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# 1 An atomistic view of the YiiP structural changes upon 2 zinc(II) binding

3  
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## 13 14 15 **Abstract**

16 BACKGROUND: YiiP is a bacterial zinc-for-proton antiporter belonging to the cation diffusion  
17 facilitator family. The zinc(II) ions are transported across the cell membrane, from the cytosol to the  
18 extracellular space.

19 METHODS: We performed atomistic molecular dynamics simulations of the YiiP dimer with  
20 zinc(II) ions in solution to elucidate how the metal ions interact with the protein while moving from  
21 the cytosol to the transport site.

22 RESULTS: We observed that of the two cavities of the dimer, only one was accessible from  
23 the cytosol during transport. Zinc(II) binding to D49 of the transport site triggered a rearrangement  
24 of the transmembrane domain that closed the accessible cavity. Finally, we analyzed the free-energy  
25 profiles of metal transit in the channel and observed the existence of a high barrier preventing  
26 release from the transport site.

27 CONCLUSIONS: The observed dynamics is consistent with the dimer-dimer interface forming  
28 a stable scaffold against which the rest of the trans-membrane rearranges.

29 GENERAL SIGNIFICANCE: Zinc(II) transporters are present in all kingdoms of life. The present  
30 study highlights structural features that might be of general relevance.

## 31 32 **Keywords**

33 zinc transporter; metal transport; metal homeostasis; CDF; cation diffusion

## 34 35 **Abbreviations**

36 CDF, Cation Diffusion Facilitator; TMD, transmembrane domain; CTD, cytosolic domain; EC,  
37 extracellular; IC, intracellular; TM transmembrane; PCA, principal component analysis.

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## 1. Introduction

Zinc is the second most abundant trace element in all living organisms and plays a fundamental role in many biological processes [1,2]. Despite sufficient intracellular zinc(II) concentration is needed to supply proteins that require zinc(II) ions as cofactor, intracellular concentrations higher than few hundred picomolar produce cytotoxic effects [3,4]. Therefore, the cellular uptake and the efflux of zinc(II) ions are tightly controlled by specific systems, present at all phylogenetic levels, to maintain its concentration in a quite narrow range [5–7]. In mammals, the Zrt-, Irt-related proteins (ZIPs/Slc39s) are responsible of the uptake of zinc(II) ions into the cell [8,9]. On the contrary, the zinc transporters of the Cation Diffusion Facilitator (CDF) superfamily (ZnTs/Slc30) mediate zinc(II) removal from the cytoplasm into the extracellular space or into intracellular compartments such as secretory vesicles [10–14]. Presently, there is no direct experimental information on the three-dimensional structure of any member of the human ZnT family [15,16]. However, this limit is partially compensated by a relatively well-characterized bacterial homolog, the YiiP transporter from *Escherichia coli* [17,18]. Among the human homologs of YiiP, Znt8 has received significant attention owing to the involvement of its W325R common variant in the onset of type-1 and type-2 diabetes [19–22].

YiiP is a cation-proton antiporter catalysing the efflux of zinc(II) against the uptake of a proton with a 1:1 exchange stoichiometry [23]. YiiP can transport several divalent metal cations but only zinc(II) and cadmium(II) with high efficiency [24]. The first X-ray structure of this transporter was solved in the outward-facing conformation [25]; in 2009 a new structure with better resolution became available (PDB ID: 3H90) [26]. These crystal structures featured an architecture shared among all CDF transporters consisting in a transmembrane domain (TMD) connected to a C-terminal, cytoplasmic domain (CTD). YiiP is a Y-shaped homodimer with six TM helices per monomer clustered in a four-helix bundle (TM1–TM2–TM4–TM5) and a helix pair (TM3–TM6). The TM3-TM6 helix pair provides the dimer interface and protrudes in the cytosolic region. Each YiiP protein chain harbours three zinc(II) binding sites (A-C). Site A is located close to the middle of the TMD and constitutes the tetrahedral zinc(II) transport site, involving D45 and D49 of TM2 as well as H153 and D157 of TM5 [24]. The CTD hosts the C sites in which the binding of two zinc(II) ions enhances the stability of the dimer [26]. The function of site B is unclear. The inward-facing state of the YiiP homolog from *Shewanella oneidensis* (PDB ID: 3J1Z) was solved at 13 Å by cryo-electron microscopy [27]. Based on the comparison of all available structures, it has been proposed that the transport of zinc(II) involves a so-called alternating mechanism [28]. This proposed mechanism involves an allosteric connection between sites A and C through which a zinc(II) ion binding in the C site can promote metal binding and transport to the TMD thanks to the reorientation of the TM3-TM6 helix pair [26]. In turn, the binding of the zinc(II) ion in the transport site A triggers the conformational switch from the inward- to the outward-facing state facilitating the release of zinc(II). Recently, a variation of the alternating mechanism has been proposed in which the motion of the four-helix bundle relative to the static TM3-TM6 scaffold is proposed to be the critical step for the zinc(II) transfer across the membrane [29]. This mechanism relies on a persistent dimer interface within the lipid membrane acting as a static scaffold versus a mobile transport domain; a similar strategy is used also by other secondary active transporters [30]. In this regard, the zinc(II) translocation to

1 the transport site depends on the accessibility of the hydrophobic cavity located in the TMD region  
2 facing the intracellular space [31].

3 In this work, we investigated the zinc-induced dynamics of YiiP from *Escherichia coli* by  
4 performing atomistic MD simulations of the inward-facing conformation in presence and without  
5 zinc(II) ions in solution, respectively. Our results pointed out how the binding of one zinc(II) ion in  
6 the transport site triggers the closing of the portion of the channel going from the cytosolic space  
7 to the transport site itself, hereafter called the TM cavity. The TM cavity closing was mainly the  
8 result of TM4-TM5 of the four-helix bundle moving towards TM3-TM6. In this regard, TM5 played a  
9 crucial role through a reorientation that brought the side chains of L152 and M197 in close contact,  
10 thus forming a hydrophobic gate as proposed by Gupta et al. Unexpectedly, we observed that zinc(II)  
11 binding triggered the selective opening of only one channel out of the two protein chains in the  
12 dimer. Our work provides a detailed view of the mechanism of zinc(II) permeation from the cytosol  
13 to the TM cavity and gives hints on the subsequent steps of the transport mechanism.

## 14 15 **2. Methods**

16  
17 The dimeric model of the inward-facing state of YiiP from *Escherichia coli* was built with the  
18 Modeller software [32] (mod9v2) exploiting two different structural templates. Since the inward-  
19 and the outward-facing state share the same conformation of the CTDs, we used the structure with  
20 the highest resolution available (PDB ID: 3H90) [26] to model the residues in the range 208-290.  
21 Instead, the inward-facing TMDs, spanning residues 7-207, were modelled on the cryo-EM structure  
22 of the homologous *Shewanella oneidensis* protein (PDB ID: 3J1Z) [27]. The membrane builder  
23 module of the CHARMM-GUI was used to embed the protein in a rectangular lipid bilayer composed  
24 by 366 DPPC phospholipids [33,34]. The system was solvated with TIP3P water molecules with a  
25 hydration ratio of 1:100 lipid to water. Two dummy atoms were bound with harmonic restraints to  
26 the two cytosolic binding sites (site C) to mimic the binuclear zinc coordination mediating the  
27 interaction between the CTDs [12]. All the histidine residues were kept neutral. The N $\delta$ -protonated  
28 tautomer was used for all the histidine residues of the zinc(II) binding sites that coordinate the metal  
29 with the N $\epsilon$  atom in the 3H90 crystal structure. Instead, the N $\epsilon$ -protonated tautomer was used for  
30 all the remaining histidine side chains.

31 The holo-simulations were performed in the presence of 0.150 M ZnCl<sub>2</sub> corresponding to 94  
32 zinc(II) ions and 174 (188 minus 14 ions to balance the negative charge of the protein) chloride(I)  
33 ions in solution. The non-bonded parameters for zinc(II) were taken in agreement with the use of  
34 TIP3P water model in combination with the classical 12-6 LJ non-bonded model [35]. In particular,  
35 the Compromise set (CM) of parameters for divalent ions was used (available from the  
36 frcmod.ions234lm\_126\_tip3p file of the AMBER18 package).

37 The No-zinc simulation was prepared by adding 0.150 M of NaCl corresponding to 104  
38 sodium(I) ions and 90 chloride(I) ions in solution. A harmonic potential was applied to prevent the  
39 insertion of sodium(I) ions in the TM cavities.

40 The E79A simulation was prepared starting from the snapshot of the Holo02 run at 50 ns,  
41 when the zinc(II) was already bound to D150 and E79. After introducing the E79A mutation, a  
42 chloride(I) ion was added to the system to balance the charge.

43 All the simulations exploited the same force fields for the lipid and the protein portions of  
44 the system, the Amber LIPID17 and the Amber ff14SB, respectively [36,37]. All the calculations were

1 performed with the AMBER Molecular Dynamics Package using the pmemd software [38,39], with  
2 the same equilibration protocol. An initial minimization step was carried out using the Steepest  
3 Descent algorithm followed by Conjugate Gradient. Langevin dynamics with a collision frequency of  
4  $1 \text{ ps}^{-1}$  was used to linearly heat the system in constant volume for 1 ns, during which the protein  
5 and the ions are restrained with a force constant of  $10 \text{ kcal/mol/\AA}^2$ . The equilibrium temperature  
6 was set to 323K, a value commonly used to overcome the phase transition (liquid to gel)  
7 temperature of the pure DPPC lipid membrane (about 315 K) [40]. The physiological density of the  
8 system was achieved by carrying out an NPT simulation in which the anisotropic pressure scaling is  
9 controlled by the Berendsen barostat (pressure relaxation time of 2 ps). Covalently bonded  
10 hydrogen atoms were constrained with the SHAKE and SETTLE (for water molecules) algorithms.  
11 The PME method with a cutoff of  $10 \text{ \AA}$  was applied to compute electrostatic interactions. The  
12 production runs were performed on Nvidia Pascal Xp GPGPU in the same NVT conditions reported  
13 above for the heating step [41]. The RMSD of the protein C $\alpha$  atoms from the starting conformation  
14 was calculated over time to check the convergence of the simulations (Fig S1).

15 We carried out umbrella sampling simulations using a harmonic biased potential along the  
16 zinc(II) diffusion pathway through the TM cavity of the channel [42]. We prepared two runs using a  
17 zinc(II) ion as probe and a third run using a sodium(I) ion. The starting structure of the simulations  
18 was chosen from the unbiased holo-trajectory when the zinc(II) ion is at the entrance of the TM  
19 cavity facing the IC space. In the calculation using the sodium(I) probe, the metal ion was moved  
20 some angstroms away from the channel entrance in order to probe also the energetics for its  
21 approach to the channel. The metal probes were pulled towards a water molecule fixed by tight  
22 distance restraints at the top exit of the channel. In this way the ion probe is forced to cross the  
23 whole TM portion of the transporter. The water molecule was restrained to the C $\alpha$  of six residues  
24 (L22, L58, L95, L131, L152 and L191) located in a stable position within TM1, TM2, TM3, TM4, TM5  
25 and TM6, respectively. The force constant used to fix the water molecule was  $20 \text{ kcal/mol/\AA}^2$ .  
26 Instead, the metal probe was pushed through the channel with a force constant of  $15 \text{ kcal/mol/\AA}^2$ .  
27 The total distance was split in windows of  $0.2 \text{ \AA}$ , each one sampled for 1 ns. The resulting trajectories  
28 were computed using the weighted histogram analysis method (WHAM) version 2.0.9 to derive the  
29 free energy profiles from the potential of mean forces of the metal probes crossing the TM channel  
30 [43]. The standard deviations or error bars in the PMF calculations were derived from both  
31 bootstrapping and block analysis but are not reported due to their very small values.

32 All MD trajectories and umbrella sampling simulations are freely available from the Zenodo  
33 website at <https://zenodo.org/record/2658142> (DOI: 10.5281/zenodo.2658142).

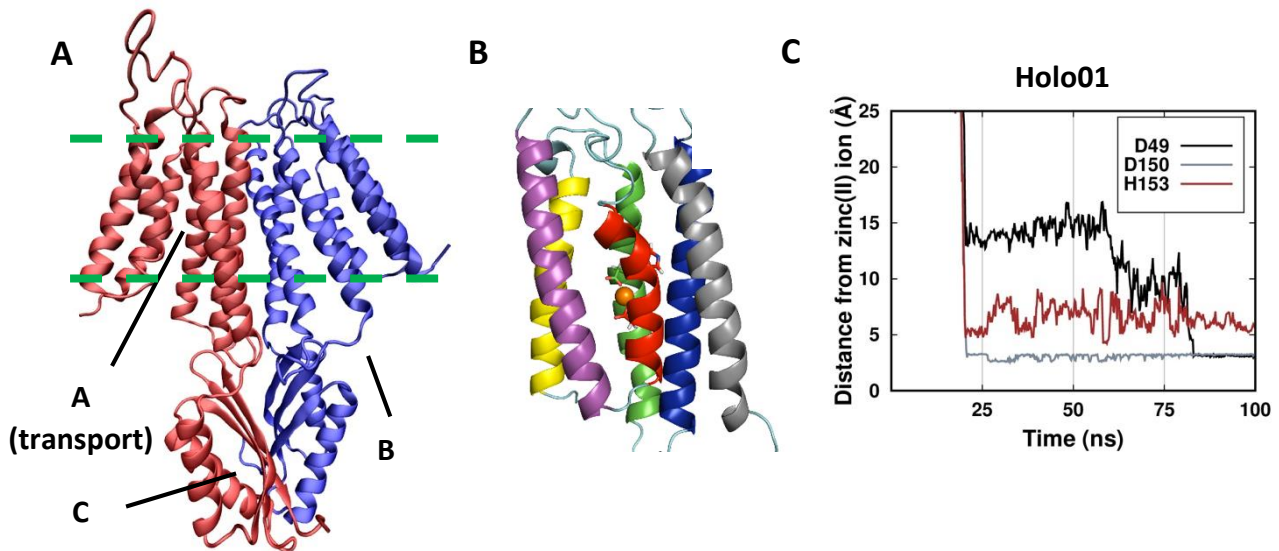
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### 35 **3. Results**

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37 Our model of the *E. coli* YiiP dimer in the inward-facing state was built by merging the model  
38 of the TMD built on the cryo-EM structure of the *S. oneidensis* homolog with the higher quality CTD  
39 obtained from the outward-facing crystal structure of *E. coli* YiiP (Fig 1A). The CTD is structurally  
40 identical in both the protein states. The resulting dimeric protein model was embedded in a lipid  
41 bilayer and solvated (see Methods). Overall, we carried out four classical MD simulations: three in  
42 the presence of 0.150 M of ZnCl<sub>2</sub> (holo) and one without zinc(II) ions in solution. The two longest  
43 holo-simulations started from the same conformation and sampled a trajectory length of 1  $\mu\text{s}$  each.  
44 Hereafter, we will refer to these two runs as “Holo01” and “Holo02”. Also the simulation of YiiP in

1 absence of zinc(II) ions (“No-zinc” hereafter) spanned a time length of 1  $\mu$ s. A further replica of the  
 2 holo system was performed for 540 ns starting from a snapshot of the “Holo01” run with the zinc(II)  
 3 ion already bound to the binding site in the channel. We will refer to this replica as “Replica01”.  
 4 Finally, we performed three umbrella simulations using the zinc(II) (2 runs) and sodium(I) ions as  
 5 probes to assess the metal-dependent free-energy barriers of the channel permeation process.  
 6



**Figure 1.** Model of the *E. coli* YiiP transporter in the inward-facing state. **A)** The three zinc(II) binding sites are indicated (A-C). The two protein chains are colored differently. **B)** Final snapshot of chain B in the Holo01 simulation. The six TM helices are colored as follow: TM1 in yellow, TM2 in green, TM3 in blue, TM4 in violet, TM5 in red, TM6 in grey. The residues in the channel featured in our work are shown as sticks and the zinc(II) ion is shown as an orange sphere. **C)** Zinc(II) interaction pathway in the TM cavity. The distances are measured from the zinc(II) ion to the C $\gamma$  atom of D49, D150 and to the N $\delta$  atom of H153.

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### 8 **3.1 Simulations of Holo-YiiP**

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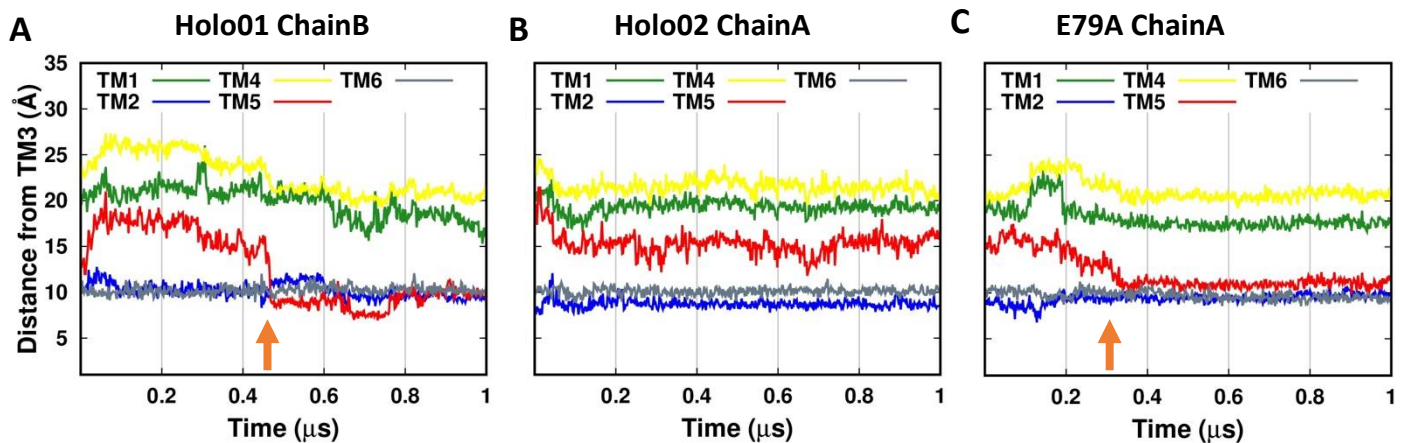
10 In the presence of zinc(II) ions in solution, the final structures of the two Holo trajectories at  
 11 1  $\mu$ s featured both sites B on the IL1 loops (Fig 1A) populated by zinc(II) ions. More importantly, in  
 12 both runs one zinc(II) ion entered the transmembrane (TM) cavity. The zinc(II) ion entered the  
 13 channel of chain B in the Holo01 run as opposed to chain A in the Holo02 run. However, only in  
 14 Holo01 the zinc(II) ion moved from the TM cavity to the transport site (Fig 1B), whereas in Holo02  
 15 the ion remained stably bound at the entrance of the TM cavity (Fig S2).

16 The distances from the residues interacting with the zinc(II) ions in the TM cavities were  
 17 measured along the simulation (Fig 1C). In the diffusion process from the cytosol toward the  
 18 transport site in the Holo01 simulation, the zinc(II) ion interacted mainly with three residues: D150,  
 19 which is part of the region of the TM5 helix facing the intracellular space, D49, which is part of the  
 20 transport site on the TM2 helix, and H153, which is part of the transport site on the TM5 helix (Fig  
 21 1B and Movie S1). After binding to the transport site, the zinc(II) ion remained coordinated by D49  
 22 and D150 until the end of the simulation.

1 In the Holo02 simulation the metal ion interacted simultaneously with D150 and E79 at the  
2 entrance of the TM cavity (Fig S3). The latter interaction was not observed in the Holo01 trajectory.  
3 Although H153 repeatedly approached the zinc(II) ion in the first half of the simulation, the two  
4 negative charges of E79 and D150 produced an electrostatic attraction on the metal ion so strong  
5 as to prevent further displacement of the ion towards the TM cavity. To analyze whether the  
6 additional carboxylate recruited by the zinc(II) ion in Holo02 with respect to Holo01 was indeed the  
7 main responsible for preventing the transit of the cation along the channel, we removed the charge  
8 of E79 by replacing it with alanine (E79A mutant) and re-started the simulation. Upon mutation, the  
9 metal ion was able to move away from the entrance of the channel and bound to D49 similarly to  
10 Holo01 (Fig S4).

11 The channel accessibility along time can be estimated by measuring a structural parameter,  
12 such as the TM cavity opening, and a biochemical parameter, such as the hydration of the channel.  
13 In this work, the channel accessibility was assessed by measuring the distances of the stable TM3  
14 (represented by I90) helix from TM1 (A20), TM2 (D49), TM4 (I130), TM5 (L152) and TM6 (M197) (Fig  
15 2). In all the holo-simulations, TM2 and TM6 showed a very stable behavior preserving a direct  
16 contact with TM3. On the contrary, at the beginning of the Holo01 run the chain permeated by the  
17 zinc(II) ion displayed a rapid increase of the TM3-TM4 and TM3-TM5 distances followed by a gradual  
18 decrease to 15 Å (Fig 2A). At 470 ns, these distances suddenly dropped due to the reorientation of  
19 TM5. In particular, the TM3-TM5 distance reached the same values as TM3-TM2 and TM3-TM6,  
20 denoting a direct contact between the TM helices. We can interpret this behavior as the channel  
21 being initially in a more exposed (open) configuration, followed by a large-scale rearrangement,  
22 induced or stabilized by the presence of the zinc(II) ion in the transport site, that eventually closed  
23 the channel. Of note, the Replica01 simulation, where a zinc(II) ion was already bound at the  
24 transport site, sampled the same large-scale rearrangement seen in Holo01 (Fig S5A). Instead, in the  
25 chain A of Holo02, where the zinc(II) ion was stably bound at entrance of the TM cavity, all the  
26 distance profiles remained fairly constant throughout the whole simulation and did not feature any  
27 relevant conformational changes, in what can be regarded as an essentially open configuration (Fig  
28 2B). Allowing the zinc(II) ion to reach the transport site by restarting the Holo02 run after introducing  
29 the E79A mutation resulted in the same reorientation of TM5 observed in Holo01 (Fig 2C).  
30 Furthermore, after this reorientation, the zinc(II) coordination became similar to the tetrahedral  
31 geometry of the outward facing configuration for some nanoseconds, with the exception of D150  
32 taking the place of H153 (Fig S4). This configuration allowed a second zinc(II) ion to approach the  
33 site from the extracellular environment, suggesting the existence of an accessible extracellular  
34 cavity.

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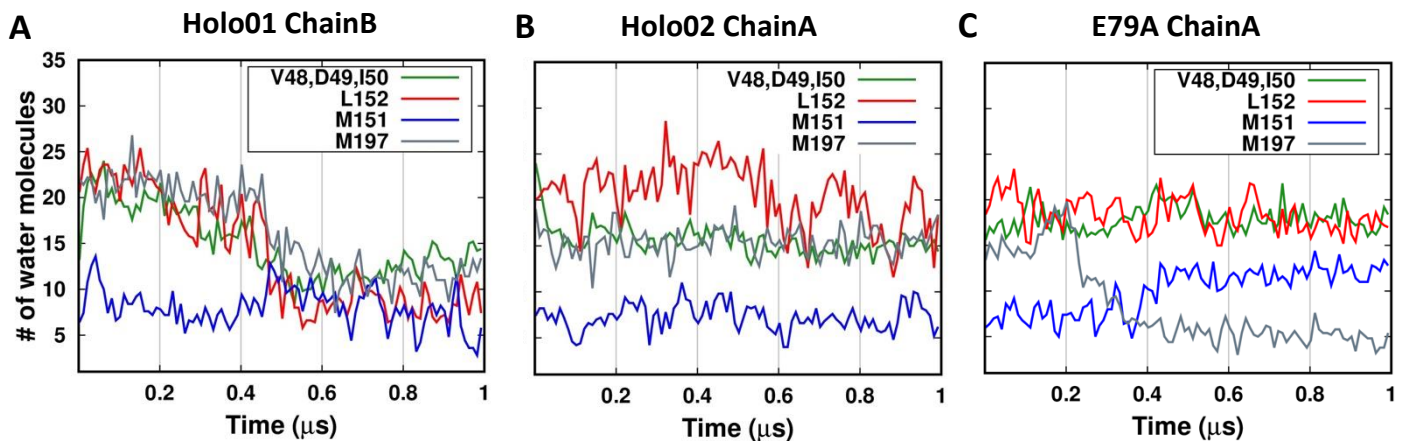
**Figure 2.** TM cavity opening and water accessibility as a function of time in Holo01 chain B, Holo02 chain A and mutant E79A chain A, respectively. The graphs show the distances from the C $\alpha$  atom of I90 in the TM3 helix to the C $\alpha$  atoms of A20 (TM1), D49 (TM2), I130 (TM4), L152 (TM5) and M197 (TM6). The reorientation of TM5 is marked by an orange arrow.

2

3 Variations of the channel hydration in response to the conformational motions caused by  
 4 zinc(II) binding were measured experimentally by Gupta et al. [31]. To obtain a qualitative  
 5 comparison with these data we measured the time evolution of the number of water molecules  
 6 (cutoff at 5 Å) surrounding the residues in the TM cavity (Fig 3). For this analysis, we selected the  
 7 residues that in experiments displayed the largest response to the presence of zinc(II) ions in  
 8 solution, namely the average of V48, D49 and I50 (these three residues are grouped together in the  
 9 experimental work), M151, L152 and M197. The profiles of the V48-D49-I50 group (TM2), L152  
 10 (TM5) and M197 (TM6) of chain B displayed a similar trend in the Holo01 and Replica01 simulations  
 11 (Fig 3A and S5B). These residues featured the highest water accessibility with TM4 and TM5 far away  
 12 from TM3. Then, their accessibility dropped when TM5 closed the gap with TM3 closing the channel.  
 13 Differently from the rest, the water accessibility of M151 (TM5) increased after the channel closed.  
 14 This effect is more evident in Replica01 and E79A than Holo01. Notably, M151 displayed an opposite  
 15 trend of experimental hydration change upon zinc(II) exposure with respect to the other residues  
 16 examined here [31]. For comparison, in the zinc(II)-bound chain A of the Holo02 simulation, V48-  
 17 D49-I50 and M197 sampled intermediate values between the poorly solvated M151 and the highly  
 18 solvated L152 (Fig 3B). Thus, the channel of chain A remained partly accessible from the IC space.  
 19 The E79A mutant sampled the same TM5 rearrangement as Holo01 and Replica01. However, here  
 20 only the water accessibility of M197 dropped (Fig 3C). This is due to the fact that in this simulation  
 21 a second cavity exposed to the extracellular environment increased the solvation of the top part of  
 22 the channel.

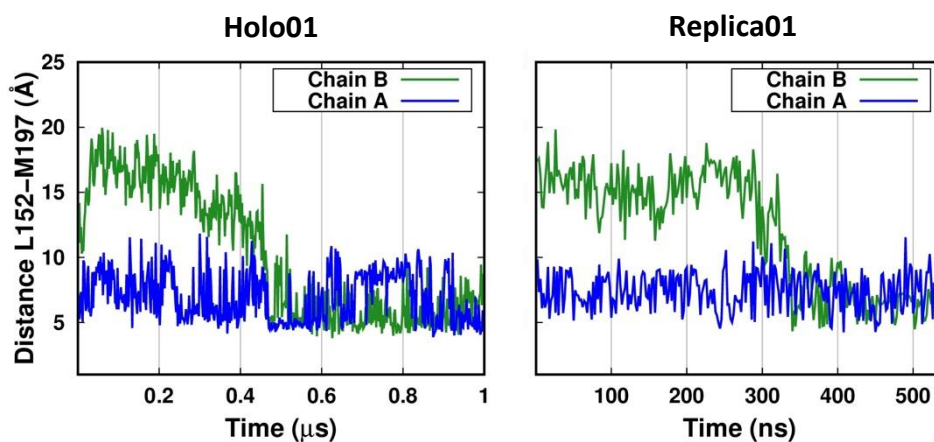
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**Figure 3.** Number of water molecules within 5 Å from the reported residues in the **A)** Holo01 chain B, **B)** Holo02 chain A and **C)** mutant E79A chain A, respectively.

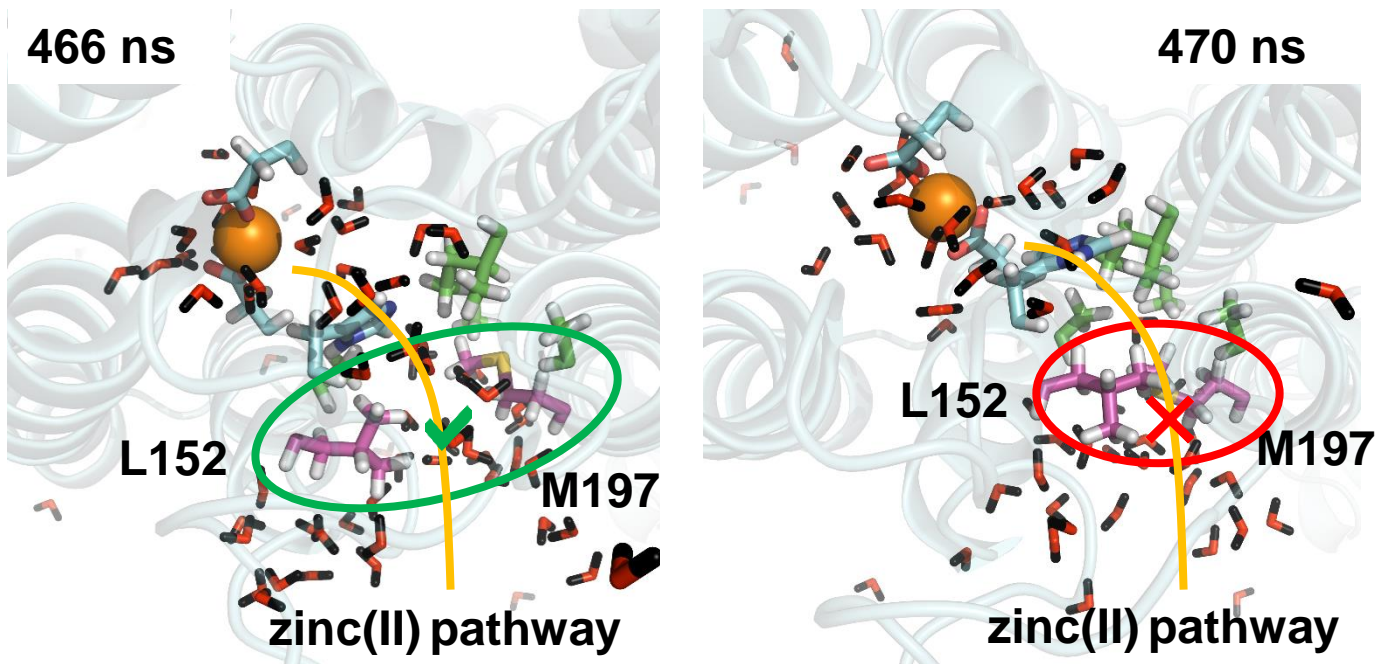
2 We then looked at the chains where zinc(II) did not enter the channel. In chain A of Holo01  
 3 TM5 sampled short distances from TM3, denoting a TM cavity constantly closed from the IC (Fig  
 4 S6A). A similar behavior was observed also for chain B in the Holo02 simulation, with the exception  
 5 of TM2 being modestly more distant from TM3 (Fig S6B). Accordingly, the presence of water  
 6 molecules in the channel was quite low for chain A of Holo01 as well as for chain B of Holo02,  
 7 with the exception of the V48-D49-I50 triad in the latter simulation (Fig S6C and S6D).



**Figure 4.** Hydrophobic gate regulating the access to the TM cavity from the IC space. The distances are measured between the C $\gamma$  of L152 and the C $\epsilon$  of M197 in both the protein chains.

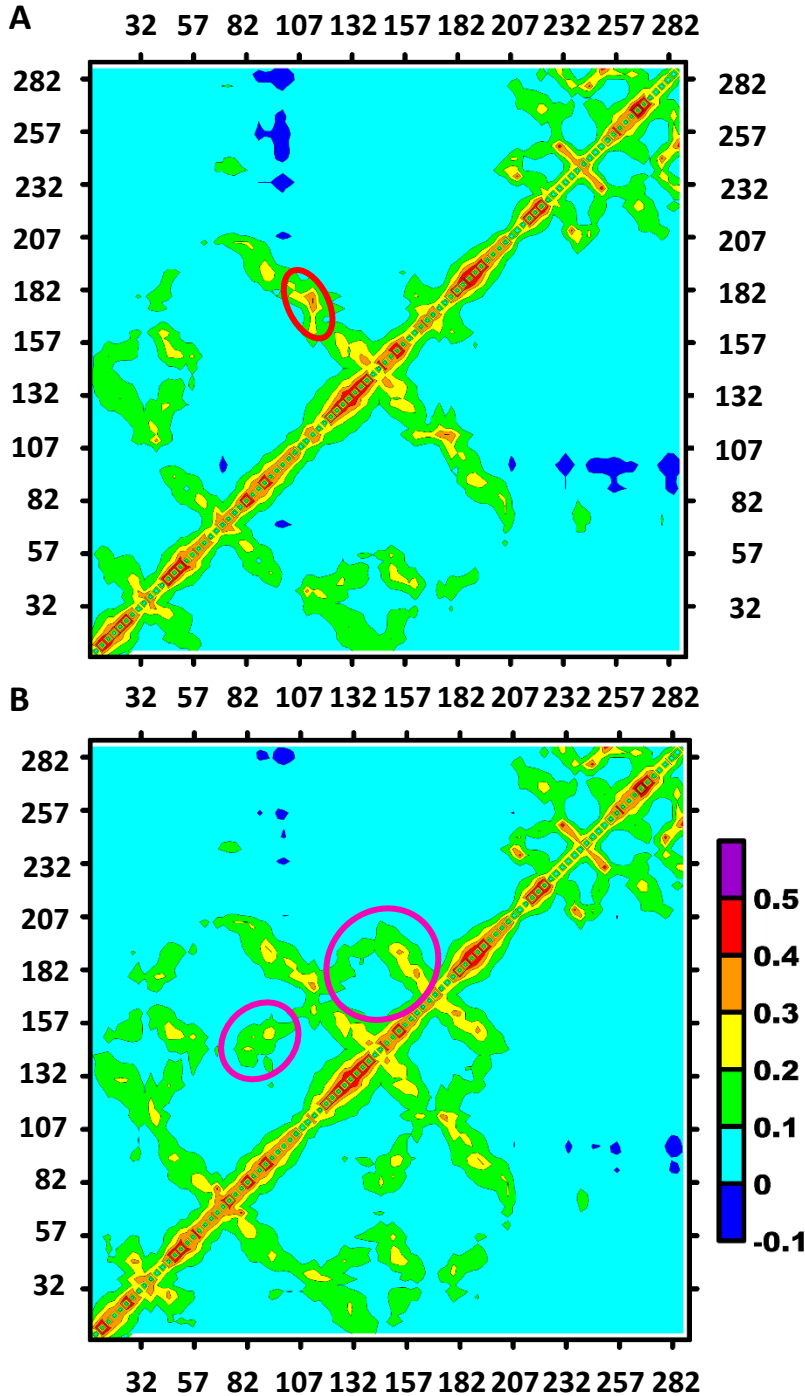
8 We monitored the accessibility of the TM cavity also through the distance between the side  
 9 chains of L152 and M197 (Fig 4). These residues were always in direct contact in chain A of the  
 10 Holo01 and Replica01 simulations, corresponding to an inaccessible cavity. On the contrary, for  
 11 chain B the interaction was suddenly achieved when the channel closed and conserved until the end  
 12 of the trajectory. The conformational change that closed the channel corresponded to a rotation  
 13 and tilt of TM5 that brought the L152 side chain in close contact with the side chains of I90 from  
 14 TM3 and of M197 from TM6 (Movie S2). This interaction created a hydrophobic gate modulating  
 15 the access of water from the IC space to the TM cavity and in turn to the transport site. The  
 16 snapshots at 466 ns and 470 ns displaying the top view of the TM cavity on the chain B of Holo01

1 point out the link between the L152-M197 distance and the presence of water molecules in the  
2 cavity (Fig 5). In particular, the snapshot at 466 ns shows that L152 and M197 were distant enough  
3 to allow water/ions to enter the TM cavity from the IC space. In fact, the TM cavity is filled by water  
4 molecules. Four ns later, the close contact between the side chains of L152 and M197 prevented  
5 the access to the TM cavity from the cytosol. As consequence, the number of water molecules in  
6 the cavity dropped. In addition to the above residues, A149 of TM5 approached A83 and I90 of TM3  
7 and to A194 of TM6 making a packed cluster of side chains that reinforced the hydrophobic barrier  
8 separating the transport site from the IC space (green sticks in Fig 5). The time evolution of this  
9 hydrophobic cluster closely paralleled the formation of the L152-M197 gate (compare Fig 4A and  
10 Fig S7).  
11



**Figure 5.** Top view of the Holo01 chain B channel at 466 and 470 ns. L152 and M197 are depicted as magenta sticks, water molecules are shown as thin sticks. The residues forming the hydrophobic cluster are shown as green sticks. The residues of the transport site coordinating the zinc(II) ion are indicated as cyan sticks.

12 To obtain further insights into the conformational dynamics of the TM helices in the Holo01  
13 simulation, we calculated the atomic correlation of motions, dividing the whole simulation into two  
14 parts: before and after the reorientation of TM5 occurring at 470 ns (Fig 6). Besides the obvious  
15 correlation among residues close in sequence, the loops EL2 (connecting TM3 and TM4) and EL3  
16 (connecting TM5 and TM6) featured the highest correlation during the first 470ns of simulation (Fig  
17 6A). This could suggest a sort of communication pathway between the TM3-TM6 helix pair and the  
18 TM4-TM5 helices of the four-helix bundle. In the second part of Holo01, the motions of TM5 and  
19 the lower part of TM4 were correlated with those of TM3 and TM6 (Fig 6B). This correlation was  
20 absent in the first 470 ns of the simulation. Thus, the re-orientation of TM5 linked directly TM4 and  
21 TM5 to the TM3-TM6 helix pair.



**Figure 6.** Atomic correlations among the C $\alpha$  atoms of the Holo01 Chain B. **A)** Correlations in the time range 1-470ns. The red circle indicates the region with the highest correlation. **B)** Correlations in the time range 471-1000 ns. The magenta circles indicate the regions with correlations arisen in this time range.

1

2 The principal component analysis (PCA) carried out on the C $\alpha$  atoms of the Holo01 simulation  
 3 highlighted the main conformational motions in a few principal components or eigenvectors. In  
 4 particular, performing the PCA on the TM helices of the Holo01 chain B (excluding the long EC loops)  
 5 together with the IL1 loop resulted in the first eigenvector containing 62% of the overall motions.  
 6 The widest motion involved the TM4-TM5 helices (Movie S3), which moved towards the TM3-TM6

1 helix pair covering a long distance. In this process TM5 not only translated toward the TM3-TM6  
2 helix pair along with TM4, but it also rotated towards the TM cavity as already described in the  
3 previous paragraphs. This is accompanied by a smaller-scale reorientation of the TM3-TM6 pair,  
4 resulting in a coordinated conformational change that modulates the packing of the four-helix  
5 bundle against the TM3-TM6 pair. We then performed a second PCA including also the CTDs (Movie  
6 S4). The first eigenvector collected 71% of the overall motions and showed again the main  
7 contributions to the mobility given by TM4, TM5 and the IL1 loop. In addition, a rotation of the CTDs  
8 was observed, thus suggesting a connection between the CTD dynamics and the conformational  
9 changes recorded in the TM region of chain B. Notably, the superposition of the TMDs of the final  
10 structures of Holo01 and Holo02 reveals that the two CTDs are tilted in opposite directions (Fig S8A).  
11 In fact, in both structures the CTD tilted toward the chain whose TM cavity was inaccessible. The tilt  
12 was greater for the Holo01 run than the Holo02 run, possibly due to the presence of a zinc(II) ion  
13 bridging site B (which is located on the IL1 loop) of chain A to the CTD, observed only in Holo01.

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### 15 **3.2 Simulation of YiiP in the absence of zinc(II) ions**

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17 The No-zinc simulation was performed without zinc(II) ions in solution for a time sampling of  
18 1  $\mu$ s. We are aware that using the structure of holo-YiiP as the starting point of the No-zinc run could  
19 be far from the correct conformational ensemble sampled by apo-YiiP. Indeed, in this simulation the  
20 global structure experienced larger fluctuations than in the Holo-simulations (Fig S1). However, the  
21 main goal of this simulation was to investigate whether the conformational changes featured in the  
22 holo simulations are zinc-dependent. To reach this goal we exploited the unique feature of  
23 molecular modelling that permits the simulation of non-physiological conditions. Thus, in this  
24 simulation a force was applied to prevent the sodium(I) and chloride(I) ions access in the TM cavities,  
25 so that the cations could not induce conformational effects similar to zinc(II), while preserving the  
26 physiological protein-sodium chloride interactions in the remaining regions of the protein.

27 To compare with the previous trajectories, we measured the distances of TM3 (I90) from  
28 TM1 (A20), TM2 (D49), TM4 (I130), TM5 (L152) and TM6 (M197) as a gauge of the accessibility of  
29 the TM cavity (Fig S9). For chain A, the profile is similar to that observed for chain B of the Holo01  
30 run, consistent with high accessibility of the cavity (Fig S9A). On the contrary, TM5 in the chain B  
31 sampled shorter distances corresponding to the partially accessible or, for short time intervals,  
32 inaccessible cavity (Fig S9B). In accord to this scenario, the L152-M197 distance sampled high values  
33 for the chain A and intermediate values for the chain B (Fig S10). By superimposing the TMDs of the  
34 final conformations of the Holo01 and the No-zinc runs, it became apparent that also in this case  
35 the CTDs tilted toward the chain whose cavity is inaccessible (chain A of Holo01 and chain B of No-  
36 zinc) (Fig S8B).

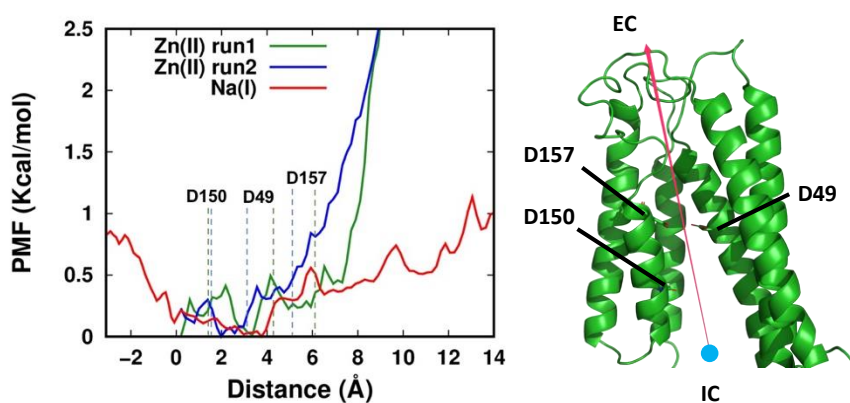
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### 38 **3.3 Umbrella Simulations**

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40 The Holo01 simulation provided us a detailed view of the zinc(II) interactions along the  
41 pathway for diffusion towards the transport site. This information can be used to perform a so-  
42 called umbrella simulation to derive the free-energy barrier encountered by the ion. The metal  
43 dependency of the energetic barriers can be estimated by replacing the zinc(II) ion with other  
44 metals. Thus, we performed three umbrella simulations using a zinc(II) or sodium(I) ion as probe on

1 the inward-facing state. The two zinc runs exploited the same starting conformation extracted from  
2 the unbiased Holo01 run when the metal ion is located at the entrance of the TM cavity of chain B  
3 (corresponding to 0 Å in Fig 7). Instead, the sodium run started with the ion placed some angstroms  
4 away from the channel entrance. The more distant starting point of the sodium(I) than zinc(II) runs  
5 is motivated by the fact that there is no evidence in the literature that sodium can enter YiiP. Thus,  
6 measuring the energetic barrier of the metal approach to the channel entrance can be relevant. The  
7 highest free-energy barrier encountered when moving from the cytosol to the transport site was  
8 similar for both metal ions (Fig 7). In correspondence of the energetic minima, the two metal ions  
9 interacted with the same residues (Fig S11). In particular, the coordination of the zinc(II) ion is the  
10 same as in the unbiased Holo01 simulation, with the carboxylates of D150 and D49 interacting with  
11 the metal (Fig S11A). Instead, the energetic profiles when going from the transport site to the EC  
12 space differed significantly between the two metals. The sodium(I) ion free-energy increased only  
13 slightly before reaching the top of the channel. Instead, in the two zinc(II) runs we observed a  
14 dramatic energy increase when detaching from D157 in the transport site to move to the top of the  
15 channel. In summary, the inward-facing conformation favored the entrance of the cations in the TM  
16 cavity and their translocation to the transport site. Here, the coordination environment of the  
17 zinc(II) ion is well defined; similar interactions with the protein have been observed also for the  
18 sodium(I) cation. In the process of release from the transport site, the zinc(II) ion experienced a  
19 dramatically high energetic barrier, which effectively prevents its free diffusion from the channel to  
20 the bulk solution. This suggests that a significant conformational rearrangement is needed to lower  
21 the energetic barrier for the exit of the zinc(II) ion. Interestingly, there is a comparatively much lower  
22 barrier for the release of the sodium(I) ion, indicating that the interactions of the latter within the  
23 transport site are somewhat weaker, albeit structurally similar, than those formed by the zinc(II)  
24 ion. This can be ascribed to the higher charge of the zinc cation.  
25



**Figure 7.** Free-energy profiles of the zinc(II) and sodium(I) ions crossing the TM cavity. The channel entrance from the IC is aligned at 0 Å. The main binding residues are reported.

26

27

## 28 Discussion

29

30 The main force that drives the zinc(II) ions toward the YiiP protein channels is electrostatic  
31 attraction. Indeed, the YiiP transporter has three negatively charged residues located at the

1 entrance of the TM cavity: E79 on TM3, D150 on TM5 and E200 on TM6. In our holo-simulations the  
2 zinc(II) ion permeating the TM cavity bound to D150 or D150 and E79. In particular, the simultaneous  
3 interaction with both D150 and E79 occurring in the Holo02 run blocked the metal ion at the TM  
4 cavity entrance for the entire simulation. Instead, in the Holo01 run the metal ion interacted initially  
5 only with D150, allowing it to subsequently reach the transport site. We thus postulated that the  
6 zinc(II) interaction with two negative charges generated a very high energetic barrier preventing the  
7 ion from moving deeper in the channel and also inhibiting the rearrangement of TM5. We tested  
8 this hypothesis by mutating E79 to alanine. In the E79A mutant, the zinc(II) ion previously blocked  
9 at the entrance of the TM cavity reached the transport site as seen in the Holo01 simulation (Fig  
10 S4). Notably, the human homologues of YiiP lack D150, which could result in a similar effect of  
11 lowering the energetic barrier for the diffusion of zinc(II) within the channel while preserving the  
12 negative electrostatic potential needed for attracting the ions from the IC space [44]. Indeed, the  
13 energetic profile of Fig 7 indicates that once the metal ion has reached the entrance of the TM  
14 cavity, its translocation to the transport site encounters modest barriers. Electrostatics is, based on  
15 our energetics calculations, a crucial factor also in preventing the free diffusion of the metal ion  
16 from the transport site towards the EC space in the inward-facing state of YiiP.

17 The possibility for the zinc(II) ion to enter the TM cavity from the IC space is related to the  
18 accessibility of the TM cavity delimited by the four-helix bundle (TM1, TM2, TM4 and TM5) and the  
19 TM3-TM6 helix pair. It has been proposed that the dynamics of TM5 with respect to the TM3-TM6  
20 helix pair is the main factor in modulating the access to the transport site [31]. Our Holo simulations  
21 demonstrated a tight correlation between the motion of helices TM5 and, to a lesser extent, TM4  
22 and the presence of water molecules in the TM cavity. The TM helices sampled short distances in  
23 the chains not permeated by zinc(II) and had low solvation, corresponding to a closed configuration.  
24 Instead, the chains whose TM cavity was permeated by zinc(II) displayed a relevant increase of the  
25 distance between the TM4-TM5 pair and the TM3-TM6 pair at the beginning of the simulation (open  
26 configuration); this was accompanied by relatively high hydration of the channel (Fig 2 and 3). After  
27 the zinc(II) ion reached the transport site by binding to D49, TM4 and TM5 slowly closed the gap  
28 with the TM3-TM6 helix pair. In parallel, the number of water molecules in the cavity gradually  
29 decreased. Then, a rapid conformational change took place that put TM5 in close contact with the  
30 TM3 making the TM cavity inaccessible from the IC space. The rearrangement of TM5 involved also  
31 a motion of TM4, so that the whole structural change can be described as the four-helix bundle  
32 achieving a tighter packing against the TM3-TM6 pair. In our Holo01 and Replica01 simulations, this  
33 led to the formation of a hydrophobic gate between L152 and M197 (Fig 4 and 5). This process was  
34 highlighted by X-ray-mediated hydroxyl radical labeling measurements, leading to the suggestion  
35 that the hydrophobic gate is responsible for the regulation of zinc(II) access to the TM cavity [31].  
36 Our simulations support this view.

37 In all Holo runs, including the E79A mutant, we found only one of the two available TM  
38 cavities was accessible, whereas the other was totally or partially inaccessible. Therefore, the usual  
39 symmetric representation of the YiiP transporter does not match the dynamics sampled by our  
40 simulations. The open cavity was the one permeated by the zinc(II) ion. In the simulation without  
41 zinc(II) ions both chains were in an open or partly open configuration. Thus, it is possible that the  
42 binding of zinc(II) initially stabilizes the chain in the open configuration while the other chain  
43 rearranges to the closed configuration. Subsequently, when the zinc(II) ion reaches the transport  
44 site also the first chain reorganizes to reach the closed configuration.

1           Based on X-ray-mediated hydroxyl radical labeling measurements, it was suggested that  
2 zinc(II) binding at the transport site with tetrahedral coordination geometry is the crucial event to  
3 trigger the conformational change of TM5 [31]. In particular, it was hypothesized that this  
4 rearrangement would cause the TM cavity to close in the portion facing the IC while opening  
5 towards the extracellular side in a sort of alternating mechanism. Here, in the Holo01 and Replica01  
6 simulations YiiP underwent a wide conformational change that caused the closing of the TM cavity  
7 upon zinc(II) binding at the transport site. To achieve this, it was sufficient for the zinc(II) ion to reach  
8 a stable interaction with only D49 of the transport site, suggesting that tetrahedral coordination is  
9 not essential to trigger the reorientation of TM5. Only in the E79A simulation we could observe the  
10 coordination sphere of the zinc(II) ion in the transport site achieve a geometry very similar to the  
11 tetrahedral geometry of the outward facing configuration. Intriguingly, this event was accompanied  
12 by other smaller-scale structural changes, which allowed a second zinc(II) ion to approach the site  
13 from the EC side of the protein. This observation is in line with a recent experimental work  
14 suggesting that a conformation of the transporter where a persistent dimer interface is combined  
15 with an inaccessible intracellular cavity and an accessible extracellular cavity is able to transport  
16 zinc(II) across the membrane [29]. Overall, our simulations and the latter experimental data indicate  
17 that the four-helix bundle rearranges with respect to a static TM3-TM6 helix pair scaffold, in a  
18 rocking-bundle mechanism common also in other active transporters as LeuT [45,46]. This  
19 mechanism was apparent in the conformational dynamics sampled in the Holo01 run (Movie S5),  
20 even though the simulation length is very short compared to the estimated timescale of the overall  
21 zinc(II) transport process [23,47]. Furthermore, our umbrella simulations showed that the IC-facing  
22 conformation permitted the translocation of zinc(II) to the transport site but not its release. Thus,  
23 we can postulate that a further conformational change involving at least the TM helices harboring  
24 the zinc(II)-binding residues (TM2 and TM5) is needed to lower the free-energy barrier of the zinc(II)  
25 release process [44].

26           In conclusion, our simulations combined with the available structural and experimental data  
27 on YiiP provide an atomistic view of various steps of the transport mechanism involving the inward-  
28 facing conformation. First of all, we observed that during zinc(II) transport the symmetry of the YiiP  
29 dimer is lost, with the channels in the two chains consistently featuring a significantly different  
30 accessibility. Zinc(II) permeation within the TM cavity of one chain is sufficient to trigger the  
31 compaction of the four-helix bundle and the TM3-TM6 helix-pair in the TMD of that chain. After the  
32 zinc(II) ion fully enters the transport site further structural changes may put the site in  
33 communication with the extracellular space without achieving a full transition of the outward-facing  
34 state. Nevertheless, our energy calculations suggest that there is a substantial energetic barrier  
35 preventing the release of the ion from the coordination environment in the transport site. Our data  
36 do not allow us to evaluate how this barrier is eventually removed, an event which may be combined  
37 with the proton antiport performed by YiiP.

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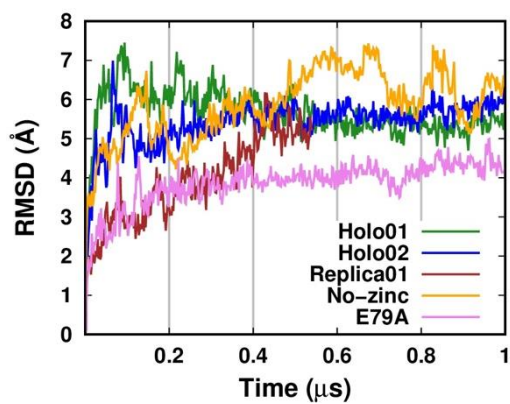
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45

1 **Supporting information**

2 **Supplementary figures and movies**

3



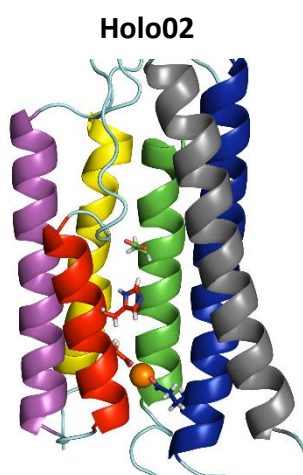
**Figure S1.** RMSD of the C $\alpha$  atoms over time.

4

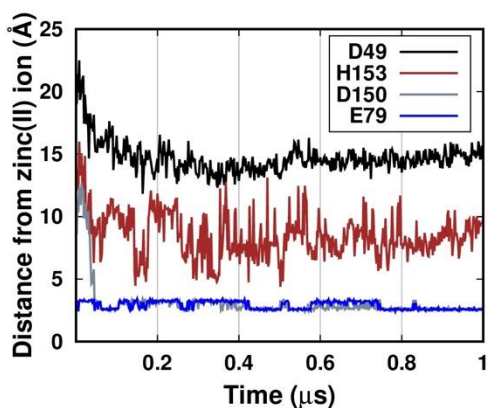
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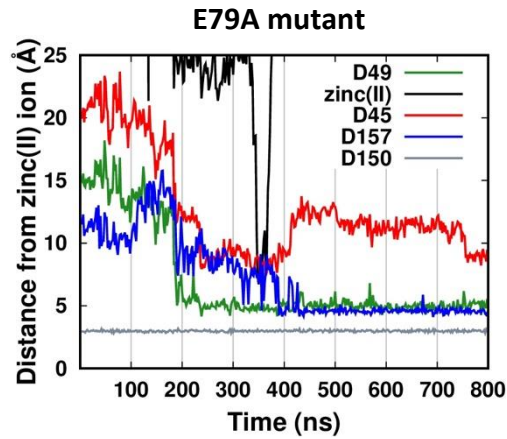
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**Figure S2.** Final snapshot of the holo02 simulation.

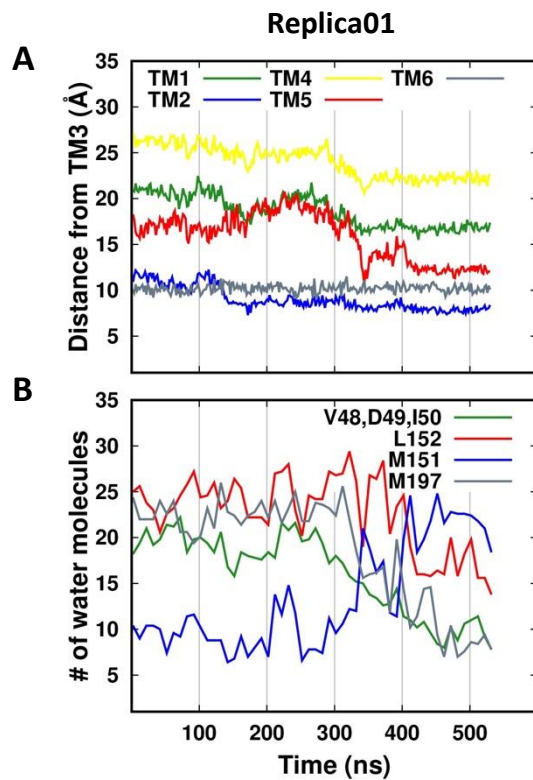


**Figure S3.** Zinc(II) interaction pathway in the TM cavity of chain A of the Holo02 run. Distances were measured from the zinc(II) ion to the C $\gamma$  atoms of D49 and D150, to the N $\delta$  atom of H153 and to the C $\delta$  atom of E79.



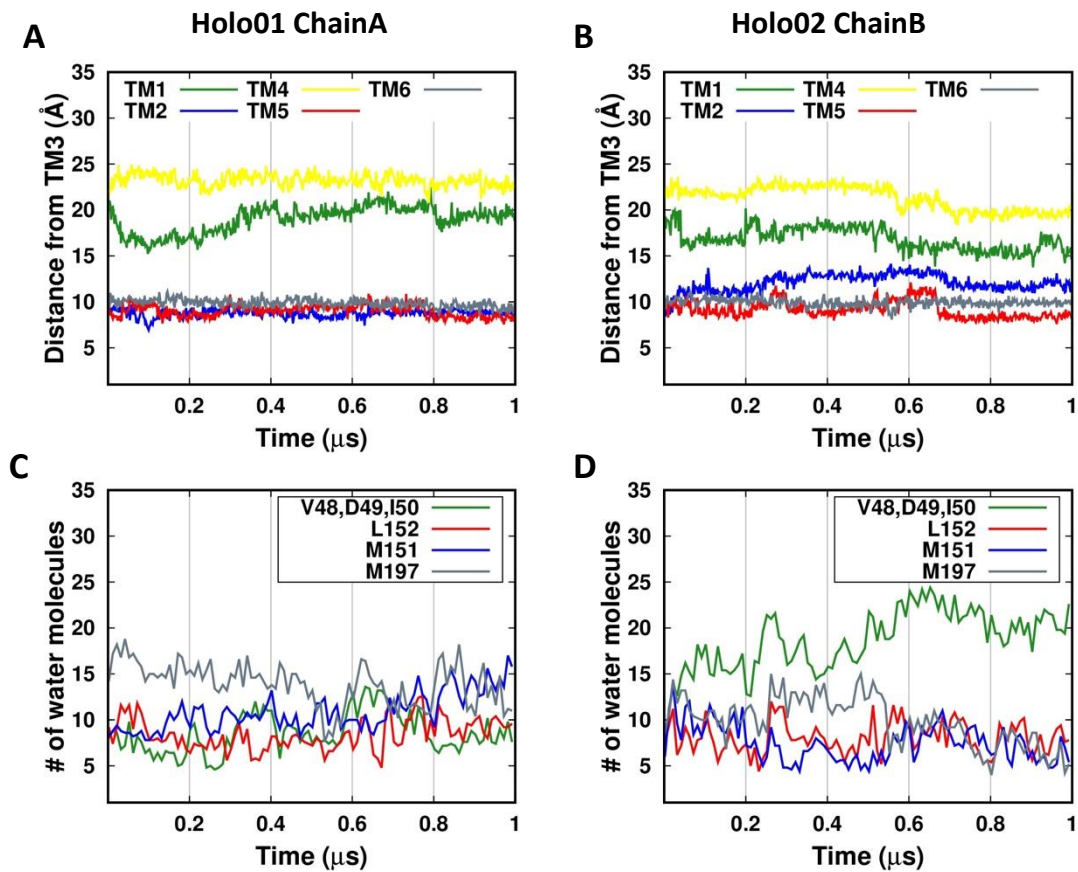
**Figure S4.** Zinc(II) interaction pathway in the TM cavity of the E79A mutant simulation. All the distances refer to the zinc(II) ion permeating the channel from the IC space. Thus, the black line indicates the zinc(II)-zinc(II) distance between the ion bound in the transport site and another ion coming from the EC space.

1



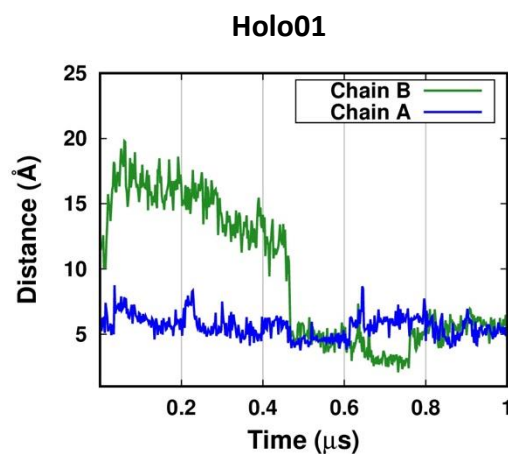
**Figure S5.** TM cavity opening and water accessibility of chain B of the Replica01 simulation. **A)** Distances from the  $\text{C}\alpha$  atom of I90 on the TM3 helix the  $\text{C}\alpha$  atom of A20 (TM1), D49 (TM2), I130 (TM4), L152 (TM5) and M197 (TM6). **B)** Number of water molecules within 5 Å from the residues.

2



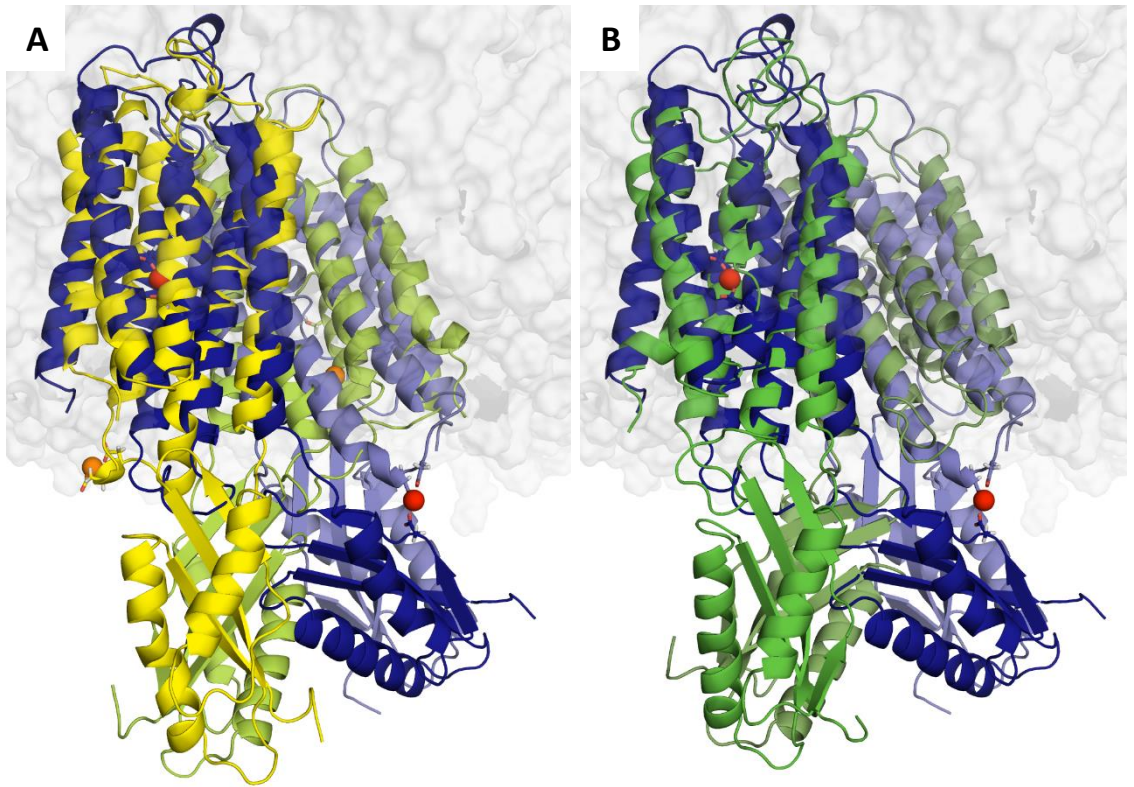
**Figure S6.** Opening and water accessibility of the TM cavity vs time in the Holo01 chain A and Holo02 chain B, respectively. **A-B)** Distances from the C $\alpha$  atom of I90 on TM3 helix to the the C $\alpha$  atoms of A20 (TM1), D49 (TM2), I130 (TM4), L152 (TM5) and M197 (TM6). **C-D)** Number of water molecules within 5 Å from the residues.

1



**Figure S7.** Distance between groups of aliphatic residues in the TM cavities of the Holo01 simulation. The distance is measured between the side chains of A149(TM5)-L152(TM5) and the side chains of A83(TM3)-I90(TM3)-A194(TM6)-M197(TM6).

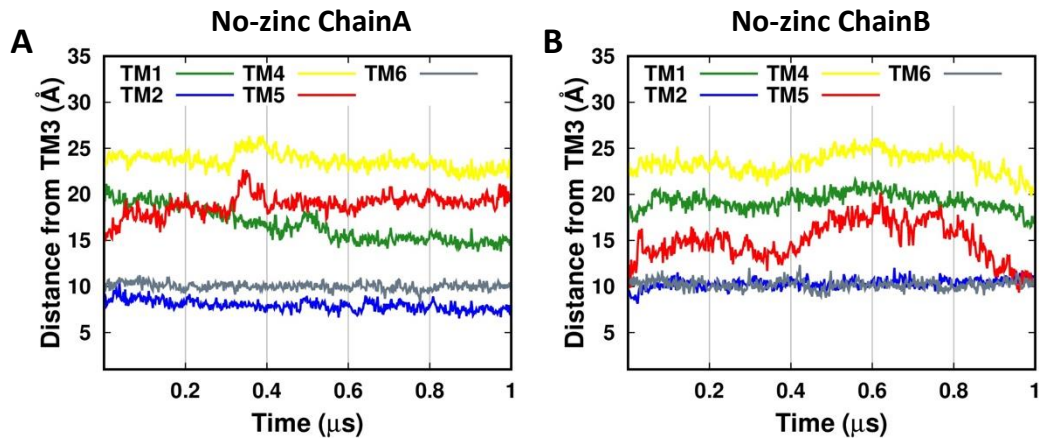
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**Figure S8.** Superimposition of the final structure of the simulations. The conformations are aligned in the TMD region. The two protein chains have different shades of the same color. The Holo01 zinc(II) ion bound to the transport site and the one bridging the Site B on IL1 and the CTD are shown as red spheres. The residues coordinating the zinc(II) ions are shown as sticks. **A)** The Holo01 and Holo02 conformations are in blue and yellow, respectively. The Holo02 zinc(II) ions bound at the TM cavity entrance and the one bound to the SiteB on IL1 are shown as orange spheres. **B)** The Holo01 and No-zinc conformations are in blue and green, respectively.

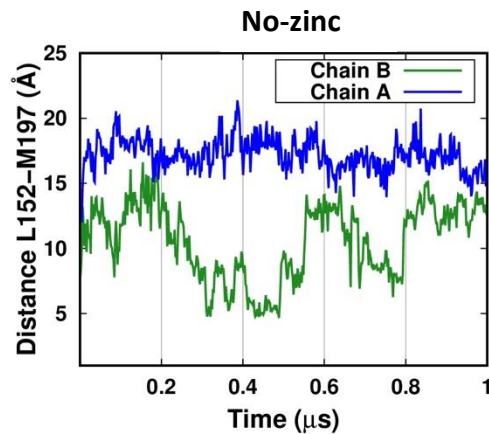
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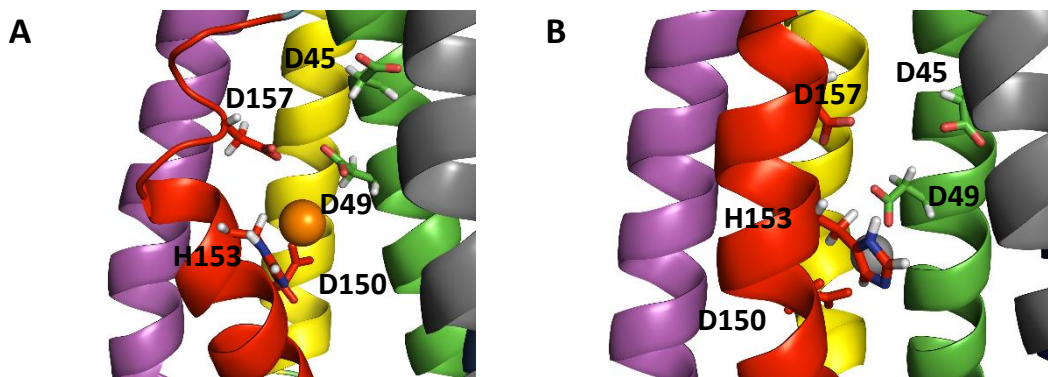
**Figure S9.** The TM cavity opening along time in the No-zinc simulation. Distances among the C $\alpha$  atoms of I90 on TM3 helix from A20 (TM1), D49 (TM2), I130 (TM4), L152 (TM5) and M197 (TM6). **A)** Chain A. **B)** Chain B.

2



**Figure S10.** Hydrophobic gate regulating the access to the TM cavity from the IC space in the No-zinc simulation. The distances are measured between the C $\gamma$  of L152 and the C $\epsilon$  of M197 in both the protein chains.

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**Figure S11.** Metal ions coordination at the energetic minimum of the corresponding umbrella simulation. **A)** zinc(II). **B)** sodium(I).

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1 **Movies**

2

3 **Movie S1** GIF showing the zinc(II) ion entrance in the TM cavity and the approach to the  
4 transport site of the Holo01 chain B. The TM5 backbone is shown as a red cartoon. The zinc(II)  
5 binding residues side chains D150, H153, D49 and the two residues side chains regulating the cavity  
6 accessibility L152, M197 are shown.

7

8 **Movie S2.** GIF showing the re-orientation of TM5 responsible of the TM cavity closing upon  
9 zinc(II) binding. The TM5 backbone is shown as a red cartoon. The main hydrophobic residues in the  
10 cavity are shown.

11

12 **Movie S3.** GIF showing the projection on the first eigenvector of the TM helices motion. The  
13 color code from blue to red indicates the descending contribution of each residue to the total  
14 motion of the first eigenvector.

15

16 **Movie S4.** GIF showing the projection on the first eigenvector of the TM helices and CTDs  
17 motion. The color code from blue to red indicates the descending contribution of each residue to  
18 the total motion of the first eigenvector.

19

20 **Movie S5.** Movie showing the whole Holo01 simulation. The zinc(II) ions bound to the known  
21 binding sites are shown as orange spheres.

22