A simulation study of the NaChBac channel: stability, Na^+ binding sites and hydration

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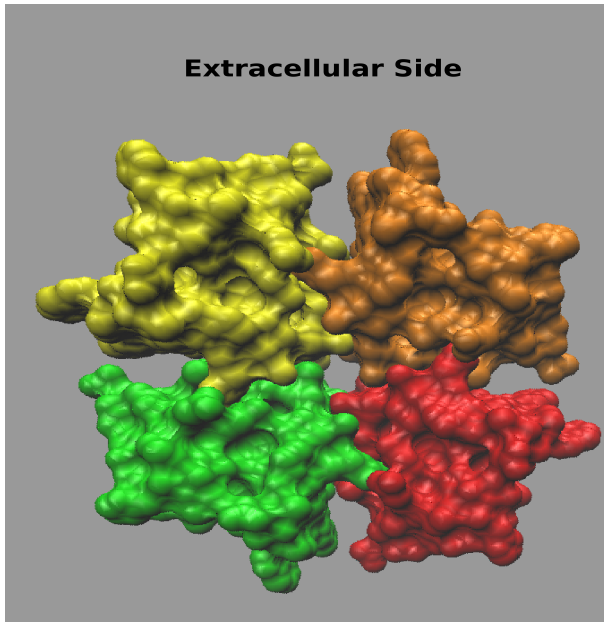
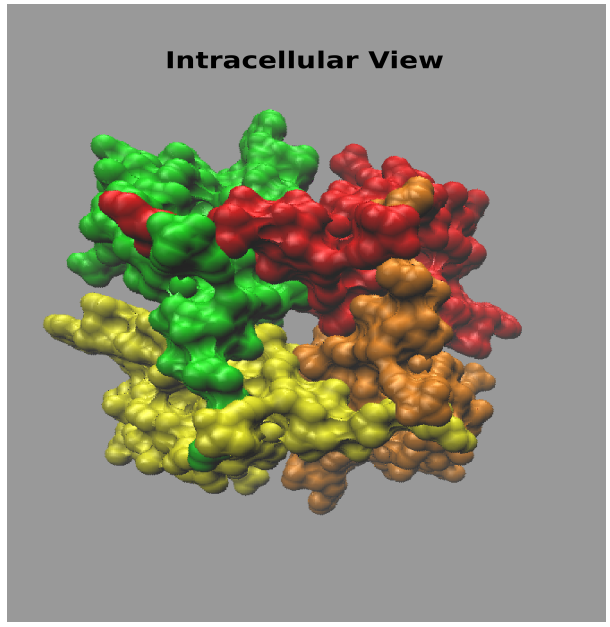
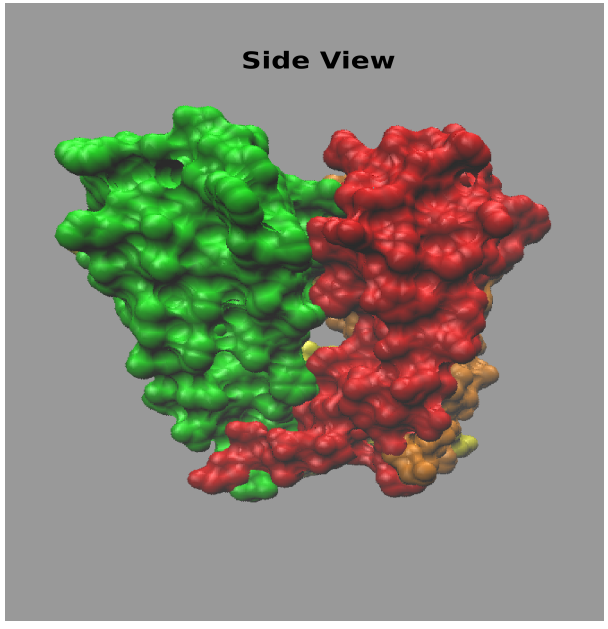
The NaChBac channel

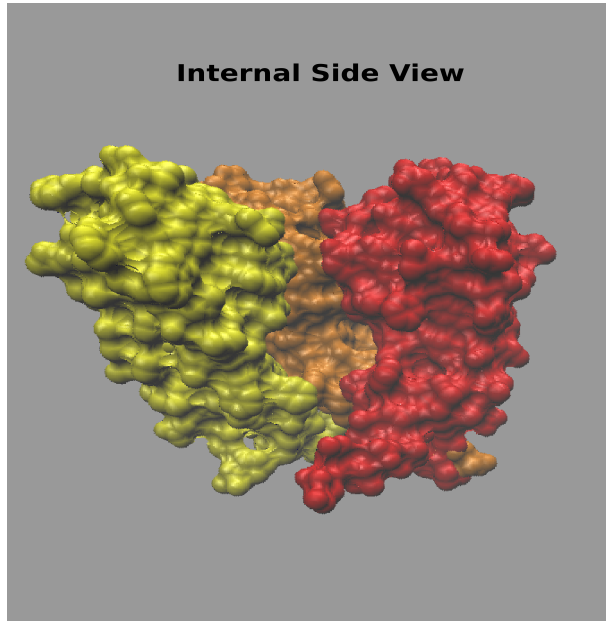
- Prokaryotic homotetrameric VGSC
- Voltage Sensor (S1-S4) and Pore Domain (S5-S6)
- Selectivity filter: TLESWAS
- Same motif as eukaryotic Ca_v channels: FxxxTxExW
- Mutations S192D and S195D increase Ca^{2+} conductivity twofold with respect to Na^{+}. The double mutant is Ca^{2+} selective.
- Homology model based on NavMs template (Seq. Id: 45.65%)
- NavMs allows to model only the pore domain

NaChBac/NavMs: modeling

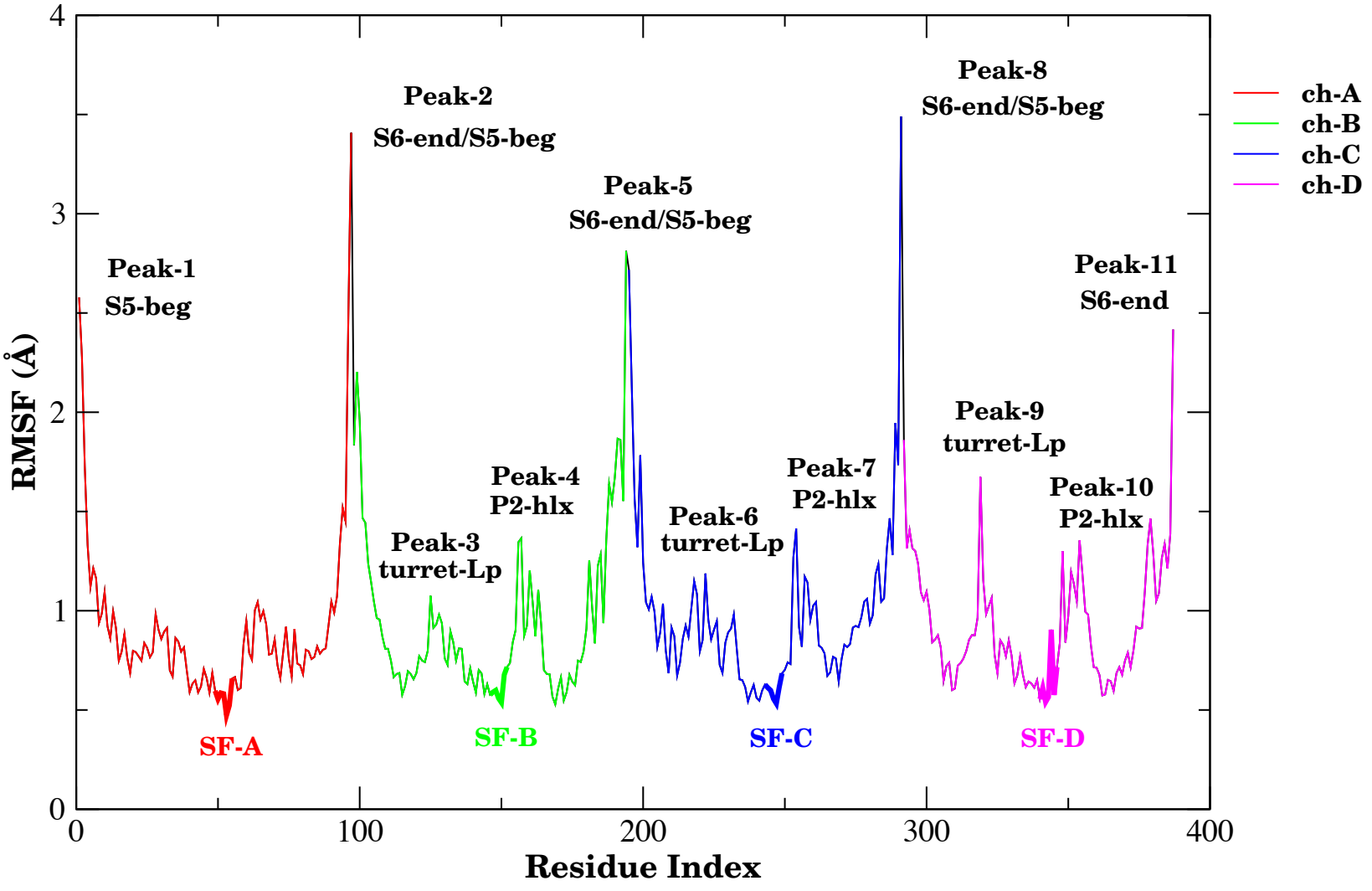




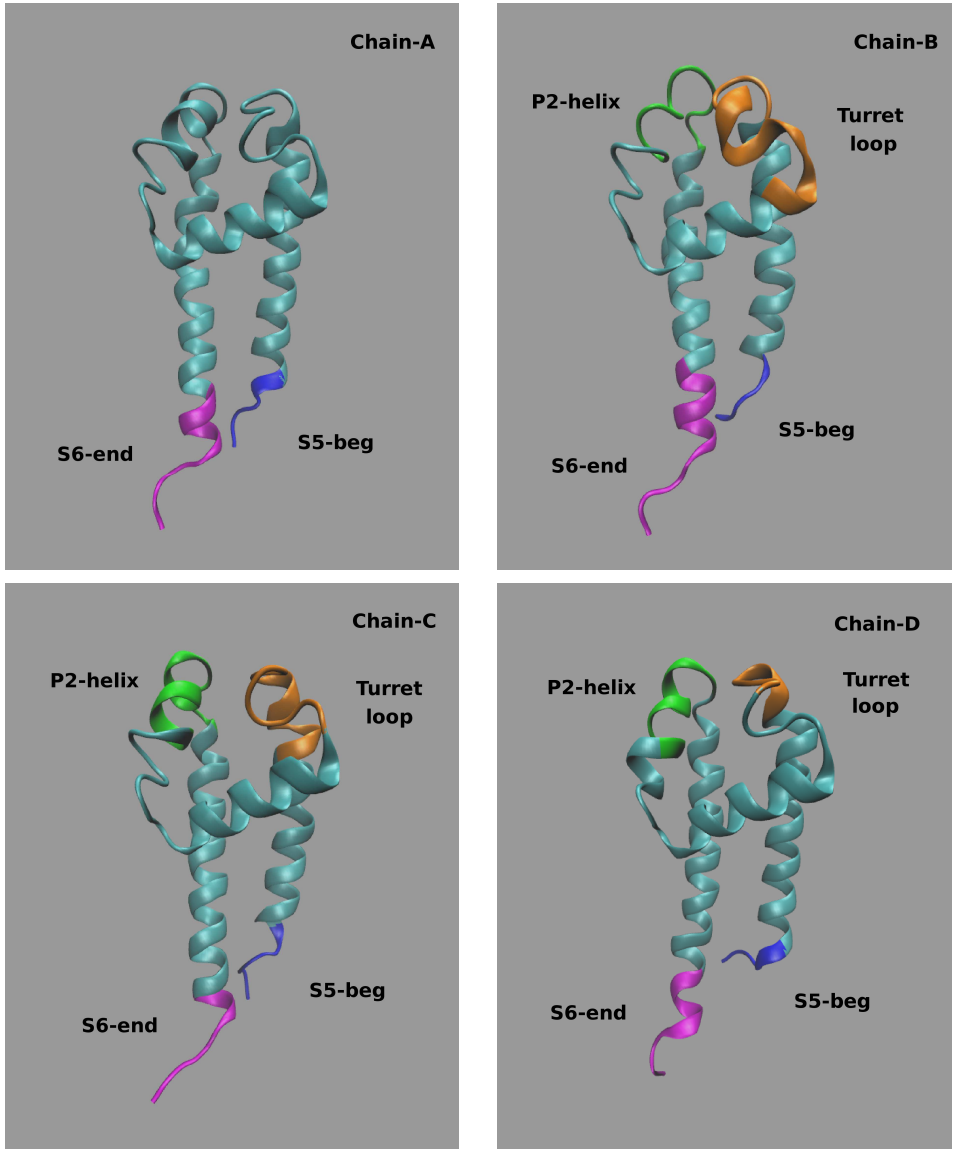




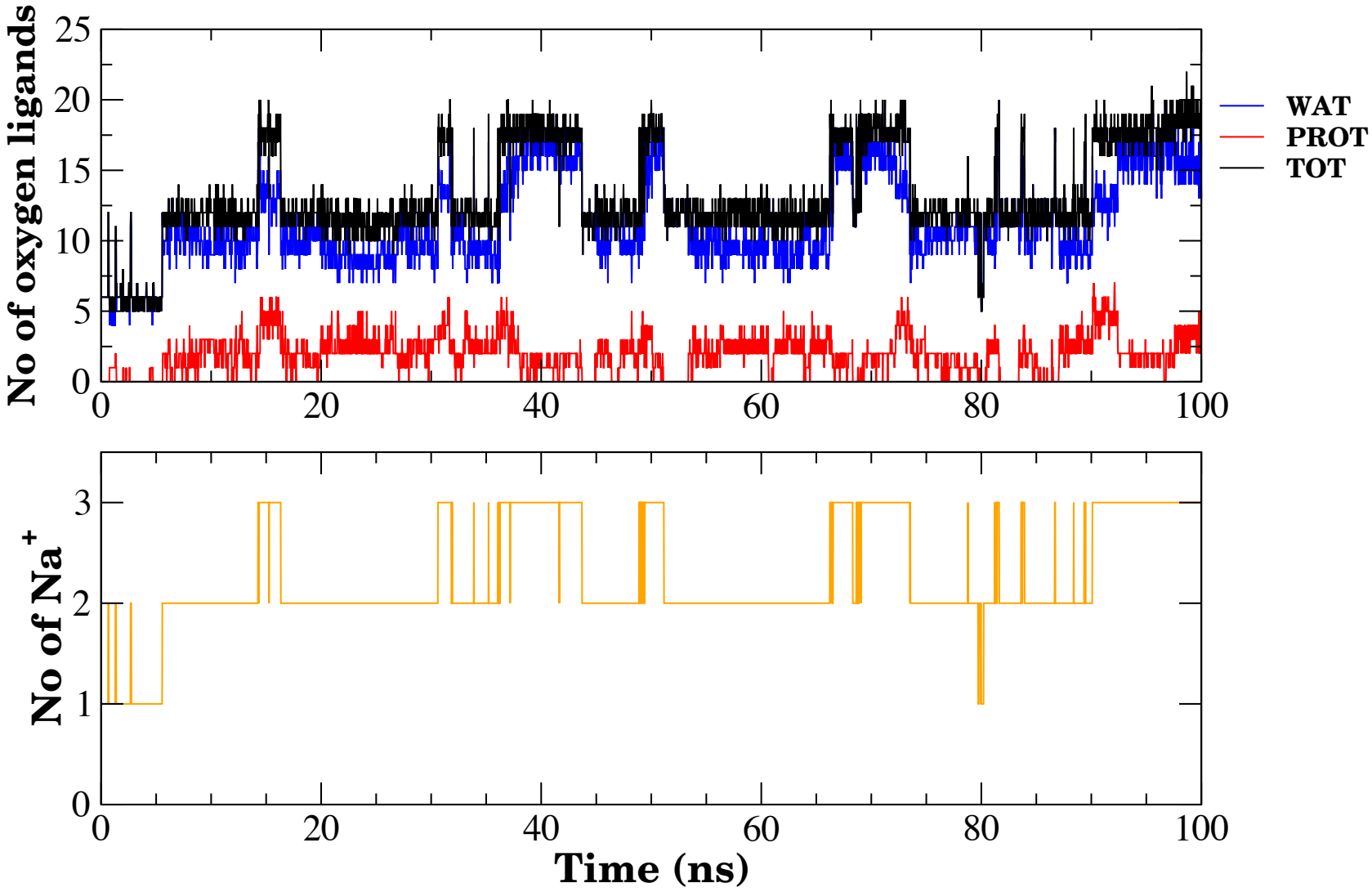
RMSF profile



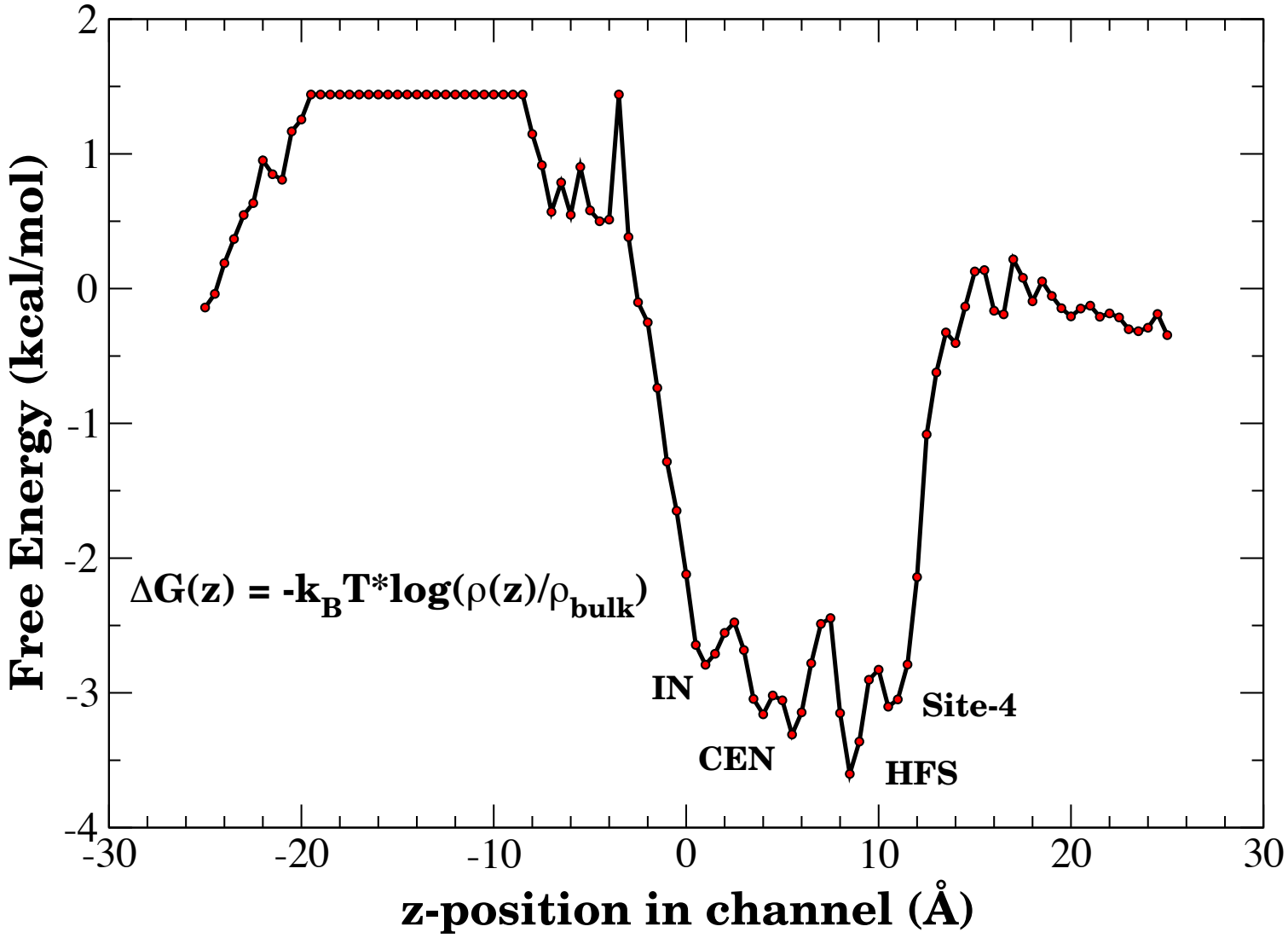
RMSF Map


Chain-A
S6-end
S5-beg
Chain-B
P2-helix
Turret
loop
S5-beg
S6-end
Chain-C
P2-helix
Turret
loop
S5-beg
S6-end
Chain-D
P2-helix
Turret
loop
S6-end
S5-beg

SF occupancy



PMF of unbiased run



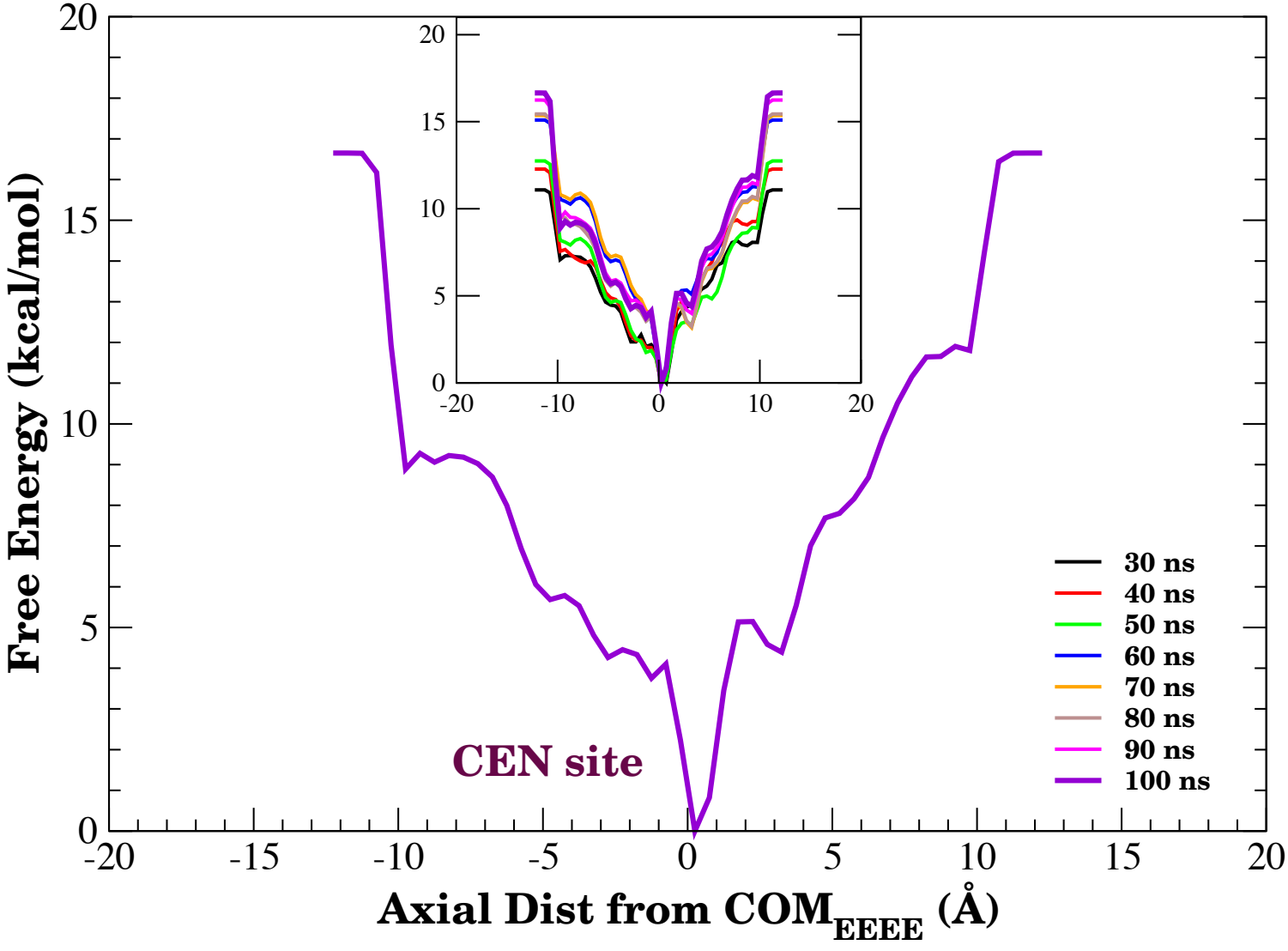
Metadynamics

- History-dependent biasing potential: sum of energy gaussians in CV space.
- In well-tempered metadynamics the height of the gaussians decays exponentially.

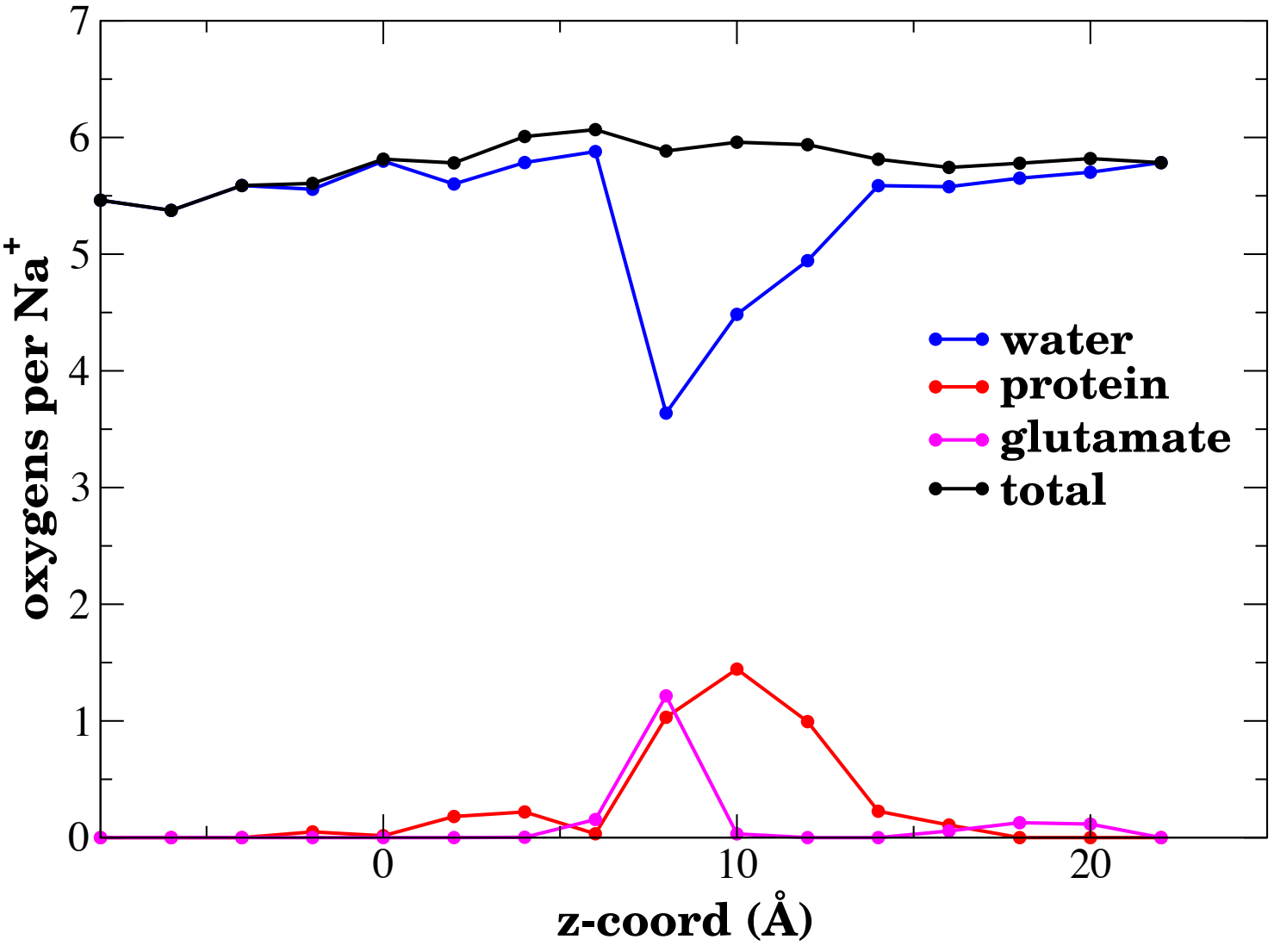
$$V(s,t) = \sum_{t'<t} W e^{-V(s',t)/\Delta T} \exp\left(-\sum_{i=1}^{d} \frac{(s_i - s_i(t'))^2}{2\sigma_i^2}\right)$$

- FES: $F(s) = -\lim_{t\to\infty} V(s,t)(T+\Delta T)/\Delta T$
- CV: z-component of distance vector between Na^+ ion in SF and COM of the C_αs of Glutamates of EEEE-ring.
- Spherical harmonic restraint to exclude other Na^+ ions from SF.

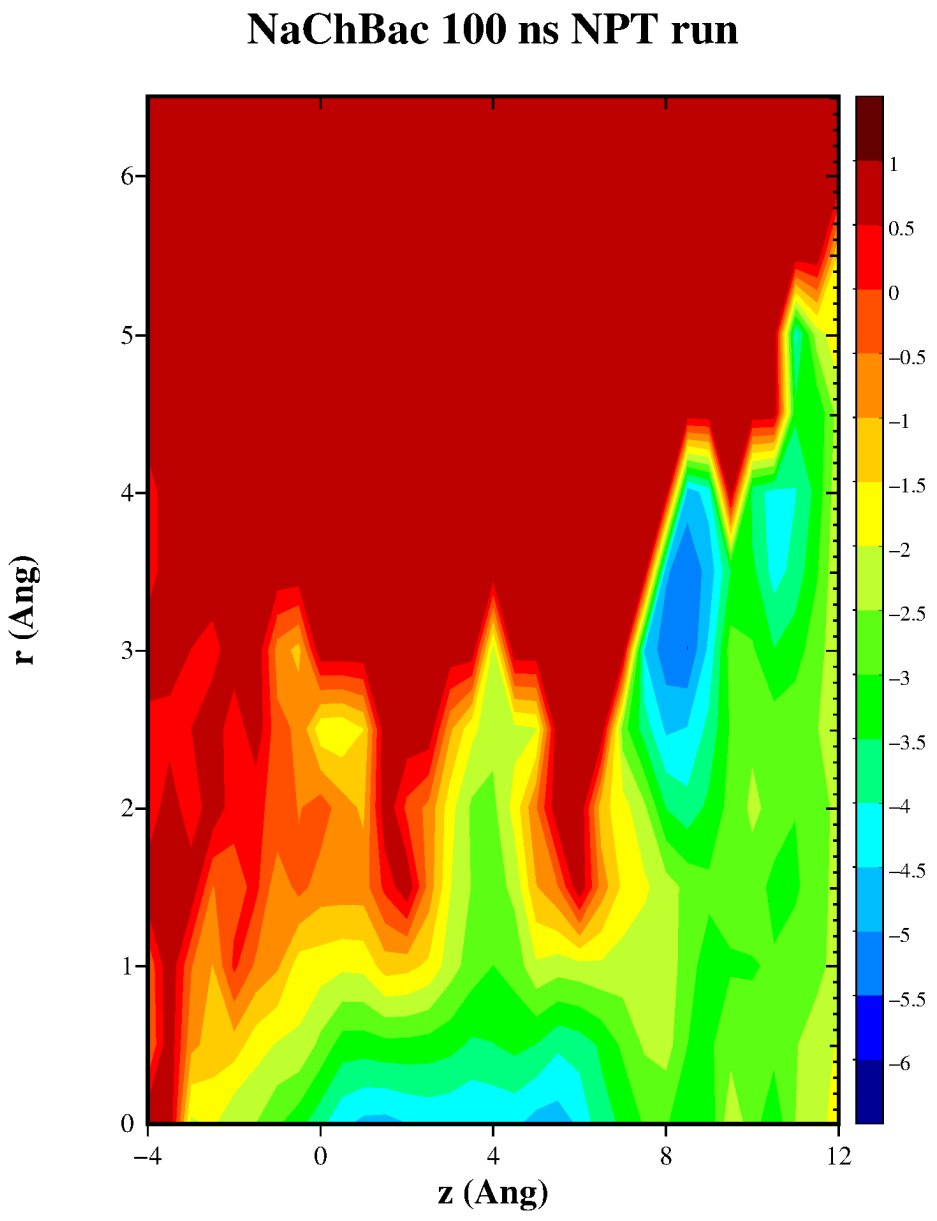
PMF from Metadynamics



Na^+ coordination



Radial-Axial PMF



MSM Building

- Define the states: single, double, triple, $\cdots$ occupancy states of sites CC, IN, CEN, HFS, S4, EX.
- Assign each frame of the trajectory to a state and compute matrix of transition probabilities.
- Repeat the calculation for different lagtimes τ to determine when the dynamics becomes Markovian.
- Goal: starting with $\tau = \Delta t$ find the smallest n such that $S(n\Delta t) = T(\Delta t)^n \Longrightarrow \mu_i(n) = \lambda_i^n$
- When this occurs the plot of implied time-scales as a function of lagtime levels out.

$$t_i = -\frac{n\Delta t}{log\mu_i(n)} = -\frac{n\Delta t}{log\lambda_i^n} = -\frac{\Delta t}{log\lambda_i}$$

Introduction to TPT: 1

- Determine transition rate matrix L from transition probability matrix S.
- Determine initial and final states A, B.
- Forward committor q_i^+: probability that a trajectory leaving state i reaches B before reverting to A.
- Backward committor q_i^-: probability that a trajectory arrived to state i comes from A rather than from B.
- By definition: $q_i^+ = 0 \; \forall i \in A$; $q_i^+ = 1 \; \forall i \in B$.
- Theory of Markov chains with absorbing states:

$$\sum_{k \in I} L_{ik} q_k^+ = - \sum_{k \in B} L_{ik}$$

Introduction to TPT: 2

- Exclude non-productive trajectories:

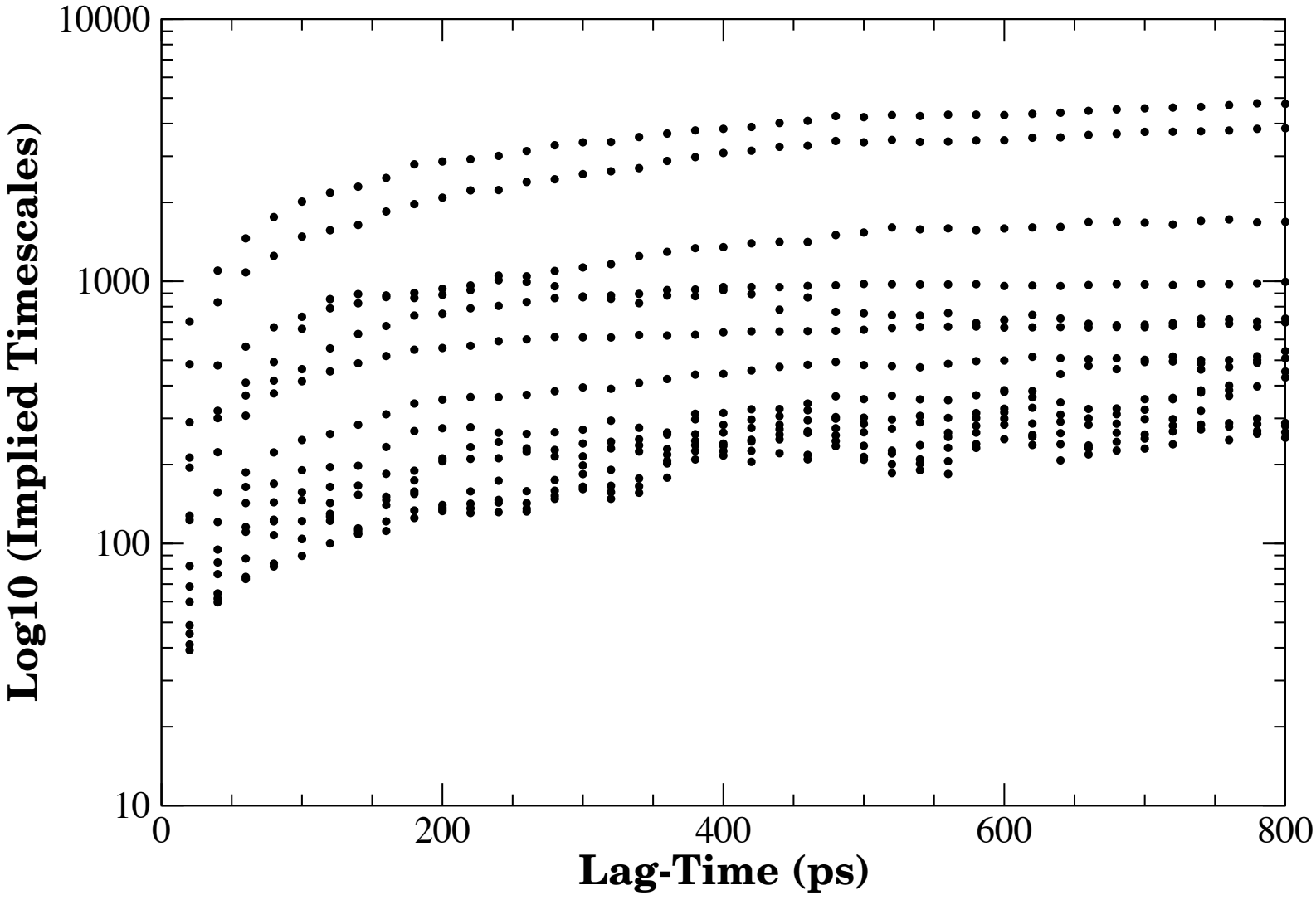
$$f_{ij} = \pi_i q_i^- L_{ij} q_j^+$$

- Exclude recrossing events:

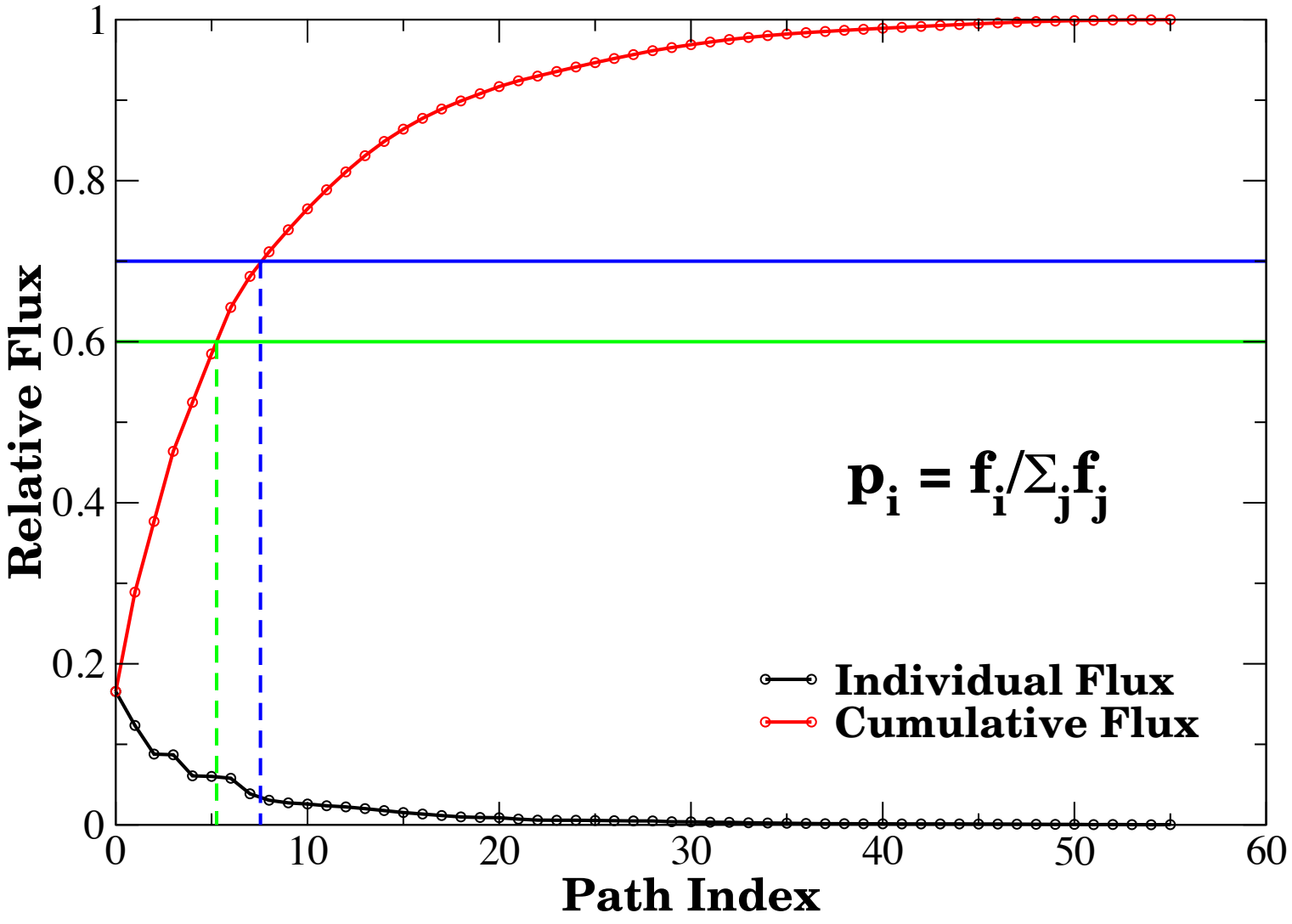
$$f_{ij}^+ = \max[0, f_{ij} - f_{ji}]$$

- Identify dominant pathways as pathways with maximal bottleneck flux.
- Use a variant of Dijkstra's algorithm.

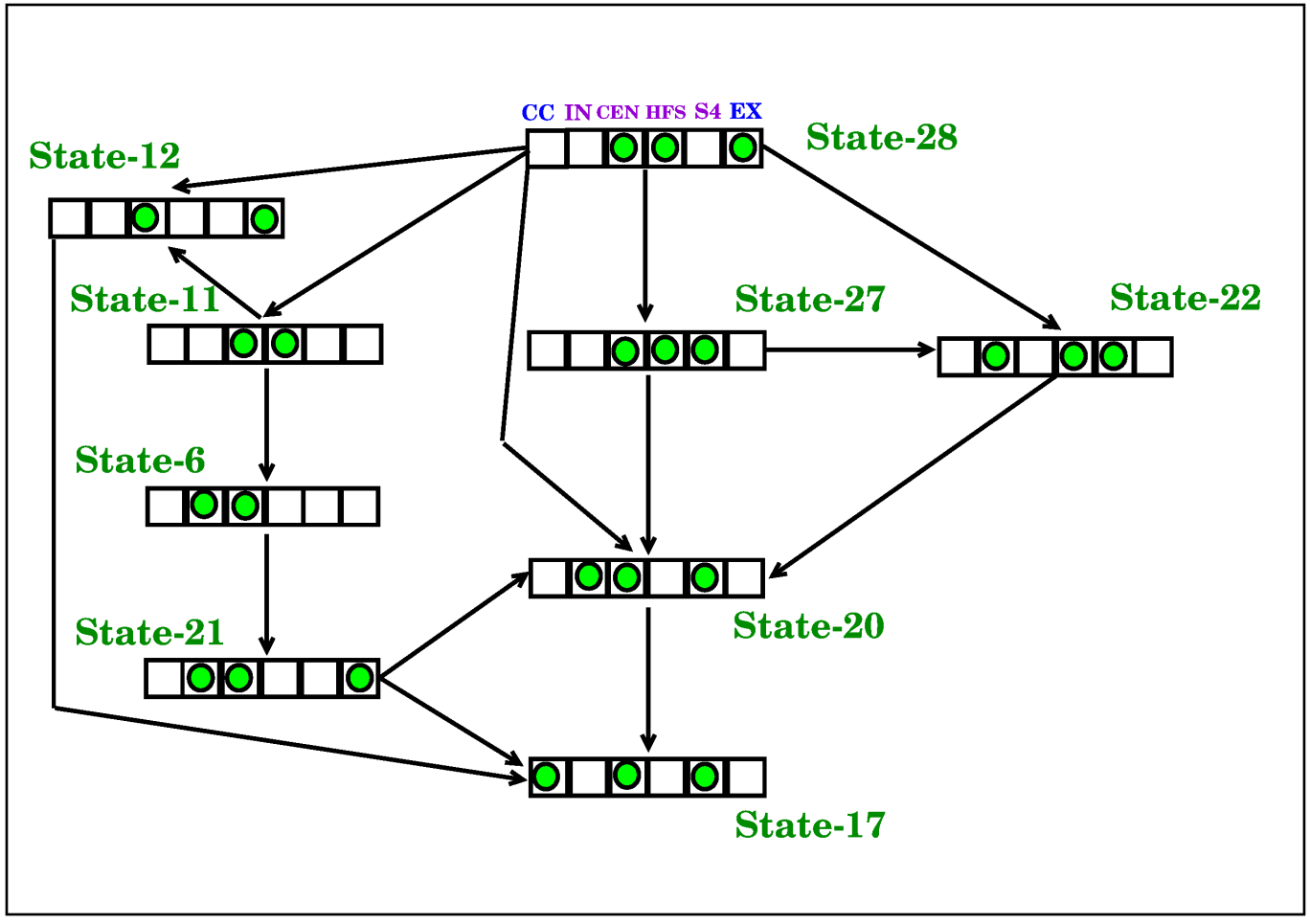
MSM Lagtime


10000
1000
100
10
0
200
400
600
800
Log10 (Implied Timescales)
Lag-Time (ps)

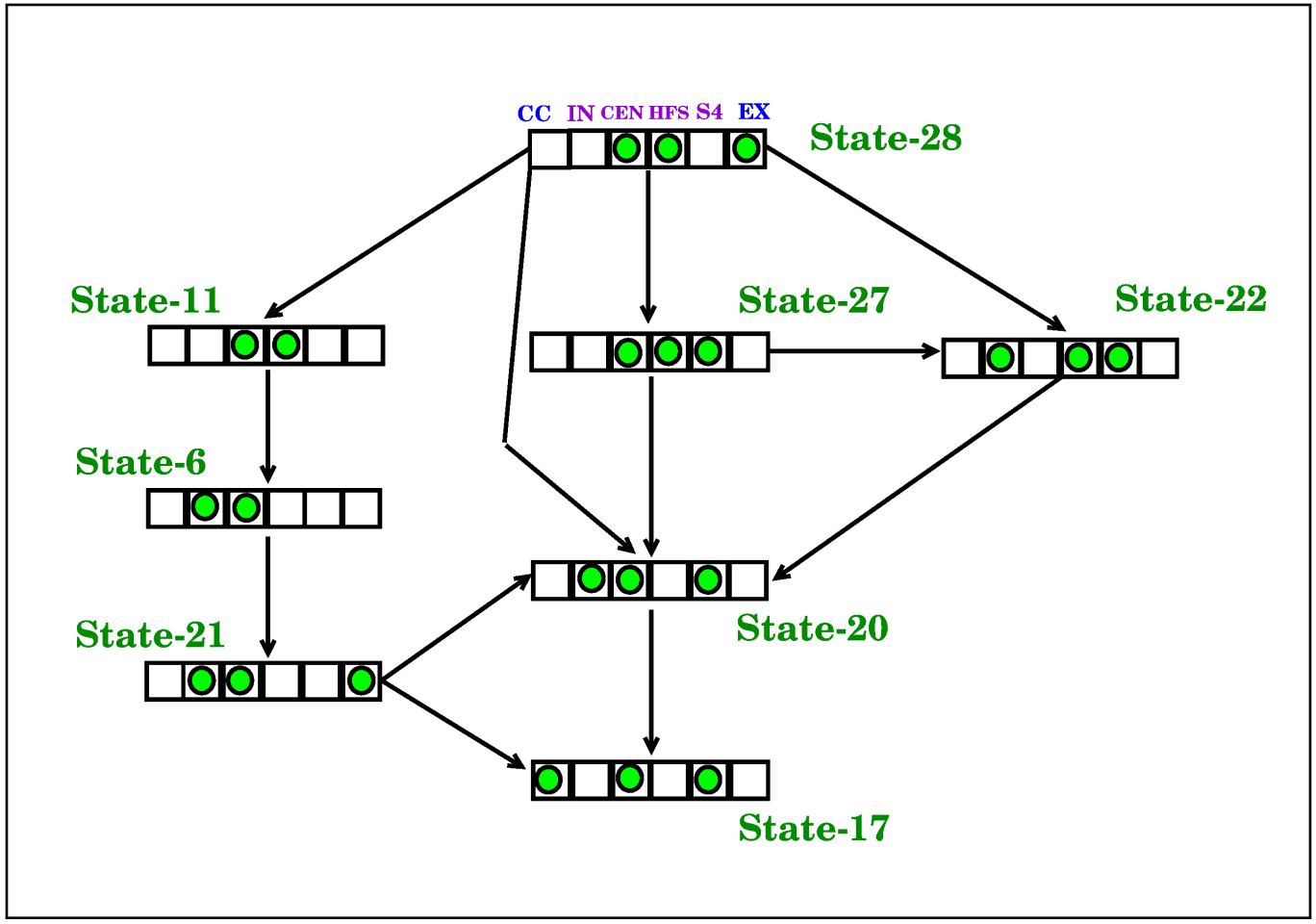
Path selection



Transition Graph



Dominant Paths



Path-1 (28-27-20-17) : 12%
Path-2 (28-22-20-17): 9%
Path-3 (28-27-22-20-17): 9%
Path-4 (28-11-6-21-20-17): 6%
Path-5 (28-11-6-21-17): 6%
Path-6 (28-20-17): 4%

Conclusions: 1

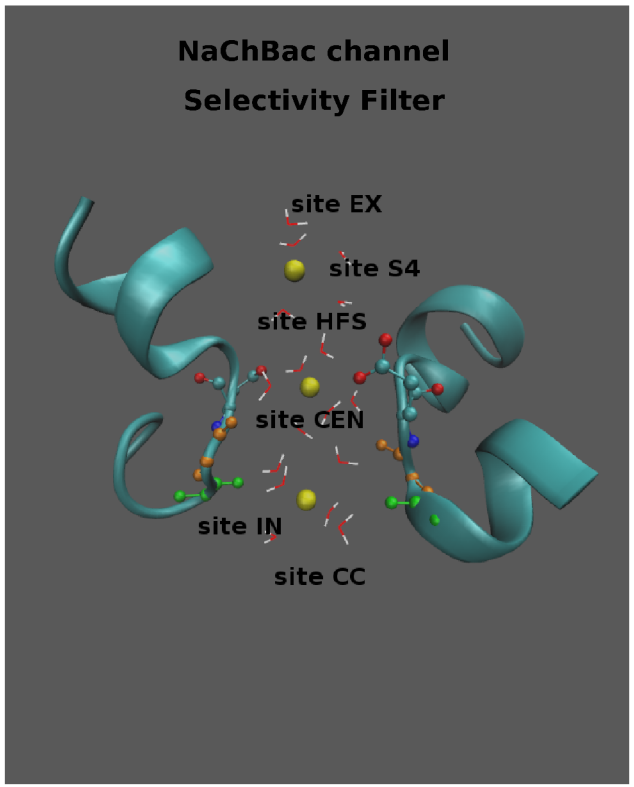
- The channel appears to be extremely stable.
- The SF is comparatively rigid occupying the minima of the RMSF profile.
- The peaks of the RMSF correspond to partially unfolded linkers that connect secondary structure elements.
- During the unbiased simulation the SF is spontaneously occupied by two ions in CEN and HFS. Many triple occupancy states can also be observed.
- The PMF of the unbiased simulation shows 4 minima three of which correspond to sites IN, CEN, HFS predicted by Catterall in NavAb.

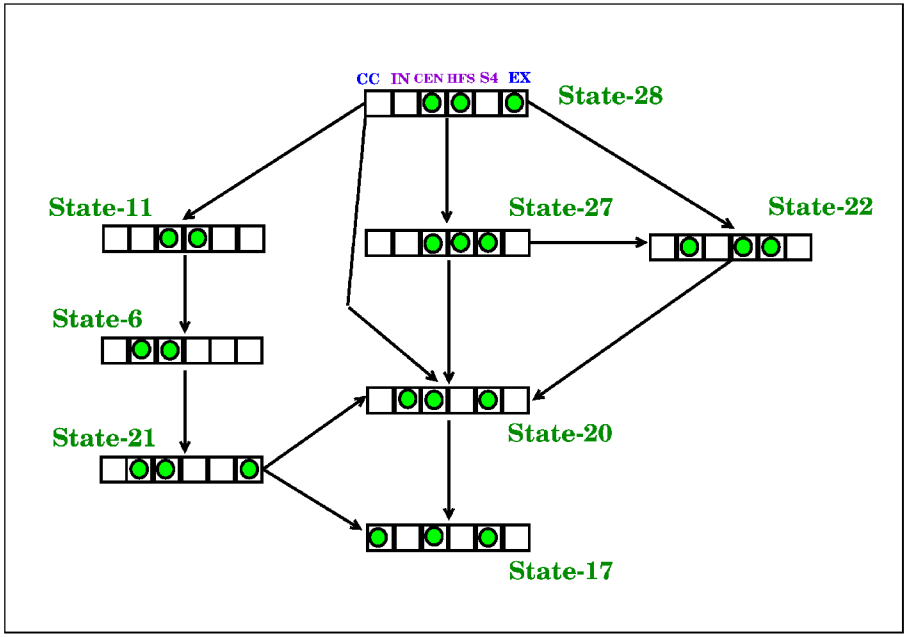
Conclusions: 2

- The metadynamics PMF reveals a single deep minimum corresponding to the upper half of site CEN.
- This possibly shows that conduction requires the presence of at least two ions in SF.
- When the ion occupies site IN or CEN it is tipycally coordinated by 6 water molecules at the vertices of an octahedron: on-axis location.
- When the ion occupies site HFS it is bound to a single glutamate and a single serine: off-axis position.
- The permeation mechanism could be described using a MSM in conjunction with TPT.

Future Directions

- 2D-Metadynamics with axial bias on two ions in SF.
  - Comparison with 1D-Meta-PMF and unbiased PMF.
  - Determine minimum free energy path through Nudged Elastic Band algorithm and compare with permeation pathway from PMF.
- NaChBac selectivity: repeat unbiased and metadynamics simulations in $CaCl_2$ solution.
- Computing currents from equilibrium simulations using linear response theory (**Biophys. J. 104 (2013) 368-376**).
- Analysis of mutants of NaChBac
  - EEEA, EEAA, EAAA, AAAA
  - EEEE$_o$,EEE$_o$E$_o$,EE$_o$E$_o$E$_o$,E$_o$E$_o$E$_o$E$_o$





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Path-4 (28-11-6-21-20-17): 6%
Path-5 (28-11-6-21-17): 6%
Path-6 (28-20-17): 4%

A simulation study of the NaChBac channel: stability, Na^+ binding sites and hydration

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1 SLIDE 1

Good morning everybody. In this brief presentation I will try to illustrate our simulation work on the NaChBac channel aiming at the characterization of channel stability, sodium ion hydration and coordination as well as the permeation mechanism.

2 SLIDE-2

NaChBac belongs to the family of prokaryotic homotetrameric VGSC. Each of the four identical subunits comprizes a Voltage Sensor domain encompassing helices S1-S4 and a Pore Domain comprizing helices S5-S6. The selectivity filter is composed by the side chains of the consensus sequence TLESWS of each chain. It is remarkable that despite being a sodium channel, NaChBac features the same selectivity signature sequence as Ca_v channels: FxxxTxExW. Moreover, mutation to aspartate of either of the two serines of the selectivity filter increases calcium conductivity twofold with respect to sodium and the double mutant is completely calcium selective.

Since no crystal structure is available we built an homology model of NaChBac based on the NavMs template that features a 45.65% sequence identity with respect to NaChBac. The downside of using the NavMs template is that it is a pore-only construct that does not allow to model the structure of the Voltage Sensor Domain but only of the Pore Domain.

3 SLIDE-3

In this slide you can see a volume filling representation of the homology model of NaChBac built using NavMs as a template. You can immediately note that not only the activation gate on the intracellular side, but also the selectivity filter on the extracellular side are wide open which suggests the possibility of an efficient permeation of sodium ions. This scenario is confirmed from the internal side view showing that the channel lumen is wide all along the channel length.

An interesting detail of the channel structure can also ne noted from the external side view. The opening that you can see on the side of the channel is a typical *fenestration*, a window that connects the membrane environment with the cavity of the pore. In the open conformation fenestrations are larger than in the closed one because a part of the fenestration boundary is represented by the S6 helix that bends when the channel opens. In the closed conformation fenestrations are occupied by the acyl chains of the membrane phospholipids while in the open conformation they are reported to remain free and they are expected to represent access pathways for small hydrophobic ligands. Fenestrations are present in the NavMs template but they also occur in NaChBac where studies by Carnevale's group suggest they may represent the hydrophobic pathway for the access of general anesthetics like isoflurane.

4 SLIDE-4 + SLIDE-5

The homology model was embedded in a bilayer containing 248 molecules of POPC and solvated on both sides with 0.5 M solution of $NaCl$. This system underwent a 100 ns fully unconstrained simulation in the NPT ensemble at the pressure of 1 atm and temperature of 300 K. The internal dynamics of the system can be analyzed through the RMSF profile of C_α atoms. The stability of the SF is revealed by the fact that the four TLESWAS stretches correspond to the minima of the profile. Conversely the peaks of the RMSF profile are typically mapped to to the initial fragment of helix S5 and the final fragment of helix S6 as well as to the turret loop and the final part of helix P2. The most flexible regions thus correspond to partially unstructured fragments acting as linkers between secondary structural elements.

5 SLIDE-6

Inn this slide you can see the time course of the number of sodium ions occupying the selectivity filter. The simulation starts from a pre-equilibrated conformation with a single ion in the SF at the level of the EEEE-ring. After 5ns of simulation, a second ion enters the SF just above the EEEE ring. The entrance of the second ion is made possible when the first sodium moves from the binding site at the level of the EEEE ring to a second binding site formed by the carbonyl groups of the four leucines of the TLESWAS sequence. The trajectory then shows several transient entering events of a third sodium ion. The longer-lived three-ions arrangements can be observed when the first sodium ion moves from the LLLL-carbonyl ting to the TTTT-carbonyl

ring, while the second sodium ion still remains in the EEEE-ring but it moves down along the channel axis.

You can also note that water provides most of the oxygen atoms coordinating the Na^+ ions. This means that at variance with potassium channels where potassium can flow through the channel only once it is stripped of its hydration shell, in the NaChBac channel sodium retains a significant portion of its hydration shell. You can also note that the content of water in the SF is correlated with the number of sodium ions. Finally, the plot also shows that sodium on average coordinates two oxygen atoms contributed by the protein.

6 SLIDE-7

In this slide you can see the Potential of Mean Force that we computed as a function of the axial position of the ions. The PMF shows the existence of four minima. The deepest minimum corresponds to a binding site at the level of the side-chains of the glutamates of the EEEE-ring. The second main minimum identifies a binding site formed by the backbone carbonyl groups of the four leucines of the SF. The third minimum, which is much higher in energy with respect to the first two, is located at the level of the backbone carbonyls of the threonines of the SF. The first three minima thus correspond to binding sites HFS, CEN and IN predicted by Catterall through crystallographic analysis of the NavAb channel and whose existence was confirmed by several simulation works. Our PMF shows the existence of a fourth high-energy minimum at the level of the serines adjacent to the

glutamates of the SF.

7 SLIDE-8

In this slide I would like to give a brief account of the metadynamics tecqnique that we have used for a more accurate estimate of the PMF of sodium ions in the NaChBac channel. Metadynamics is a technique designed to overcome the multiple minima problem of protein energy landscape. In metadynamics the energy function is supplemented by an history dependent biasing potential represented by a sum of gaussians laid in the most frequently visited regions of the conformational space. In this way the biasing potential descourages the visit of regions already explored and pushes the simulation towards unexplored areas of the conformational space. In the limit of a very long simulation, the biasing potential fills all energy minima and the free energy profile can be simply attained by reversing the sign of the biasing potential. Ideally, a metadynamics simulation should be stopped when all energy minima have been filled. Since it is difficult to identify this moment, there may be problems of overfilling that limit the accuracy of the free energy calculation. This kind of problems can be avoided by using well-tempered metadynamics where the height of the energy gaussians decreases exponentially during the simulation and it is possible to tune the fraction of the energy basins that will be filled.

Also our metadynamics run was started from a pre-equilibrated conformation with a single ion in the SF at the level of the EEEE-ring. The biased collective variable was defined as the z-component of the distance

vector between the ion in the SF and the center of mass of the C_αs of the glutamates of the EEEE-ring. Moreover a spherical harmonic restraint was implemented to prevent the access of other sodium ions to the SF.

8 SLIDE-9

In this slide you can see the PMF we computed through Metadynamics. In the inset the PMF profile is computed at intervals of 10 ns. As the simulation progresses the curves become closer and closer which is a signature of the approach to convergence. It can also be noted that, at variance with the PMF profile computed in the unbiased simulation, we have a single very deep minimum instead of four. Since the axial position of the COM of the EEEE-ring is at about 5.5 Å, the $[-0.5 : 1.5]$ Å range of the metadynamics PMF minimum can be mapped to the $[5.0 : 7.0]$ Å range of the unbiased PMF which corresponds to the upper part of the CEN binding site. The discrepancy between the PMF profiles yielded by metadynamics and the unbiased simulation may be due to the fact that in the metadynamics run we biased a single ion in the SF. This is consistent with a number of computational studies on the NavAb channel showing the existence of high energy barriers (5.0 kcal/mol) in the SF so that conduction involves the participation of another ion. In fact, in the unbiased simulation that yielded a PMF with four distinct minima, we assisted to a spontaneous occupation of the SF by two ions. This issue will be clarified through metadynamics simulations with axial bias on two ions that are currently underway in our lab.

9 SLIDE-10

In this figure we study the hydration and coordination of sodium ions plotting the average number of oxygen atoms interacting with Na^+ ions in transversal bins with height of 2.0 Å. It can be noted that for $z < 0.0$ and $z > 18.0$ all the oxygens coordinating sodium are provided by water. In particular, sodium ions are surrounded by a full hydration shell comprizing 6 water molecules arranged at the vertices of an octahedron. In the $[0 : 18]$ range the channel narrows determining a decrease of the number of water-supplied oxygen along with an increase of the protein-supplied coordinating oxygens. The $[0 : 6]$ interval corresponds to sites IN and CEN. Only in 5% of the frames whereby sodium occupies this region, the ion interacts with a single Threonine or Leucine residue whereas in the other cases it is surrounded by six water molecules. It follows that in the IN and CEN sites Na^+ is likely to occupy an on-axis position. In the $[8 : 10]$ interval sodium is coordinated by four water oxygens, an oxygen provided by glutamate and an oxygen supplied by another protein residue (typically Serine). Since the ion is bound to a single glutamate, it will likely occupy an off-axis position. Finally, in the $[10 : 12]$ range, corresponding to site S4, sodium interacts with one or two serines in 40% of cases, while it is only coordinated by water in 60% of cases. In this position the ion prevalently occupies an on-axis position while the off-axis configurations are not negligible.

10 SLIDE-11

Since the issue of the on- or off-axis placement of sodium in VGSC is a matter of debate in the literature, we also computed a 2D-PMF as a function of the z-position along the channel axis and of the radial distance from the axis. Two main minima can be immediately spotted. The minimum in the $0 < z < 6$ Å axial range and $0 < r < 1$ Å radial range corresponds to IN and CEN minima. The position of this minimum confirms that when sodium is fully surrounded by water, it tends to occupy an on-axis position. The second minimum in the 7.0-10.0 Å axial range and 2.0-4.5 Å radial interval corresponds to the HFS minimum. The position of this minimum confirms that when sodium interacts with a single glutamate, it is forced to occupy an off-axis position.

11 SLIDE-12

The mechanism of permeation of sodium in the NaChBac channel can be determined by building a Markov State Model and applying Transition Path theory. In this slide I quickly review our protocol for MSM building. First of all it is necessary to define a set of states involved in the transitions. Our states will be the single, double, triple, $\cdots$, occupancy states of sites CC, IN, CEN, HFS, S4 and EX. Sites IN, CEN, HFS, S4 are the binding sites in the SF corresponding to the minima of the PMF, while sites CC and EX are two regions respectively below and above the selectivity filter. The next step is to assign the framed of the MD trajectory to the corresponding states and to compute a matrix of transition probabilities. This calculation must

be repeated for different lagtimes to determine when the dynamics becomes Markovian. The idea is to start with a lagtime $\tau = \Delta t$ corresponding to the sampling interval of the MD simulation and to determine the smallest n such that $S(n\Delta t) = T(\Delta t)^n$. When this occurs the plot of implied timescales as a function of the lagtime levels out.

12 SLIDE-13 + SLIDE-14

The transition probability matrix S computed during the Markov model building can be used to derive a matrix of the transition rates simply discretizing the Master equation. It will be also necessary to choose a set of initial and final states A, B that in our case represent configurations of the system before and after the permeation event respectively. The key quantities involved in TPT are the committor probabilities. The forward committor is the probability that a trajectory leaving state i reaches B before reverting to A. The backward committor on the other hand, is the probability that a trajectory arrived to state i comes from A rather than from B. Based on these definitions and on the theory of Markov chains with absorbing states, it is possible to compute the committor probabilities of all states. It must be considered that the transition rate L_{ij} includes many non-productive trajectories such as those that never reach the final state B and those that reach B without originating from A. This is why it is convenient to define a probability flux along edge (i, j) contributing to transition $A \to B$: $f_{ij} = \pi_i q_i^- L_{ij} q_j^+$. Since this quantity still includes recrossing events, a net flux along arc (i, j) will be defined as $f_{ij}^+ = \max[0, f_{ij} - f_{ji}]$. Once a net

flux has been computed for all arcs of the transition graph, the dominant paths can be identified as those paths with a maximal bottleneck flux, using a variant of Dijkstra's algorithm.

13 SLIDE-15

In this slide the first 15 implied timescales of the transition probability matrix were computed for 40 different lagtimes from 20 ps to 800 ps at intervals of 20 ps. The implied timescale level out at about 200 ps identifying the lagtime where the transition dynamics becomes Markovian.

14 SLIDE-16

In this slide the probability of each permeation pathway was identified as the relative flux and both individual and cumulative flux are plotted. It can be noted that the 8 most probable pathway account for about 70% of the total flux.

15 SLIDE-17

In this slide you can see the top 8 paths describing the permeation process. In the choice of the initial and final state we were guided by the trajectory of the unbiased simulation. Since the SF becomes soon occupied by two ions in the CEN and HFS sites and many triple occupancy states were observed, our initial states was chosen to be the one with three ions in CEN, HFS and EX respectively. In a simlar way, we observed that in the few permeation

events occurring in our trajectory, when a sodium ion leaves the selectivity filter to enter into the central cavity, the SF remains occupied by other two ions in CEN and S4. This is why our final state is characterized by three ions in CC, CEN and S4.

You can see that in this graph some paths do not represent genuine permeation events. For instance in paths 28-12-17 and 28-11-12-17 in the last step the ion appearing in CC does not come from the SF but from the inner part of the channel. These two paths must thus be pruned from our graph.

16 SLIDE-18

In this slide you can see the pruned transition graph including the 6 permeation pathways with the highest flux. The most probable path is 28-27-20-17. In this pathway the ion in the EX position advances by one position occupying the S4 site. This pushes forward the ions in CEN and HFS that occupy sites IN and CEN respectively. Finally the ion in site IN leaves the SF and enters into the central cavity of the channel. It can also be noted that five out of six pathways transit through state-20 before reaching the final state. This is consistent with our analysis of the conditional PMF. If an ion mut enter the CC region, it is reasonable that in the previous step it had to occupy the lowermost position of the SF, site IN. However, we showed that an ion in IN determines a high probability of occupation of sites CEN and S4, just as in state-20.

17 SLIDE-19 + SLIDE-20

We can finally draw the conclusions of our work. The channel appears to be extremely stable since the RMSD with respect to the first frame oscillates in a very narrow band. The SF turns out to be comparatively rigid since the TLESWAS sequences of the four subunit correspond to minima of the RMSF profile. The peaks of the RMSF plot, on the other hand, correspond to partially unfolded linkers that connect secondary structure elements.

During the unbiased simulation the SF is spontaneously occupied by two ions in CEN and HFS. Many triple occupancy states can also be observed. The PMF of the unbiased simulation shows 4 minima, three of which correspond to sites IN, CEN and HFS predicted by Catterall in NavAb. The metadynamics PMF on the other hand, is characterized by a single deep minimum corresponding to the upper half of site CEN. This possibly shows that conduction requires the presence of at least two ions in the SF.

When the ion occupies site IN or CEN, it is typically coordinated by 6 water molecules at the vertices of an octahedron: on-axis location. When the ion occupies site HFS, it is bound to a single glutamate and a single serine: off-axis location. Finally, the permeation mechanism could be described using a MSM in conjunction with TPT.

18 SLIDE-21

In this slide we outline a number of potential lines of future activity. The most urgent task is to complete the 2D-metadynamics run with axial bias on two ions in the SF. If our hypothesis that conduction requires at least two ion

in the SF is correct, then the 2D-metadynamics PMF should be consistent with the one generated from the unbiased simulation revealing the a number of minima corresponsing to sites IN, CEN, HFS and S4 at variance with the single minimum exhibited by the PMF profile from 1D-metadynamics. From the 2D-PMF using the Nudged Elastic Band algorithm, it will be also possible to derive a minumu free energy path that will be comapred with the permeation path computed through TPT.

Another possible line of investigation focuses on the selectivity of NaChBac channel. In this case Both the unbiased and metadynamics simulations will have to be repeated on the system bathed by a $CaCl_2$ solution.

Another important task in view of a comparison of camputational results and electrophysiological experiments concerns the implementation of a progam to compute currents from equilibrium simulations using linear response theory. This analysis tool will also enable a more direct benchmarking of the Coulomb Blockade Theory.

Once this tool is developed, it will be used for the analysis of selected mutants of NaChBac. One possibility to change the charge of the SF is to design mutants where a glutamate of the EEEE0ring at a time is mutated to alanine. In this way the total charge will change but the charged residues will remain in their natural locations. This should guarantee the structural stability of the SF. An even less aggressive approach foresees to replace charged glutamate residues with protonated ones. This is the most conservative approach that could be used to modify the charge of the SF.