / Cyclohexanol	1	0,001	329	slot/lower
	1	0,002	1000	N/A
	1	0,003	1000	N/A
	1	0,004	566	slot
	2	0,001	405	**Unfold. loop** [1)]
	2	0,002	527	upper
	2	0,003	1000	N/A
	2	0,004	1000	N/A
LinBwt / 2-bromoethanol	1	0,001	352	slot
	1	0,002	378	upper
	1	0,003	357	slot
	1	0,004	164	upper
	2	0,001	1000	N/A
	2	0,002	251	slot
	2	0,003	96	upper
	2	0,004	216	lower
LinBL177W / Cyclohexanol	1	0,001	896	lower
	1	0,002	1000	N/A
	1	0,003	725	lower
	1	0,004	1000	N/A
	2	0,001	630	slot
	2	0,002	885	**A7-A8/A3** [2)]
	2	0,003	153	lower
	2	0,004	1000	N/A
LinBL177W / 2-bromoethanol	1	0,001	1000	N/A
	1	0,002	1000	N/A
	1	0,003	288	slot
	1	0,004	363	lower
	2	0,001	937	slot
	2	0,002	485	lower
	2	0,003	931	lower
	2	0,004	696	lower

1) Unorthodox pathway U2 described below. 2) Unorthodox pathway U3 described below.

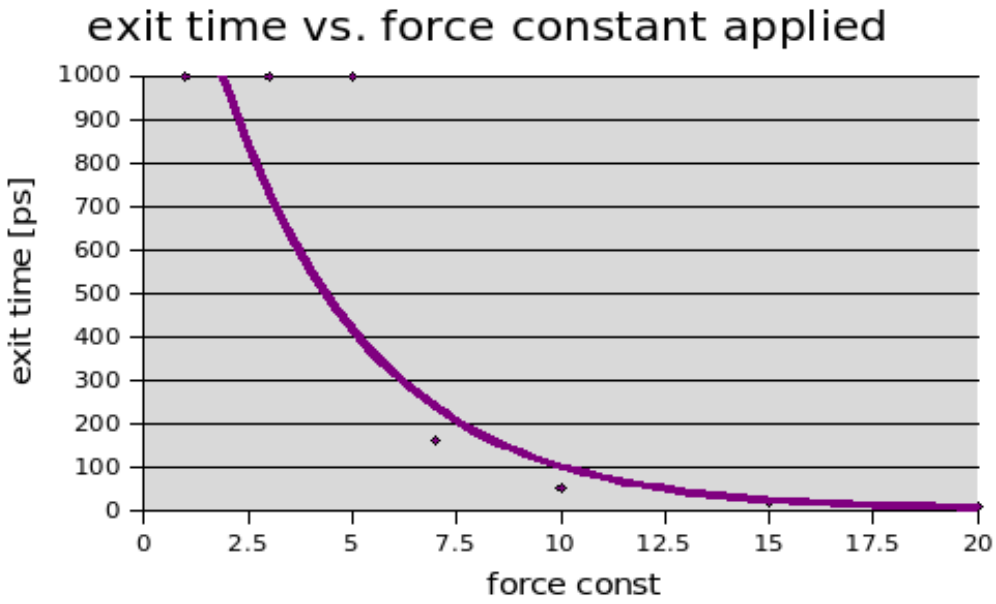

Fig. 1. Dependence of the simulation time on the force constant applied in the in LinBL177W/cyclohexanol system.

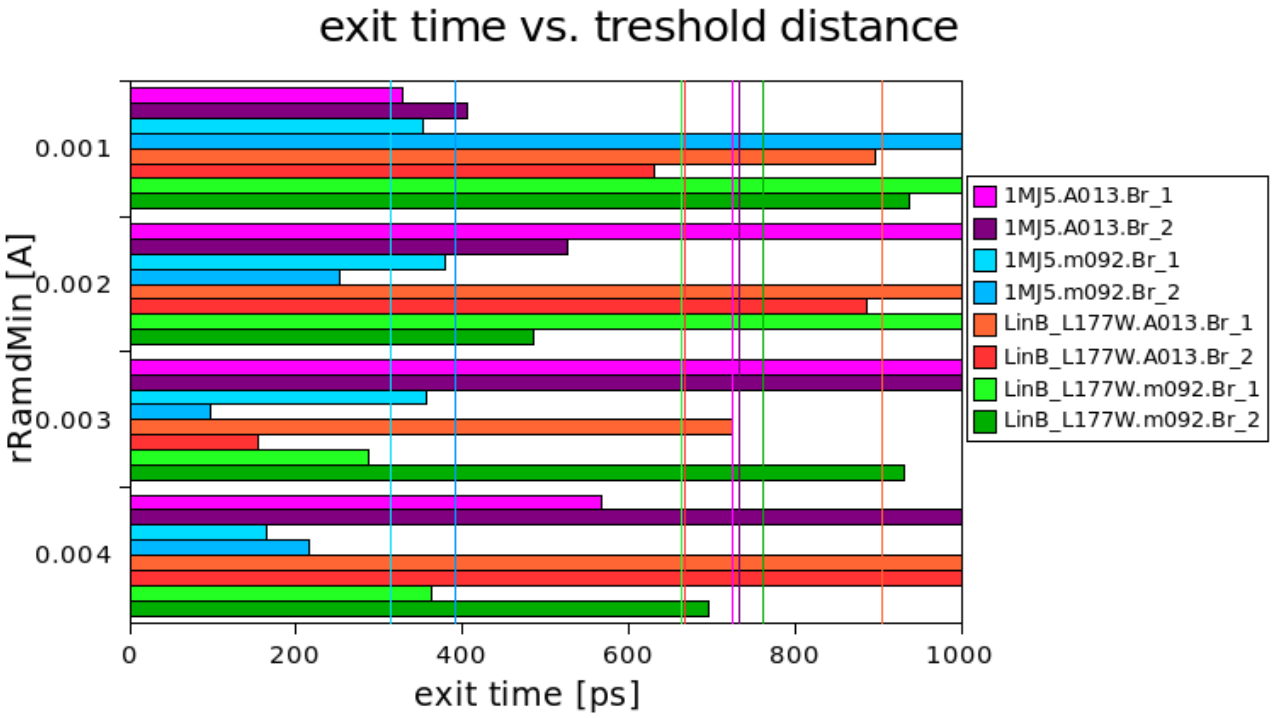

Fig. 2. Simulation time plotted against the value of distance threshold (rRamdMin parameter). For each system the average simulation time is indicated as a vertical line.

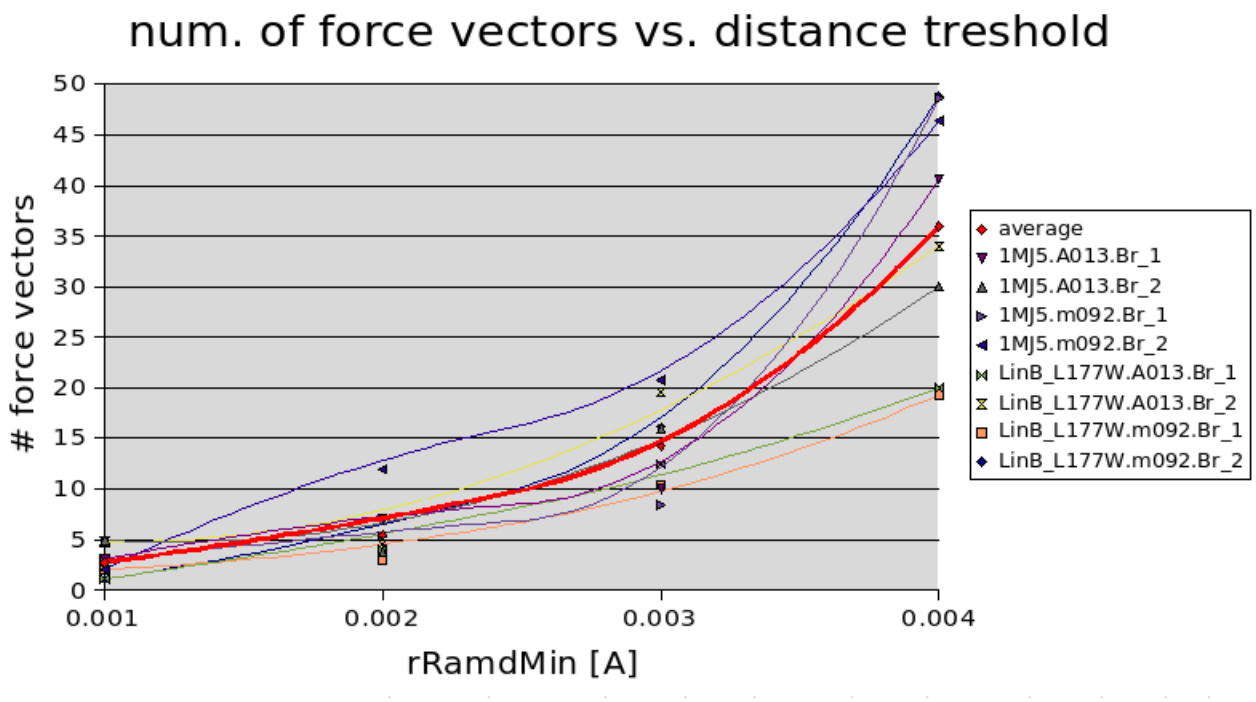

Fig. 3. Dependence of the number of new force vectors applied during simulation on the value of distance threshold (rRamdMin parameter).

Tunnel annotation:

Comparing to the previous report, a more precise analysis of the exit routes was performed, analyzing the residues contacted during ligand exit, leading to a different annotation of the observed exit pathways in some cases. The annotation was based on the similarity to the tunnels observed in the crystal structure of LinB (PDB code 1MJ5) using Caver, see Figure 4. The naming of the three tunnels leading through the cap domain of LinB follows that of the Caver paper [9].

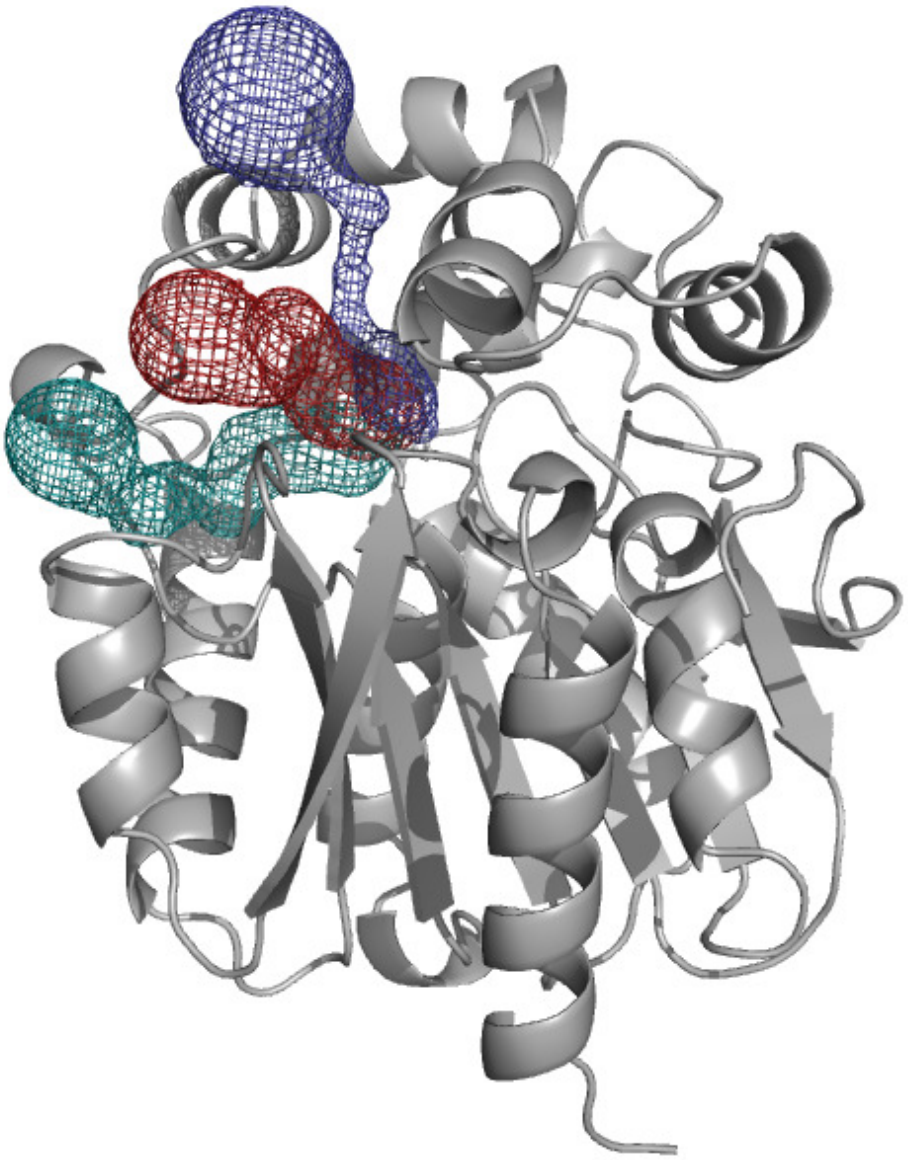

Fig. 4. Tunnels in LinB crystal structure (PDB code 1MJ5), visualized by Caver [9, 10].
Blue – upper tunnel, red – lower tunnel, cyan – slot.

RAMD results:

Within the range of the distance threshold (rRamdMin parameter) values tested, there was at least one trajectory without ligand exit for each of the systems, see Table 2. The most frequently observed pathway is the lower tunnel (8 times), followed by the slot (7 times), the upper tunnel (4 times) and the slot/lower tunnel pathway (once), see Table 2 and Figure 5. Other pathways were observed in 2 simulations and no ligand exit event was observed in 10 out of the 32 simulations.

Exit through the upper tunnel was only observed in the WT enzyme, which means that the L177W mutation effectively blocked this tunnel in the LinBL177W mutant. The cyclohexanol was observed to use several “unorthodox” pathways, described in detail below. The current observations are in contradiction to the assumption of Negri et al. [11] that the alcohol exit in LinBwt happens through the “slot” only, (based on single 8 ns trajectory of 2-bromoethanol in LinBwt).

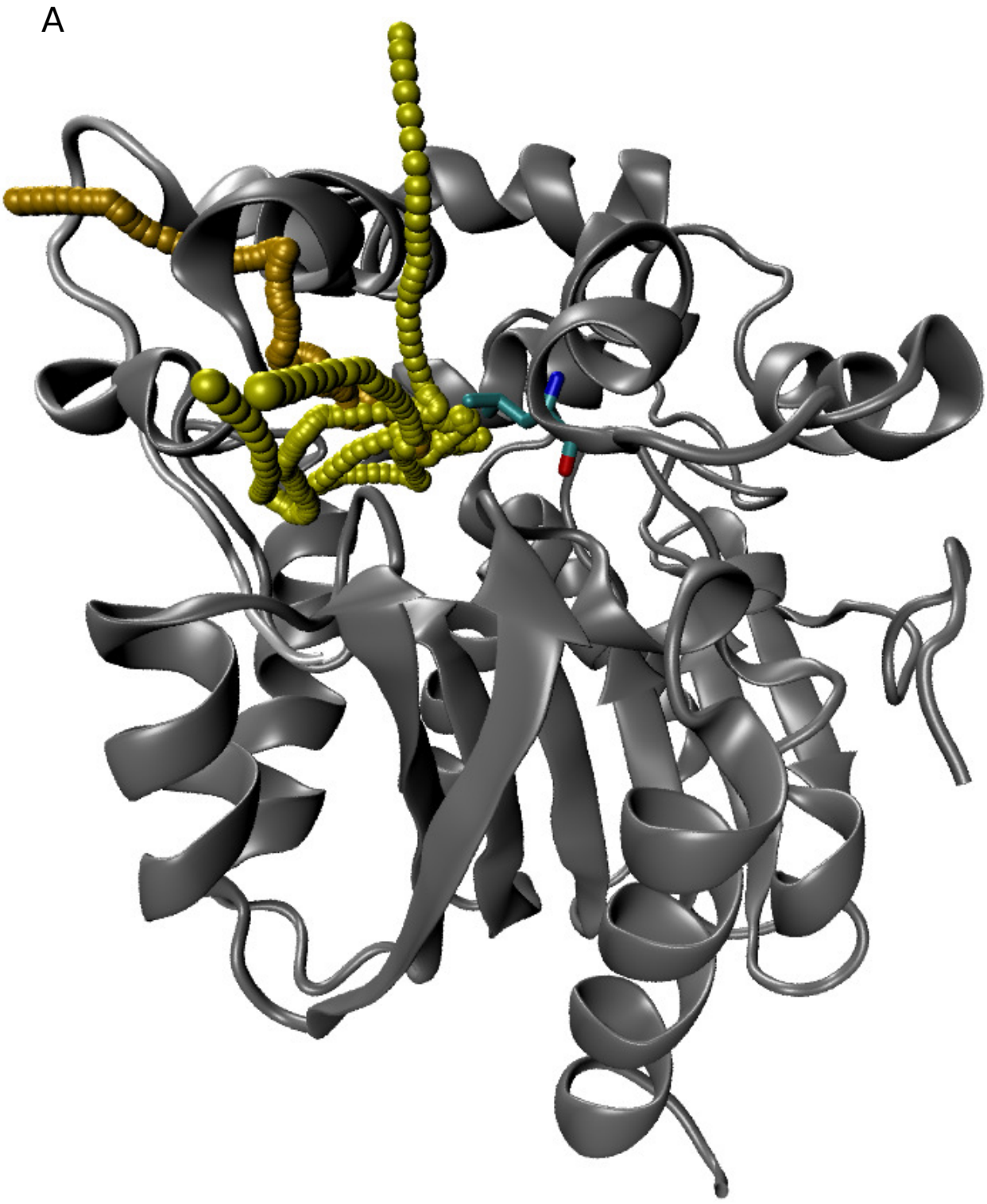

Fig. 5A. Pathways in LinBwt / Cyclohexanol system. Position of cyclohexanol during simulation is represented by a yellow CPK of the carbon bearing the hydroxyl group. A darker shade is used for the pathway where loop unfolding has been observed. The final position of the loop in this trajectory is depicted by light gray cartoon. Residue 177 is in licorice representation. Bromide anion is shown in green.

B

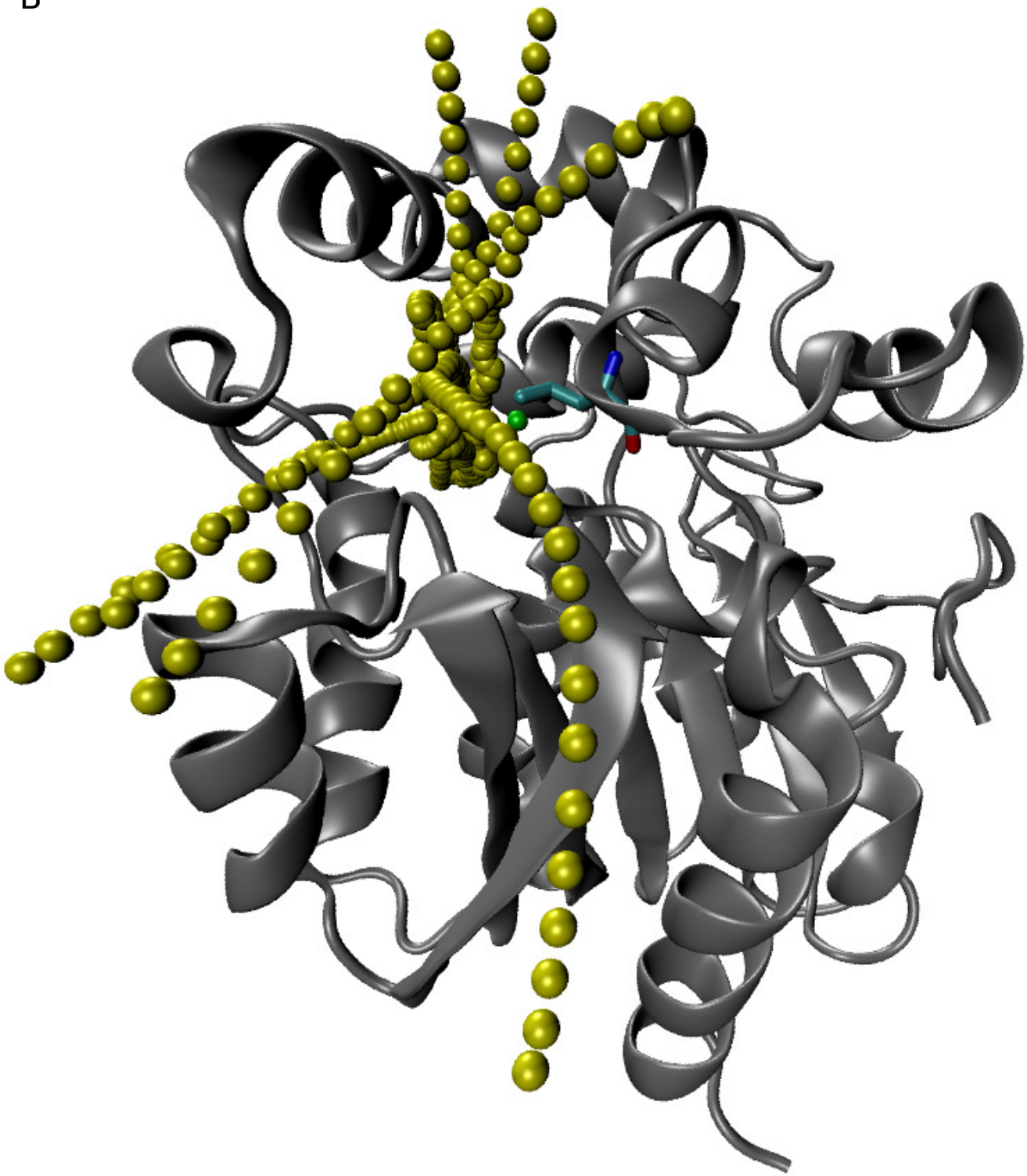

Fig. 5B. Pathways in LinBwt / 2-bromoethanol system. Position of cyclohexanol during simulation is represented by a yellow CPK of the carbon bearing the hydroxyl group. Residue 177 is in licorice representation. Bromide anion is shown in green.

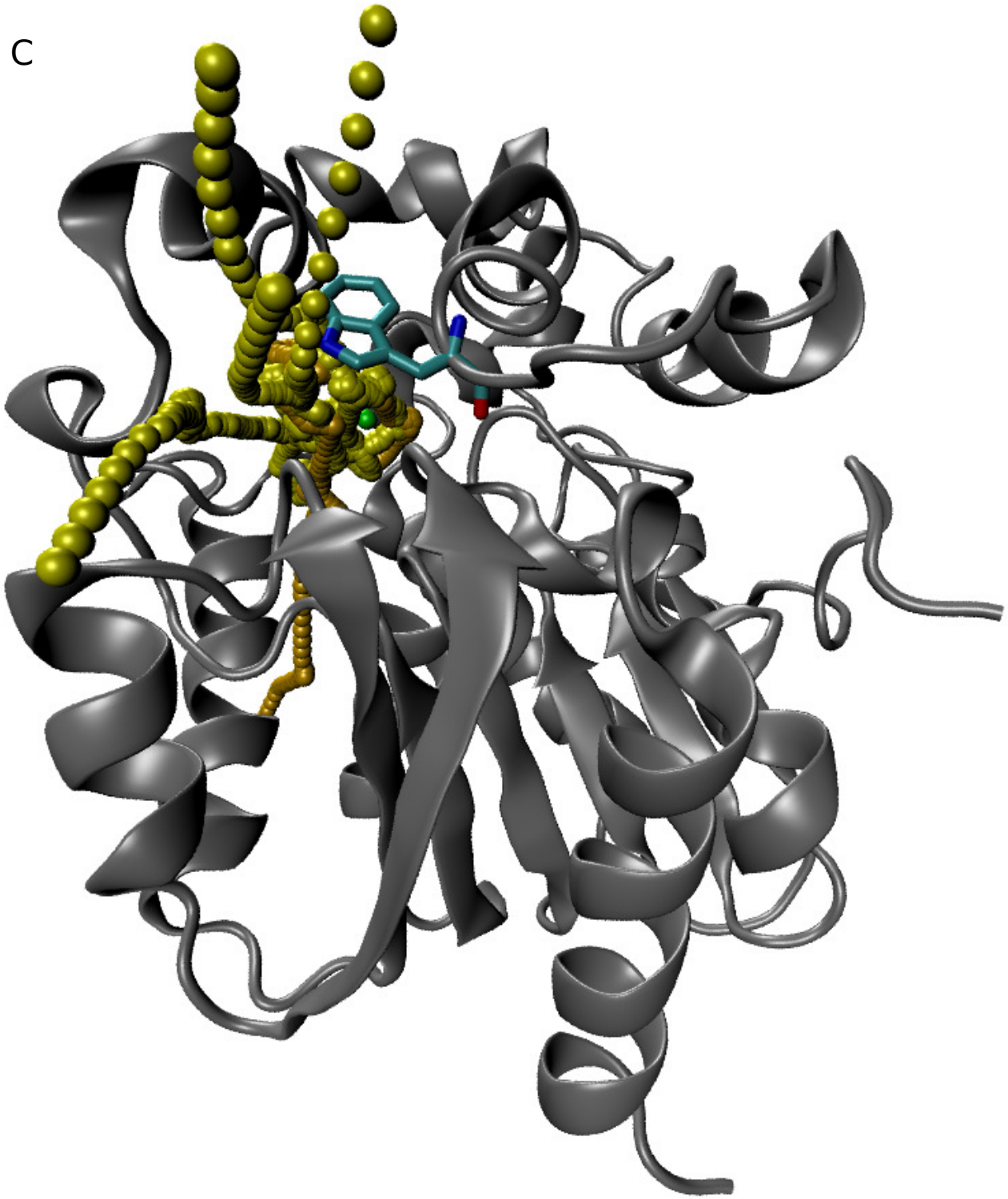

Fig. 5C. Pathways in LinBL177W / Cyclohexanol system. Position of cyclohexanol during simulation is represented by a yellow CPK of the carbon bearing the hydroxyl group. A darker shade is used for the "unorthodox" pathway between helices A7-A8/A3. Residue 177 is in licorice representation. Bromide anion is shown in green.

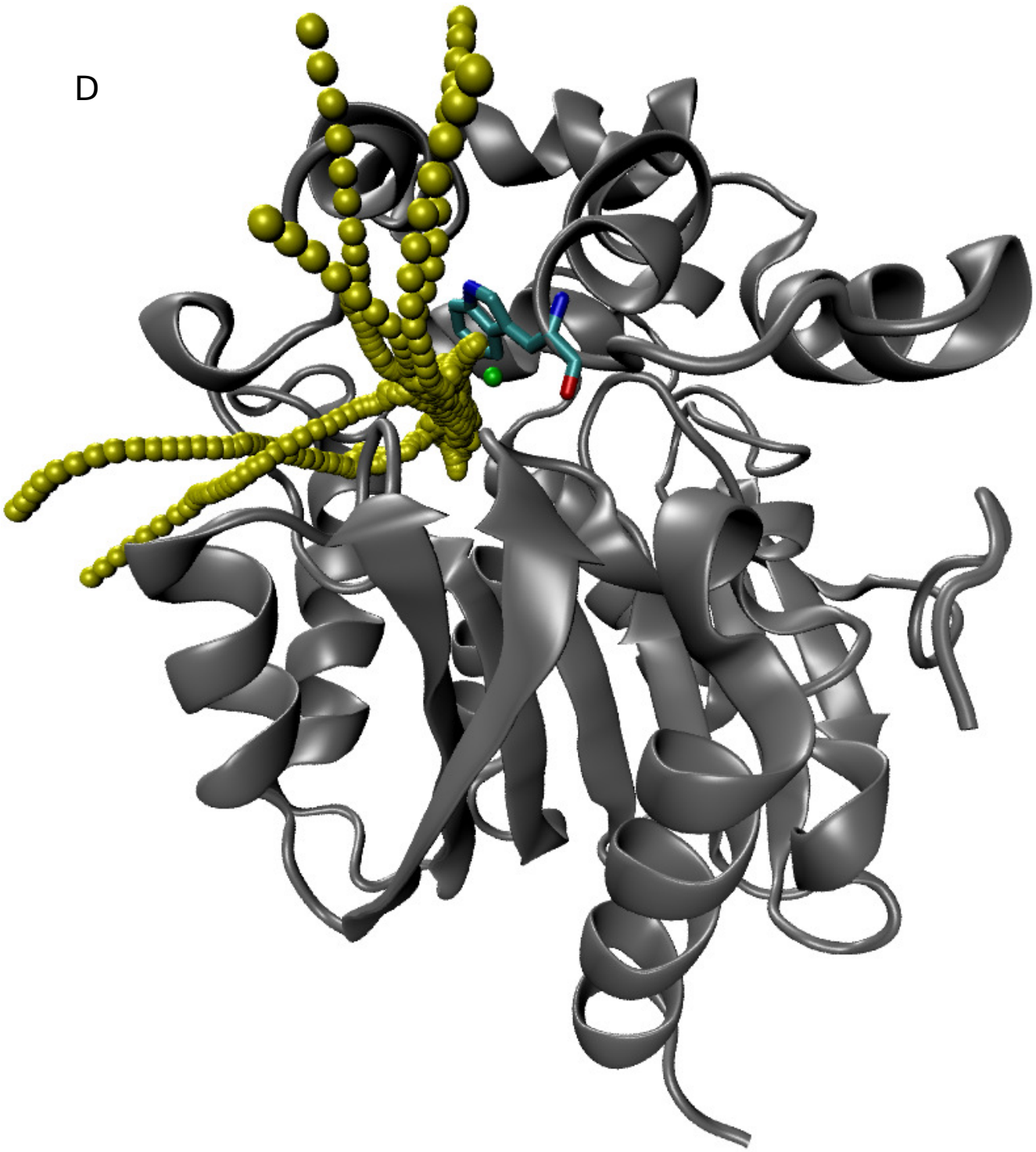

Fig. 5D. Pathways in LinBL177W / 2-bromoethanol system. Position of cyclohexanol during simulation is represented by a yellow CPK of the carbon bearing the hydroxyl group. Residue 177 is in licorice representation. Bromide anion is shown in green.

“Unorthodox” pathways:

In the simulations so far, three ligand exit pathways have been observed which cannot be described as following any of the tunnels in the cap domain - upper tunnel, lower tunnel or slot. These pathways are therefore described here in more detail.

- Pathway U1: System LinBL177W/cyclohexanol - exit below helix A4.

(Simulation code ramd1/LinB_L177W.A013_1_10_7_002)

The cyclohexanol follows a straight path between the helix A4 and the loop before A4. The main obstacle in this path represents Trp139, which forms a "lid" closing the path. In order to exit, the cycloxehanol has to push the Trp139 sideways and flip the Phe142 aromatic ring by about 130 degrees, see Figure 6.

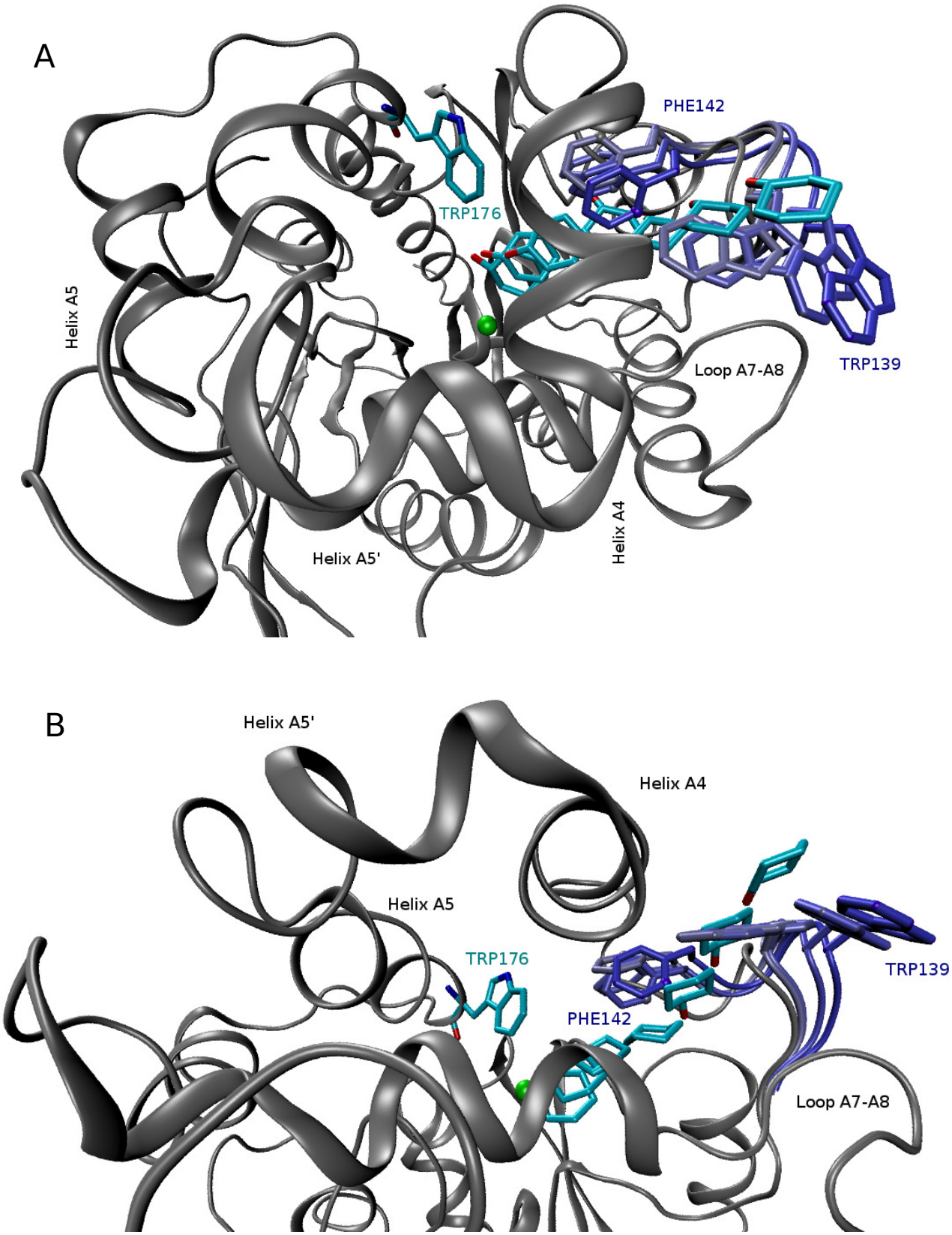

Fig. 6. Unorthodox pathway U1 A) top view, B) side view. The CD2 atom of Phe142 is indicated by a ball to illustrate the flipping of this residue.

- Pathway U2: System LinBwt / Cyclohexanol - unfolding loop before helix A4 (res. 142-147). (Simulation code ramd1/1MJ5.A013_2_10_5_001)

The cyclohexanol reorients its OH group and follows a direct path toward the loop before helix A4, formed by residues 142-147, causing it to unfold and open a pathway in the upper part of the "slot" tunnel, see Figure 7. It is clear that there is a barrier associated with this process, but it seems to be smooth, without major jumps, the unfolding happening in one continuous movement.

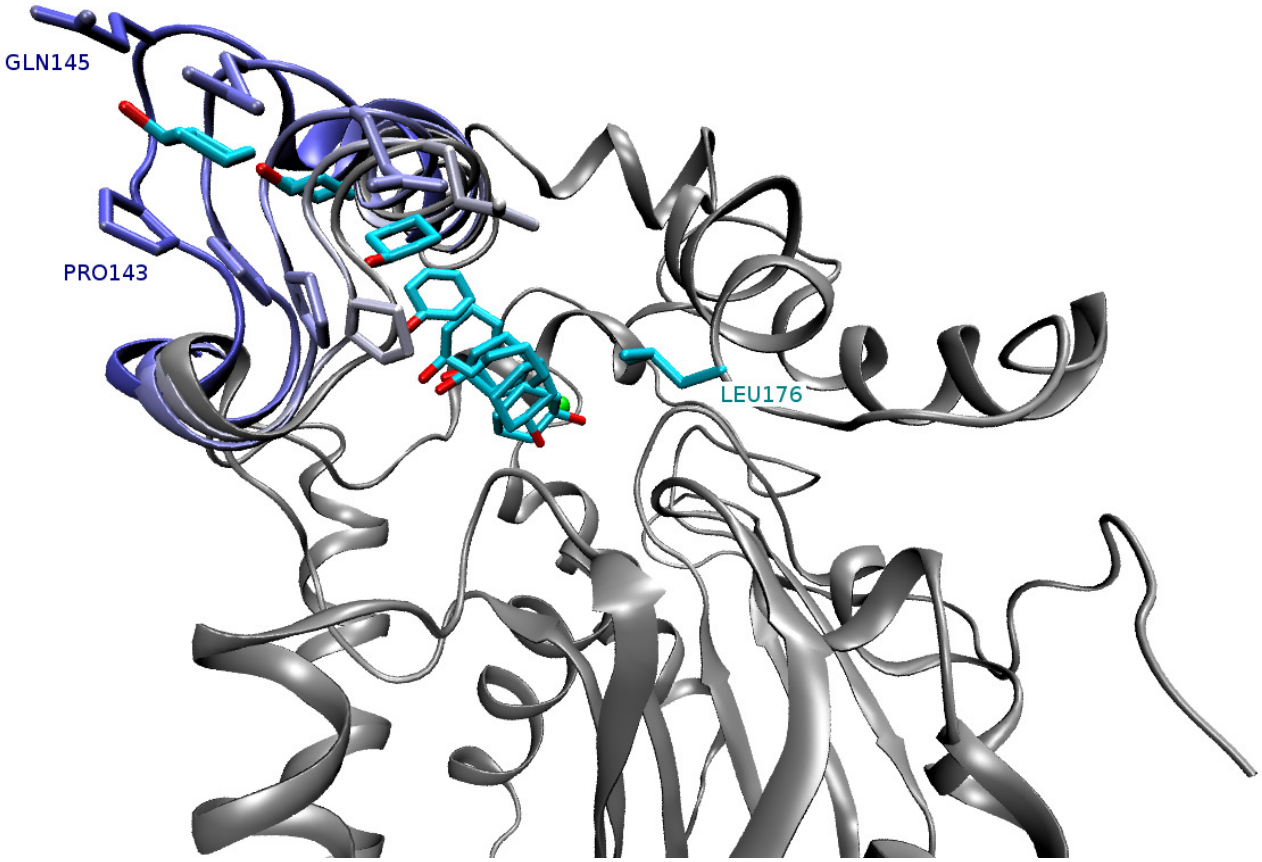

Fig. 7. Unorthodox pathway U2

- Pathway U3: System LinBL177W / Cyclohexanol - exit along helix A3 and under helix A8. (Simulation code ramd1/LinB_L177W.A013_2_10_5_002)

After wiggling in the active site cavity for a while, the cyclohexanol molecule follows a path along helix A3. There are at least two major barriers, the first being the Ile133. Pushing of this residue to the side makes room for the cyclohexanol to pass along and simultaneously causes the Tyr226 residue, located further down the pathway, to slightly turn its ring (by about 30 degrees). This opens a free pathway for cyclohexanol and enables it to move fast forward. The last obstacle is than Ile222, which is pushed outwards, while the Ile133 and Tyr226 relax slightly towards their original positions, see Figure 8.

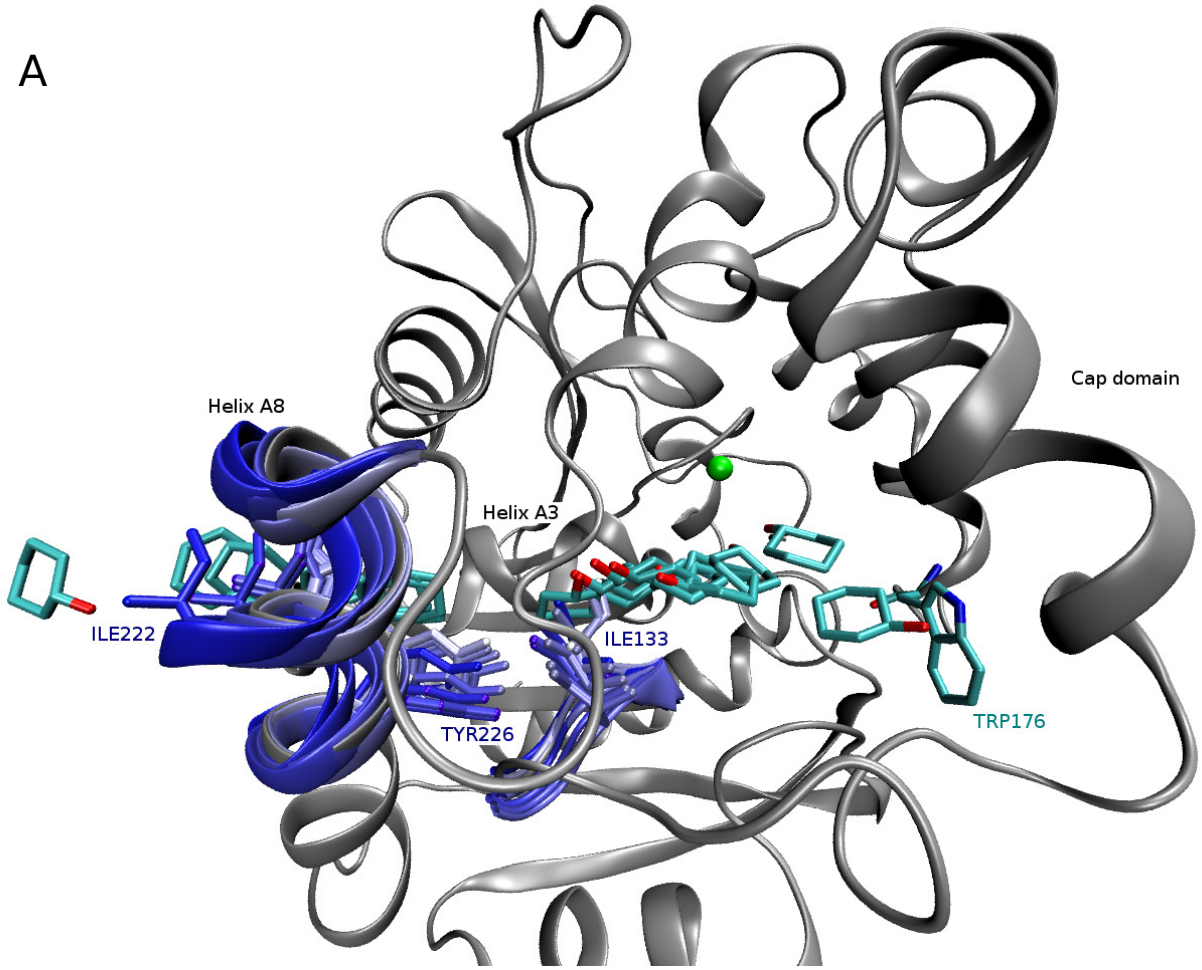

Fig. 8A. Unorthodox pathway U3 – side view.

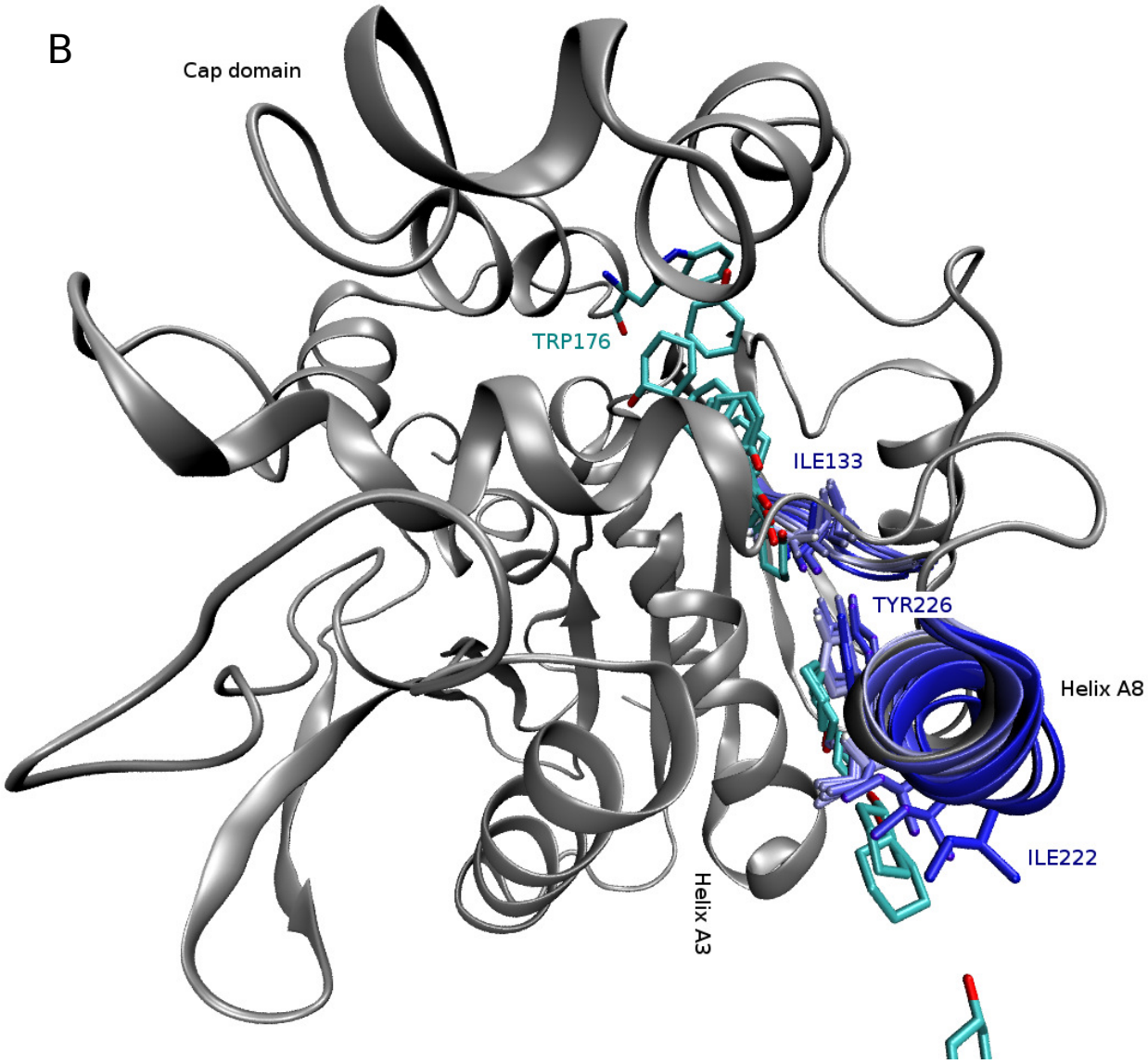

Fig. 8B. Unorthodox pathway U3 – back view.

Bromide exit:

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. [11], 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 simulation times used here and about one order longer simulation times would be needed if a spontaneous halide exit is to be observed.

In the ~2 ns MD simulations of Klvana et al. [12] 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.

NAMD implementation:

The problems with RAMD implementation in NAMD v.2.7, reported in the previous report [1], seem to have been successfully resolved by NAMD developers.

Conclusions:

- The RAMD parameters identified previously have been validated on a second set of RAMD simulations using the same systems, LinBwt and LinBL177W in complex with cyclohexanol or 2-bromoethanol.
- The ligand exit pathways in these systems have been analyzed. Fist conclusions about the effect of L177W mutation can be drawn. This mutation obviously prevents the ligand exit through the upper tunnel, while only slightly hindering the exit through the other tunnels - longer simulation times are observed for the ligand exit through these tunnels in the L177W mutant comparing to the WT enzyme.
- Residues forming the exit pathways have been identified (data not shown), which enables to select hot spots for direct evolution experiments.
- Several unexpected ("unorthodox") pathways have been observed for the exit of cyclohexanol, which have been analyzed in more detail.
- Some analysis of the trajectories still remains to be done, e.g. monitoring of water dynamics inside the tunnels, the changes of the tunnel properties with ligand passage using Caver, comparison of normal modes with the conformational changes induced in the protein by ligand egress etc.
- NAMD version 2.7 seems to work fine with RAMD now.

References:

1. Biedermannova, L., *Monthly report July 2009: Initial RAMD simulations of LinBwt and LinBL177W*. 2009.
2. DeLano, W.L., *The PyMOL Molecular Graphics System*. 2009, The PyMOL Molecular Graphics System. DeLano Scientific LLC, Palo Alto, California, USA. http://www.pymol.org.
3. Morris, G.M., et al., *Automated docking using a Lamarckian genetic algorithm and an empirical binding free energy function.* Journal of Computational Chemistry, 1998. **19**: p. 1639-1662.
4. Vriend, G., *WHAT IF: A molecular modeling and drug design program.* Journal of Molecular Graphics, 1990. **8**: p. 52-56.
5. Jorgensen, W.L., et al., *Comparison of simple potential functions for simulating liquid water.* Journal of Chemical Physics, 1983. **79**(2): p. 926-935.
6. Phillips, J.C., et al., *Scalable molecular dynamics with NAMD.* J Comput Chem, 2005. **26**(16): p. 1781-802.
7. Luedemann, S.K., V. Lounnas, and R.C. Wade, *How do substrates enter and products exit the buried active site of cytochrome P450cam? 1. Random expulsion molecular dynamics investigation of ligand access channels and mechanisms.* Journal of Molecular Biology, 2000. **303**: p. 797-811.
8. Humphrey, W., A. Dalke, and K. Schulten, *VMD – Visual Molecular Dynamics.* Journal of Molecular Graphics, 1996. **14**: p. 33-38.
9. Petrek, M., et al., *CAVER: A new tool to explore routes from protein clefts, pockets and cavities.* BMC Bioinformatics, 2006. **7**: p. 316.
10. Damborsky, J., et al., *Identification of tunnels in proteins, nucleic acids, inorganic materials and molecular ensembles.* Biotechnology Journal, 2007. **2**: p. 62-67.
11. Negri, A., et al., *Stepwise dissection and visualization of the catalytic mechanism of haloalkane dehalogenase LinB using molecular dynamics simulations and computer graphics.* J Mol Graph Model, 2007. **26**(3): p. 643-51.
12. Klvana, M., et al., *Pathways and Mechanisms for Product Release in the Engineered Haloalkane Dehalogenases Explored using Classical and Random Acceleration Molecular Dynamics Simulations.* Journal of Molecular Biology, 2009: p. accepted.