Computational Study of Ion Permeation Through Claudin-4 Paracellular Channels

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ABSTRACT
Claudins (Cldns) form a large family of protein homologs that are essential for the assembly of paracellular tight junctions (TJs), where they form channels or barriers with tissue-specific selectivity for permeants. In contrast to several family members whose physiological role has been identified, Cldn4 remains elusive, despite experimental evidence suggesting that it can form anion-selective TJ channels in the renal epithelium. Computational approaches have recently been employed to elucidate the molecular basis of Cldns' function, and hence can help in clarifying Cldn4 role. In this work, we use structural modeling and all-atom molecular dynamics simulations to transfer two previously introduced structural models of Cldn-based paracellular complexes to Cldn4, in order to reproduce a paracellular anion channel. Free energy calculations for ionic transport across the pores allow us to establish the thermodynamic properties driving the ion-selectivity of the structures. While one model shows a cavity permeable to chloride and repulsive to cations, the other forms barrier to the passage of the major physiological ions. Furthermore, our results confirm the charge selectivity role of the residue Lys65 in the first extracellular loop of the protein, rationalizing Cldn4 control of paracellular permeability.

KEYWORDS: Tight junctions, Claudins, Pore models, Ion-selectivity, Molecular Dynamics, Free energy calculations, Paracellular space.

INTRODUCTION
Claudins (Cldns) form a family of 27 homologous proteins with a pivotal role in endothelial and epithelial tight junctions (TJs)[1–6] structure and function. Available structural information shows that Cldns fold in a four-helices bundle (named TM1 to TM4) that embeds in the cellular membrane and anchors two extracellular loops (ECL1-2) and a loop in the cytoplasmic region, where the two terminal domains are also contained. Cldns assemble in TJs via: (i) the formation of strands of protomers lining on the same cell membrane, held together by *cis* interactions, and (ii) intercellular aggregates involving *trans* interactions between the ECLs from opposing protomers, which seal the thin layer separating two neighboring cells, the paracellular space. The ECLs arrange in a β-sheet layer and, typically, ECL1 affects the TJ permeability, while ECL2 determines the *trans* interactions between Cldns belonging to two adjacent cells[7]. These proteins regulate the paracellular transport of ions and molecules via highly selective mechanisms[8,9], and for this reason their dysfunction is directly associated with clinical disorders[10,11].
While the physiological role of few Cldns has been extensively characterized[6,7], others are still to be completely understood. A remarkable example is Cldn4, highly expressed in the kidney but also present in other tissues[12–19]. Recent investigations suggest that Cldn4 may play a relevant role in the dynamics of tumor growth, and its

attitude to bind the *Clostridium perfringens* enterotoxin (CPE), as a receptor, can open new approaches for drug delivery strategies[20–22]. Several studies suggest that Cldn4 is responsible for anion reabsorption in the collecting ducts, forming highly selective channels for chloride[23–27], most likely due to a positively charged residue, Lys65, belonging to the ECL1 domain[23,28,29]. However, Cldn4's ability to form TJ strands has been found to depend on the cellular system in which it is expressed[26,30–32]. While biochemical data reported by Hou et al.[23] suggest that heterotypic interactions between Cldn4 and Cldn8 are required for a functional localization of the TJ in the kidney epithelial cells, homotypic Cldn4 interactions have also been observed[33].

In the last years, structural modeling and molecular dynamics (MD) simulations, have become instrumental to grasp the fine details of biophysical processes, such as protein-protein aggregation, protein conformational transitions and selectivity of ion channels[34]. More recently, MD simulations allowed the atomic description of the structural and functional features of various Cldn-based paracellular aggregates, including Cldn15, Cldn5, and Cldn2[35–40]. A remarkable step toward a detailed description of TJ proteins was the introduction of a first model for Cldn15-based channels in Ref. 41, often referred to as "Suzuki model". The proposed arrangement is built from the crystal structure of the isolated Cldn15 monomer (PDB ID: 4P79)[42] and is consistent with cys-scanning mutagenesis experiments and freeze-fracture microscopy imaging. Linear strands of neighboring Cldns are held together by *cis* interactions and join strands in the facing cell to seal the paracellular space, with the ECLs of each four protomers forming a β-barrel super-secondary structure that results in a pore of radius smaller than 5 Å. Subsequent computational works contributed to refine and validate the original Cldn15-based model[37,38,40,43], also extending the pore configuration to channels of other members of the Cldns family and assessing its consistency with the tissue-specific physiological properties[36].

A second model of a Cldn-based pore was suggested in Refs. 35,39. Accordingly, two protomers belonging to the same cell assemble in a dimer via interactions between the TM2 and the TM3 helices[44]. Focusing on Cldn5, the authors emphasized that the structural stability of the dimer is due to the formation of a *leucine zipper* involving residues Leu83, Leu90, Leu124 and Leu131 in TM2 and TM3, supported by two homophilic π- π-interactions between the aromatic residues Phe127 and Trp138 on the opposing TM domains. This specific arrangement for Cldn5 protomers matches experimental results by Rossa et al.[45]. As described in the computational investigation of Refs. 35,39, two copies of these dimers, belonging to two opposing cells, can associate across the paracellular space to form a pore via a network of *trans*-interactions. Coarse-grained (CG) self-assembly simulations suggested that this second model is suitable for the assembly of Cldn4-based TJs[35] as well, but the application to other homologs is highly questionable due to experimental evidences[46].

In this work, we present a computational investigation of Cldn4 in the two pore arrangements, in order to assess their transferability to this member of the Cldn family. Consistent with the notation of Refs. 35,39,44, we refer to the two models described above as Pore I and Pore II, respectively. The two tetramers are simulated in explicit double membrane bilayer and water environments, and free energy (FE) calculations are performed for single ion or water molecule permeation across the two paracellular cavities. Results show that Pore I is attractive for chloride, since its energetic profile displays a minimum of -2.5 kcal/mol in the inner portion, where the Lys65 residues[23] belonging to the four protomers form a cage that functions as a selectivity filter. Indeed, the passage of cations is prevented by barriers of ~4 kcal/mol and ~8 kcal/mol for monovalent and divalent cations, respectively, with peaks framed by the same basic residues. In contrast, due to the drastically different distribution of charged pore-lining residues, Pore II is impermeable to all ions, showing double FE barriers of ~3 kcal/mol and ~6 kcal/mol for monovalent and divalent cations, respectively, and a single barrier of ~5 kcal/mol for the anion.

The results reveal that the specific pore arrangement can strongly affect the selectivity properties of Cldn-based TJ pores. In particular, the thermodynamic features of ionic permeation in the different Cldn4 pore models suggest that only the Pore I structure is consistent with the anionic selectivity of Cldn4 paracellular pores.

METHODS

Pore I assembly. Pore I was assembled with four monomers of Cldn4, following the protocol illustrated in Ref. 37 and matching the structure published by Suzuki et al.[41]. The high-resolution Cldn4 structure is unlikely to be representative of the native conformation of the monomer, due to the binding to the C-terminal fragment of the *Clostridium perfringens* enterotoxin (PDB ID: 5B2G)[47]. For this reason, the monomers of Cldn4 were homology modelled starting from Cldn15, available in the isolated form (PDB ID: 4P79)[42]. The SWISS-MODEL platform[48,49] was used to generate the Cldn4 structure. Subsequently, the model was refined with the server ModRefiner[50] available at https://zhanggroup.org/ModRefiner . The resulting protomer of Cldn4 was replicated in four units and superimposed on the template of the Suzuki pore model[41] with the UCSF Chimera[51] Matchmaker tool. Afterwards, a further refinement of the system was performed with the GalaxyRefine algorithm available at the GalaxyWeb server[52,53] provided by the Seok's Lab at http://galaxy.seoklab.org. The *cis*-interactions occurring between two protomers on the same cell are shown in **Fig. 1A**, and the tetrameric structure sealing the paracellular space is depicted in **Fig. 2A,B**.

Pore II assembly. Because of the lack of a reference model based on experimental data of the pore to use as template, the strategy applied to reproduce the tetrameric architecture of Pore II configuration was different from that adopted for Pore I. First, we generated and equilibrated the *cis*-dimer reproducing the leucine zipper (**Fig. 1B**). A dimer of Cldn4 monomers with the required protein-protein interface was obtained from the MEMDOCK server[54]. Following an additional refinement with the DOCKING2 tool of Rosetta online server[55–57], the structure was equilibrated in a single membrane bilayer with ~250 ns of all-atom MD simulation performed with the NAMD 3.0 program[58] and the CHARMM36m force field[59]. The ClusPro docking algorithm[60–64] was used to build the tetramer, starting from two replicas of the equilibrated dimer configuration and searching for the putative poses. Positional restraints to keep the TM domains of opposing dimers far from each other, anchored within the respective membranes and to ensure the interaction via the ECLs domains. Afterwards, the structure was relaxed according to the algorithm of GalaxyRefine algorithm[52,53] (**Fig. 2C,D**).

Double Bilayer set-up. Each tetrameric system was embedded in a double bilayer of pure 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), solvated with explicit three-point (TIP3P)[65] water molecules and neutralized with counterions, as described below. The pore axis was oriented along the y-axis. The CHARMM pdb file of the protein complex was generated with the PDB manipulator tool of CHARMM-GUI server[66,67]. A single hexagonal bilayer of pure POPC, inscribed in a square of 128.0 X 128.0 Å, was provided by the *membrane builder* tool of the same platform[67,68]. Two equilibrated replicas of the membrane were used to embed the transmembrane domains of the pore. After the removal of steric clashes between protein and lipids, the resulting hexagonal box was filled with explicit TIP3P[65] water. When needed, counterions were added in both the cytosolic and paracellular layers to ensure the system neutrality. The 4 disulfide bridges between residues Cys54 and Cys64 were preserved. The CHARMM36m[59] force field was used for all the simulations.

Equilibration and unbiased MD simulation. After an initial short energy minimization, 30 ns of equilibration were performed by progressively releasing positional restraints on the heavy atoms. The system was simulated in the NPT ensemble at T = 310 K and P = 1 bar, maintained by a Langevin thermostat and a Nosé-Hoover Langevin piston, respectively[69,70]. The NAMD 3.0 program[58] in combination with CHARMM36m force field[59] was used. The hexagonal box was inscribed in a square of sides about 120.0 X 120.0 Å and of height around 160.0 Å. Periodic boundary conditions were used to replicate the system and remove surface effects. Long-range electrostatic interactions were computed using the Particle Mesh Ewald (PME) algorithm[71]. Chemical bonds involving hydrogen atoms and protein heavy atoms were constrained with SHAKE[72], while those of the water molecules were kept fixed with SETTLE[73]. The transmembrane domain of each protomer was held fixed by harmonically restraining the Cα atoms of residues 11, 14, 25, 28, 78, 81, 99, 102, 116, 119, 143, 146, 166, 169, 183, 186 to their starting positions. Additional harmonic restraints on the Cα atoms of residues 30, 35, 40, 45, 50, 55, 60, 65, 70, 150, 153, 156, 159, belonging to the ECLs, were added to Pore II during the production phase. The use of restraints on these atoms mimics the constriction exerted on the structure by the neighboring protomers in the TJ strand, which are not included in the simulation of the single-pore systems. For each of the two system, 250 ns of standard MD simulations were performed as additional equilibration, maintaining the restraints on the Cα atoms only. The final configurations were adopted for the FE calculations.

Pore size analysis. The size of the paracellular channel was monitored along the trajectory with the HOLE program[74,75]. This algorithm maps the radius of a channel along a given axis (here, the paracellular channel was oriented along the y-axis) by fitting a spherical probe with the Van der Waals radii of the pore-lining atoms. A threshold of 10 Å was chosen to define the boundaries of the channel.

Free energy calculations. Each FE profile of the permeation events was calculated with the Umbrella Sampling (US) method[76], where a restraining potential term is added to the MD potential to confine a *collective variable* (CV) in selected regions, named *windows*, thus allowing proper sampling also of high-energy regions. Here, the CV is represented by the coordinate of the tagged permeating ion along the pore axis, which was oriented along the cartesian y-axis, and the restraining potential $V_i(y)$ in each window i is:

$$V_i(y) = \frac{1}{2}k(y - y_i^0)^2$$

where y_i^0 indicates the value in Å at which the CV is restrained in the window (called *center*) and k is a constant that is appropriately chosen in order to ensure a sufficient overlap of the CV distributions of adjacent windows (in this work, we used k = 2.0 $kcal/(mol\ \text{Å}^2)$ for all simulations). In each window, the displacement of the ion orthogonal to the pore axis is confined within a disk of radius $r_0 + \delta$, where r_0 is the pore radius as determined by the HOLE program[74,75] and δ= 2 Å. The equilibrated conformation of the system was used as starting structure of all US windows, and the ion was manually positioned at each center y_i^0**.** The Pore I channel axis was split into 60 windows spaced 1 Å from each other. After an initial minimization, 16 ns-long trajectories were carried out using the same set-up and parameters of the unbiased MD simulation. The first 1 ns was excluded from the analysis for all windows. Due to a more elongated channel, sampling the Pore II model required 75 1-Å-spaced windows. The minimization, equilibration and production procedures followed the same protocol of Pore I and up to 20 ns per window were simulated in order to achieve proper convergence of the FE profile.
The full FE profiles were obtained adopting the *weighted histogram analysis method* (WHAM)[77–79] using the code available from the Grossfield's group (http://membrane.urmc.rochester.edu/content/wham)[80], which calculates the statistical error associated to the FE estimation using the bootstrap method.

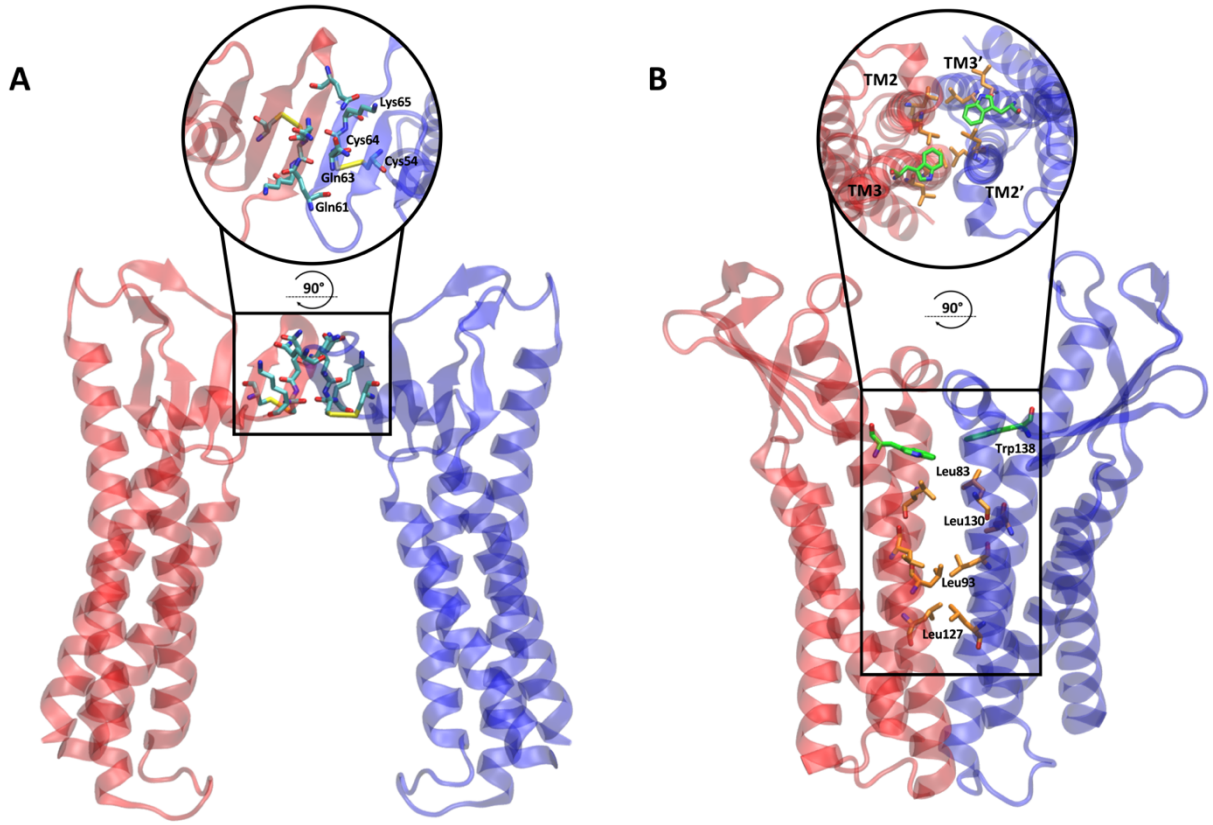

Figure 1. Dimeric cis-interfaces of the two pore models. *A) In the Pore I model, two protomers of the same cell interact at the level of their ECLs, resulting in a highly hydrophilic interface. The apical zoomed view of the interface is shown in the circle. B) In the Pore II model, two protomers of the same cell interact at the level of the TM domain giving rise to a hydrophobic interface resulting from a leucine zipper made by residues Leu83, Leu130, Leu93 and Leu127 and supported by the π - π interactions between the aromatic sidechains of the Trp138 residue. A zoomed apical view of the interface is shown in the circle.*

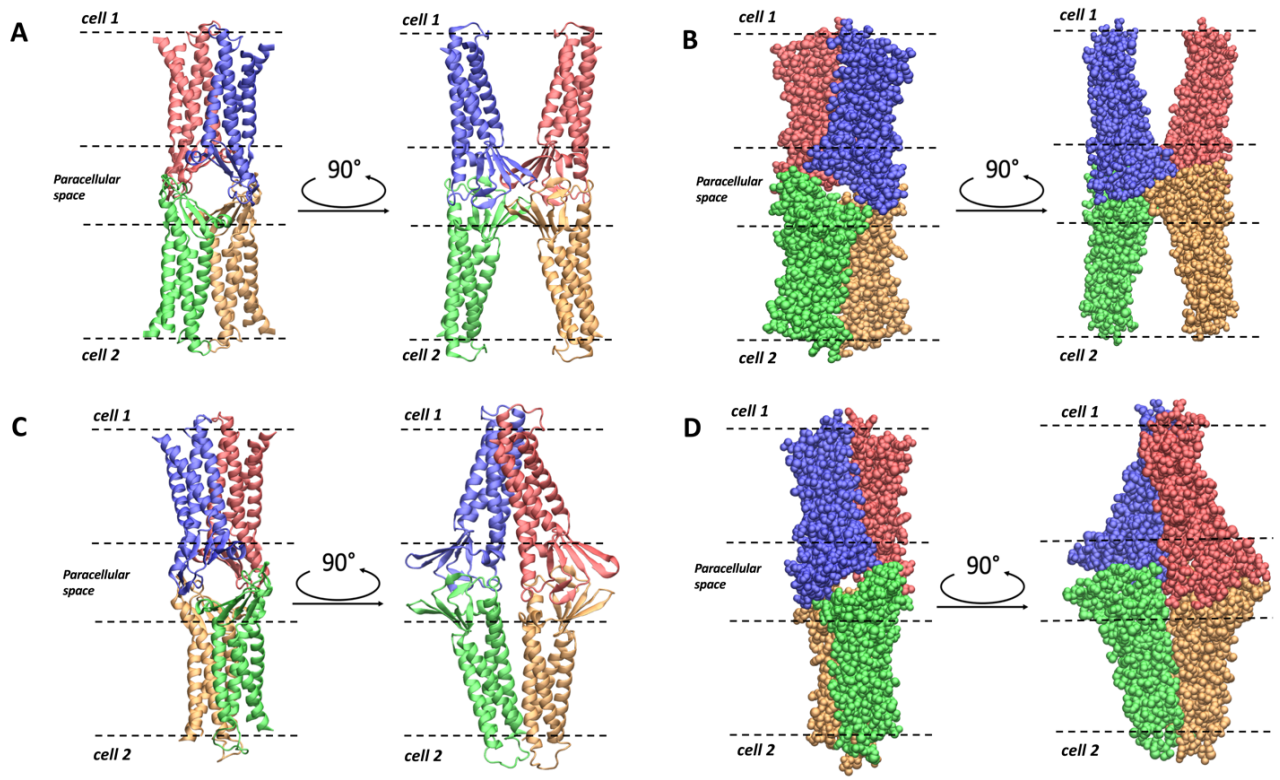

Figure 2. Representations of the Pore I (A,B) and Pore II (C,D) models. *The dashed lines identify the boundaries of the membranes of two opposing cells separated by the paracellular space. A) Ribbon representation of the Pore I in apical/basolateral and lateral view. B) Van der Waals representation of the Pore I in apical/basolateral and lateral view. C) Ribbon representation of the Pore II in apical/basolateral and lateral view. D) Van der Waals representation of the Pore II in apical/basolateral and lateral view. The four protomers of Cldn4 are distinguished by their coloring.*

RESULTS

By comparing the two Cldn4-based pore models, the location of the amino acids along the channel axis changes completely as a result of the opposite relative orientations of the monomers (**Fig. 3**). In Pore I, we observe an uncharged region in the middle of the channel, where two pairs of glutamine residues of the ECL1 loops (Gln61 and Gln63) face each other, with their sidechains pointing toward the lumen (**Fig. 3A,B**). This differs from the Pore I structure of Cldn15, characterized by the charged cage of Asp55[58] in its inner section. Moving away from the center along the axis, two pairs of Lys65 are next to the glutamines, followed by other acidic (Asp48, Asp68 and Asp146) and basic residues (Arg31 and Arg158). In Pore II, the positions of all these amino acids are inverted, with the glutamines at the channel's mouths and the charged residues towards the middle (**Fig. 3C,D**).

In our unbiased MD simulation, the Pore I model revealed a remarkable stability of the paracellular β-barrel super-secondary structure, with the sidechains of the residues lining the center mostly maintaining their relative orientation. As illustrative examples, we report the distances between the amino C-atom of Lys65 and the amide C-atoms of Gln63 and the Gln61 sidechains and between the amide C-atoms of two neighboring Gln63 sidechains in **Fig. 4**. Stationary profiles around ~5 Å and ~6 Å are obtained for the distance between the Lys65 and the Gln63 sidechains and between the Lys65 and the Gln61 sidechains, respectively. Larger fluctuations are observed for the separation between the Gln63 residues from 50 ns to 150 ns of the simulated trajectory, but stationarity around ~4 Å is obtained before and after that time window. Conversely, in the Pore II this interacting network is not found due to the location of the Gln61, Gln63, Lys65 residues of interacting monomers at opposing sides of the channel.

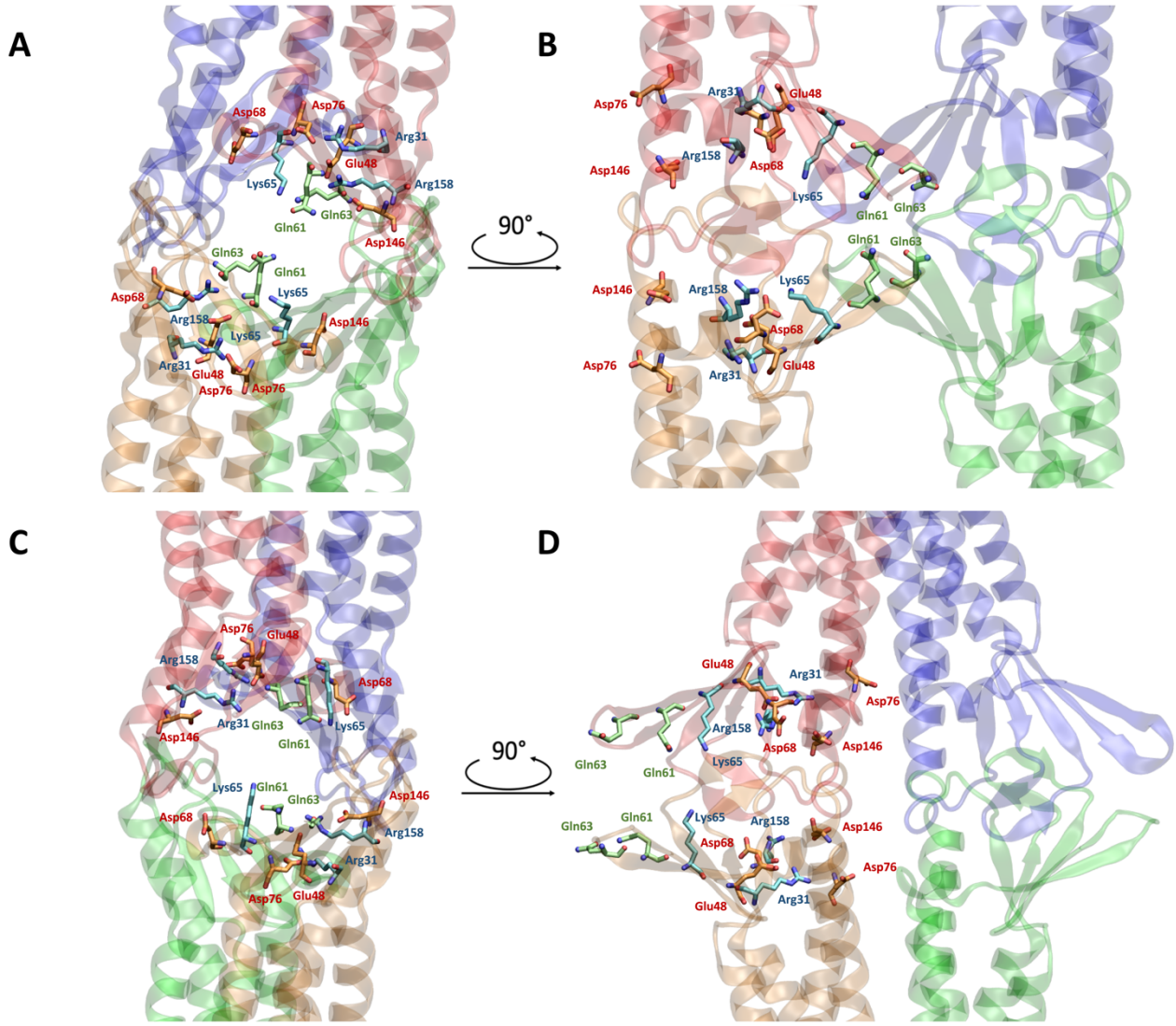

Figure 3. Representations of relevant residues in the two pore models. *A,B) Apical/basolateral (A) and lateral (B) views of the Pore I configuration. The β-barrel arranged by the ECLs of the 4 protomers is visible. C,D) Apical/basolateral (C) and lateral (D) views of the Pore II configuration. The reverse orientation of the pore-lining residue compared to the Pore I is visible. The pore-lining residues are indicated for two opposing protomers with respect to the paracellular plane. Acidic residues are depicted in orange, basic residues in light blue and neutral residues in green. Oxygen and nitrogen atoms are shown in red and blue, respectively.*

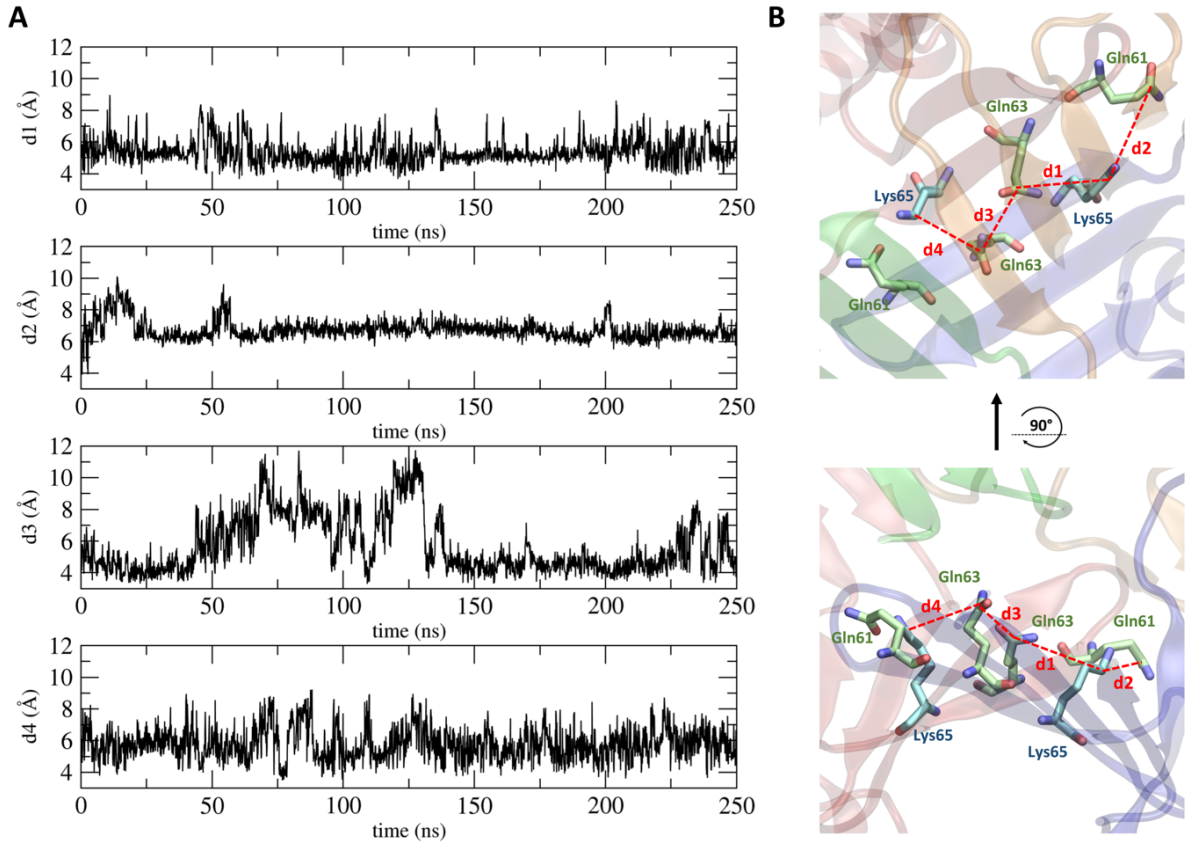

Figure 4. Distances between residues in the central region of the Pore I model. *A) Time evolution of distances between the amide C-atom of the Gln residues and the amino C-atom of the Lys residues. B) 3D representation of the computed distances.*

Free energy calculations. The physiological roles of Cldn-based TJ pores are differentiated via their capacity to form channels or barriers to the passage of ions and molecules. In this work, we investigated the permeability to water and physiological ions of the two pore structures built with Cldn4 protomers. FE profiles were calculated for both systems using the US method[76]**.** Results are shown in **Fig. 5** and **Fig. 6**, for Pore I and Pore II, respectively. To uncover the role of the pore-lining residues as pivotal determinants of the ion-selectivity, their positions along the pore axis are overlaid to the FE profiles. In the Pore I model (**Fig. 5**), a flat energy profile is obtained for the passage of water molecule. On the contrary, barriers of ~4 kcal/mol and 8 kcal/mol are observed for monovalent and divalent cations, respectively, all with a symmetrical shape with respect to the pore center, where Gln61 and Gln63 sidechains are located. In this region, surrounded by the side chains of pore-lining Lys65 pointing towards the pore lumen (**Fig. 3A,B**), the pore radius reaches a minimum value (~3 Å, **Fig. 7A**). The four positively charged sidechains are symmetrically paired with respect to the cavity center, with two residues at ~25 Å and two at ~37 Å along the channel axis. In contrast, the FE profile of the chloride is characterized by a broad flat minimum, ~2.5 kcal/mol deep, with inflection points between the Arg31 and Arg158 residues, which are located closer to the pore entrances with respect to the central glutamines (**Fig. 3A,B**).

In the Pore II structure, the FE profile for water permeation is also flat (**Fig. 6**). Conversely, all the profiles for cations show two peaks localized at the opposite entrances of the channel. Compared to the single peaks obtained for the Pore I model, smaller barriers oppose to the passage of both monovalent (~2.5-3 kcal/mol) and divalent ions (~5.5-6 kcal/mol). The two pairs of Lys65 residues are now closer to the pore boundaries (**Fig. 3C,D**), at ~15 Å and ~62 Å along the pore axis, but their position still correlates with the features of the FE profiles. Furthermore, the two energy peaks are located at the narrowest region of the channel, where the radius is at its minimum value (~2.5 Å, **Fig. 7B**). Between the two FE maxima, the profiles for the cations show minima at the

cavity center due to the presence of a wide negatively charged chamber formed by the Asp146 and Asp76 residues. In this region, the Pore II configuration exhibits its maximum pore radius (~6 Å, **Fig. 7B**) and the electrostatic environment is defined by the acidic sidechains pointing towards the lumen of the channel and located between 32 Å and 45 Å along the pore axis (**Fig. 3C,D**). The FE profile of the chloride shows a symmetrical barrier almost 5 kcal/mol high at the cavity center, correlating with the positions of the negatively charged side chains of the residues Asp76 and Asp146 (**Fig. 5**). Note that, in Pore I, the acidic residues Asp76 and Asp146 do not obstacle the chloride flux because they are positioned in a broader region of the pore, closer to the mouth, and thus have minimal interactions with the anion, whose passage is mainly driven by the inner, positively charged Arg31, Arg158 and Lys65 sidechains (**Fig. 3A,B**).

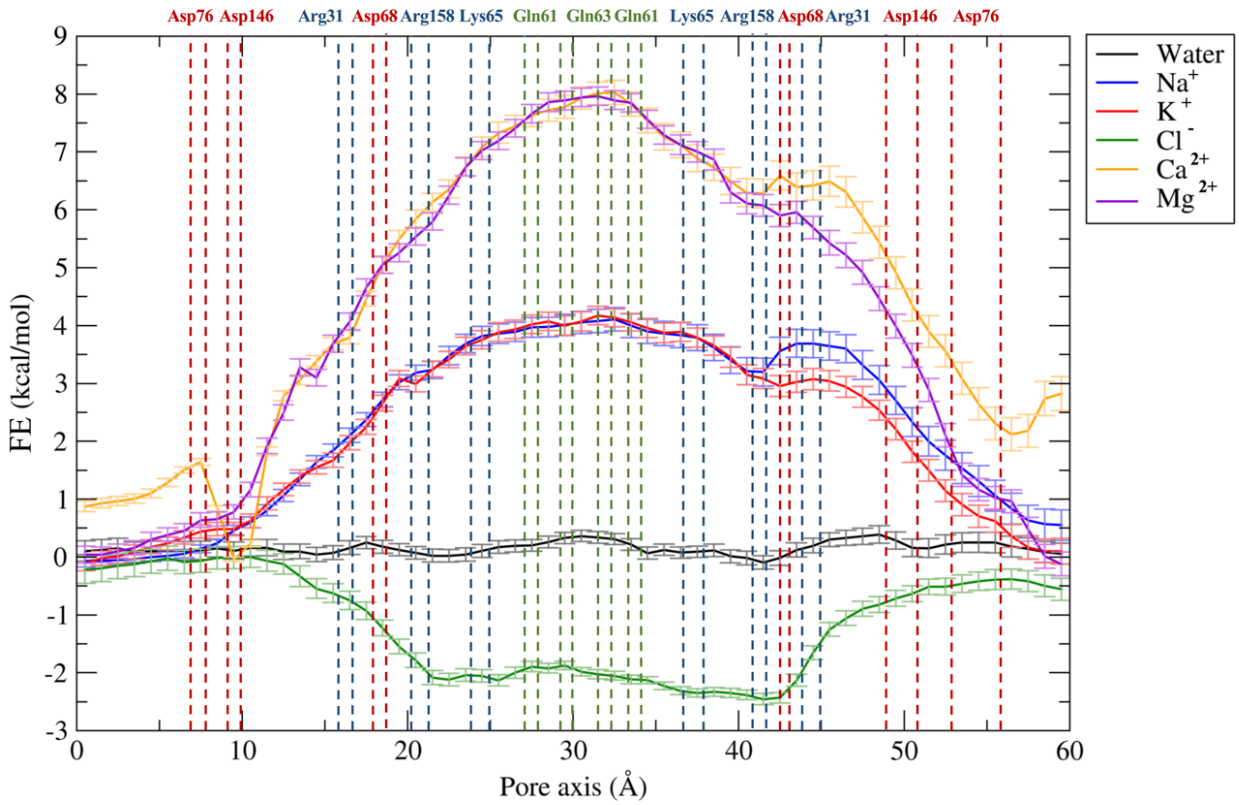

Figure 5. FE profiles for the permeation of water and ions across the Pore I model. *The position of pore-lining residues along the pore axis coordinates is indicated as dashed vertical lines. Acidic residues are colored in red, basic residues in blue and neutral residues in green.*

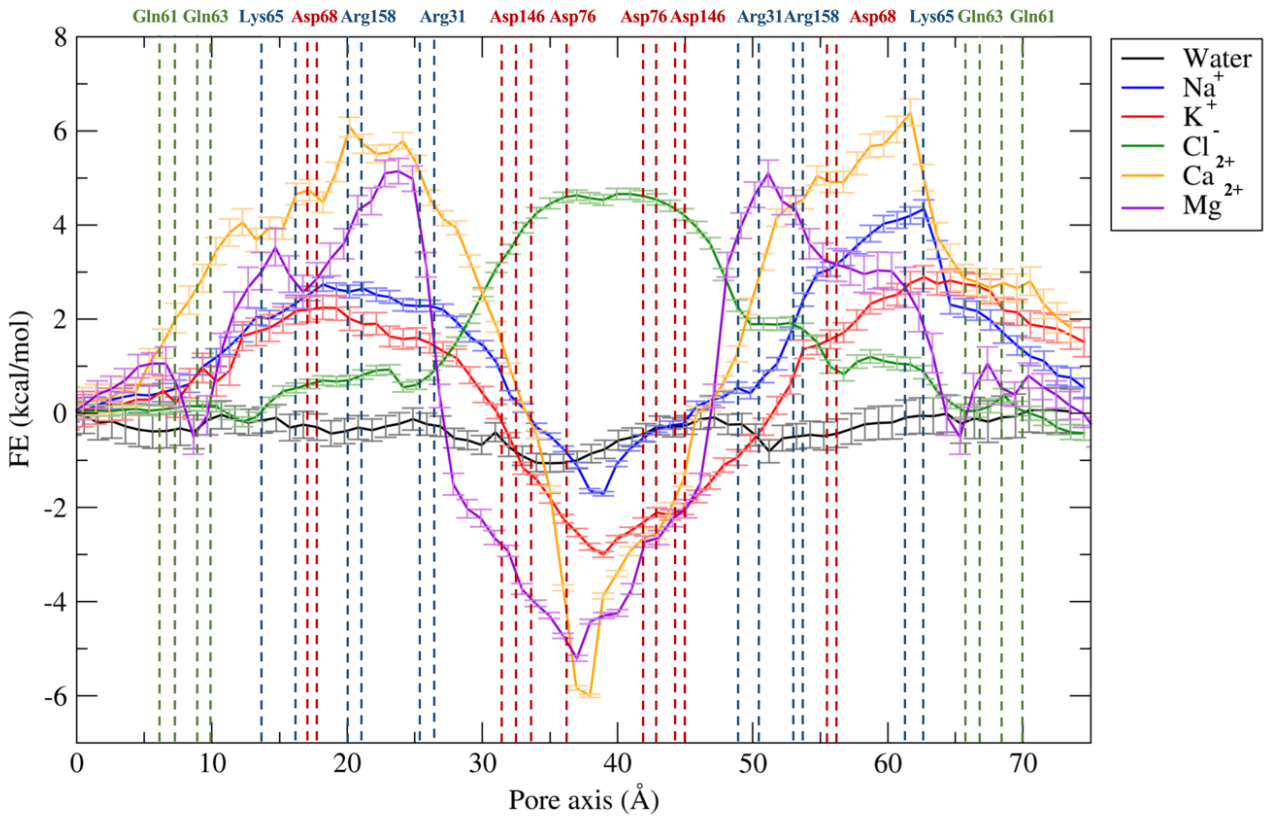

Figure 6. FE profiles for the permeation of water and ions across the Pore II model*. The position of pore-lining residues along the pore axis coordinate is indicated as dashed vertical lines. Acidic residues are colored in red, basic residues in blue and neutral residues in green.*

Hydration scheme of chloride. As a further investigation of the microscopic mechanism associated with ion permeation through the two pore models, we computed the average coordination number of chloride in each simulated US window (using a threshold radius of 3.5 Å[81–83]). Results are reported in **Fig. 7** as a function of the pore axis coordinate. It was previously suggested that the passage of ions through the narrow region of cation selective Cldn15[38,84] and Cldn2[82] pores is associated with the loss of one or more hydrating water molecules. The hydration profiles we calculated reveal fluctuations in the number of coordinating water molecules surrounding the anion due to pore size variation and contact with the charged pore-lining residues in the two pore models. In Pore I (**Fig. 7A**), whose radius varies smoothly and with limited differences in the inner region, minimal variations in the hydration sphere of chloride are observed: on average, the anion loses half water molecule in correspondence of the positions of residues Arg31, Arg158 and Lys65. The stabilizing interactions with the positively charged pore-lining residues fill the slight depletion of the solvation sphere of the ion and favor its passage through the channel of the Pore I model, consistently with the energetic minima observed in **Fig. 5**. Conversely, in the Pore II configuration, the anion undergoes a more pronounced dehydration. Chloride loses up to two water molecules in correspondence of the tight regions of the channel, where interactions with the sidechains of the positively charged residues (Lys65, Arg31, Arg158) occur. On the contrary, in the inner part of the pore, the anion is fully hydrated, consistently with the passage through the widest region of the cavity and the occurrence of unfavorable interactions with the negatively charged pore-lining residues Asp146 and Asp76 (**Fig. 7B**), responsible for the FE barrier hindering the permeation of the anion in the Pore II model (**Fig. 6**).

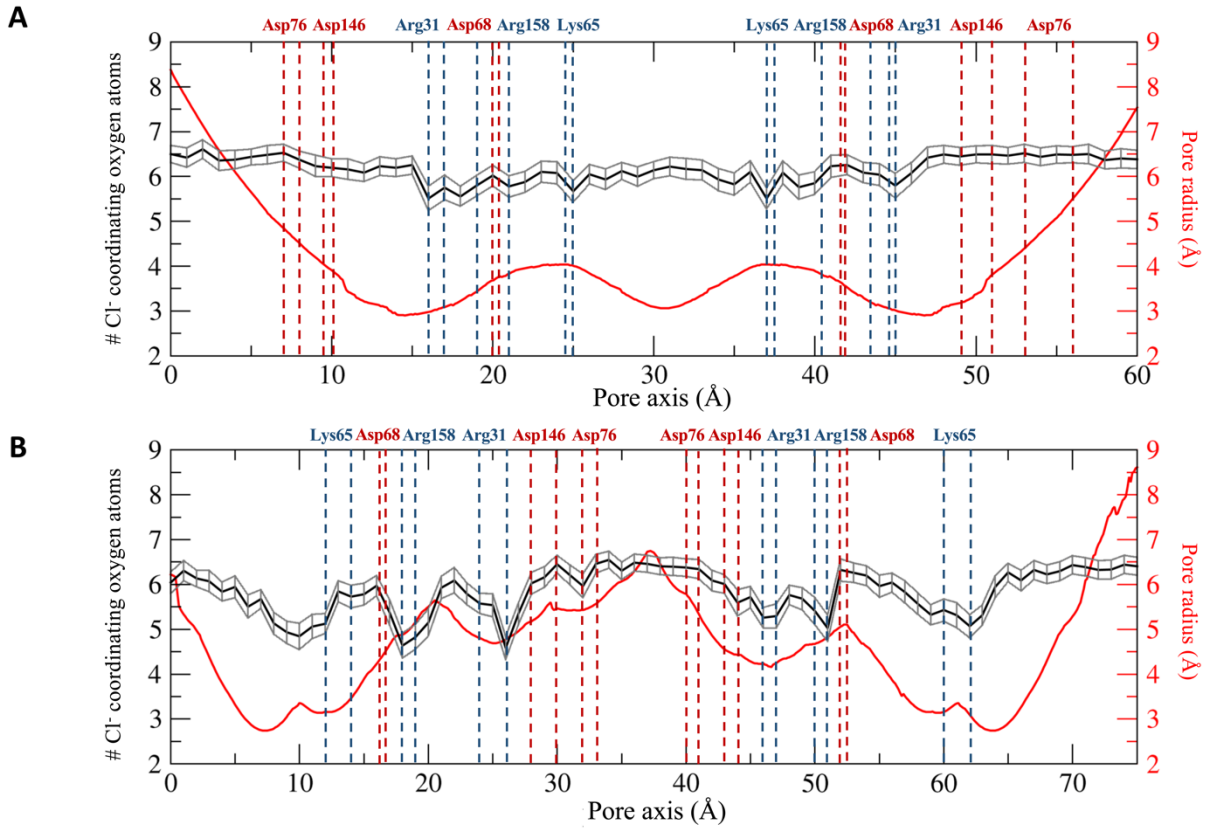

Figure 7. Hydration patterns of chloride and pore radius profile for the Pore I (A) and the Pore II (B) models. *The orientation of the pore-lining residues driving the ion selectivity is indicated. Acidic residues are shown in red and basic residues in blue.*

DISCUSSION

The study of the ionic selectivity is a pivotal task to unravel the physiological function of biological channels. While in the literature there is a consolidated state-of-art for the computational investigation of transmembrane ion channel selectivity[85–93], the study of paracellular channels is still limited to few works only[37,39,40]. In this work, we compared the features of two different paracellular pore models as putative TJ arrangements formed by Cldn4 proteins.

In both configurations, two copies of Cldn dimers, belonging to two neighboring cells, interact with each other across the paracellular space. The Pore I architecture was postulated in the work of Suzuki et al.[41], describing a homophilic strand of Cldn15-based TJs starting from the crystal structure of the isolated protomer (PDB ID: 4P79)[42]. Notably, it was also proposed independently by Irudayanathan et al.[39] for Cldns 3 and 5 from docking dimers spontaneously formed in CG MD simulations, first obtained for Cldn5[44]. This configuration was further investigated and refined in various studies for Cldn15[37,38,40,43], Cldn2[35,36] and Cldn5[36]. Conversely, the Pore II configuration was first introduced for Cldn5[39], again by docking dimers assembled in CG MD simulations[44]. Comparisons of Pore I and II conformations were also made for Cldn10b and Cldn3[46], and for Cldn2 and Cldn4[94], based only on structural models, with no refinement by extensive MD simulations. The study in Ref. 94 identified Lys65, Asp68 and Arg158 as key residues for Cldn4 anion selectivity.

Here, we used MD simulations and FE calculations to investigate the reliability of the two putative configurations as pore structures for Cldn4, and to further evaluate their transferability among different Cldn homologs in terms of preservation of tissue-specific physiological functions, governed by the non-conserved residues in the ECL1[30,95] loops.

From a structural point of view, the two models exhibit significant differences at the intracellular, *cis*-interfaces. In the Pore I architecture, two Cldn4 protomers of the same cell interact via a highly hydrophilic interface defined by residues Gln61, Gln63 and Lys65 on the ECL1 in the paracellular domain (**Fig. 1A**). Oppositely, the dimers originating the Pore II configuration are stabilized by a hydrophobic pattern over the TM domain, mainly involving a leucine zipper supported by the $\pi - \pi$ interactions provided by the Trp138 residues on the TM3 helices (**Fig. 1B**).

As previously described in Ref. 39 for Cldn5, although similar *trans*-interactions are observed in both models, they lead to opposite arrangements of the pore-lining residues. The Pore I model is characterized by a neutral central region formed by Gln61 and Gln63 pairs, surrounded by the two pairs of positively charged Lys65 residues symmetrically positioned with respect to the center. Conversely, the arrangement of the residues in the Pore II model generates an alternating charge environment with Lys65 closer to the pore mouths, and two rings of negatively charged sidechains (residues Asp76 and Asp146) in the central region. Consistent with the values proposed in Refs. 36,39 for the same pore models of other Cldns, the two configurations display a similar minimum pore radius between 2.5 and 3.0 Å (**Fig. 7**). The Pore I duct shows a smooth profile, identifying two bottlenecks symmetrically paired at 15 Å and 45 Å on the pore axis, and a central constriction with a radius of 3 Å (**Fig. 7A**). In contrast, the Pore II channel is tighter at the extremities and shows a central bulge with a radius of 6 Å (**Fig. 7B**).

In order to provide a quantitative assessments of the selectivity of the two configurations, we used the US method[76] to perform one-dimensional FE calculations for the permeation of water and several physiological ions through the channels. In the Pore I model, we observed a wide barrier to the passage of cations located at the cavity center with magnitude proportional to the ionic charge (**Fig. 5**). The barriers encountered by the cations peak in the central regions of glutamines, surrounded by the two pairs of Lys65 residues. The overlap of the FE profiles of cations with same charge indicates a major role of the electrostatic interactions with respect to the steric effects in driving the passage of ions through the channel, similarly to what previously suggested by Alberini et al.[38] for Cldn15 cation-selectivity. In contrast, chloride is attracted through the channel by a 2.5 kcal/mol deep minimum spanning a region comprised between Arg31 and Arg158, and including the two pairs of Lys65 (**Fig. 5**). Analysis of the hydration state of the anion along the permeation pathway revealed a slight dehydration in correspondence of the positively charged pore-lining residues, able to compensate for the missing water interaction (**Fig. 7A**).

On the contrary, the Pore II configuration shows barriers to the passage of all the ions. The pore-lining residues sequence highlights a possible role of the Lys65 residues in blocking the permeation of cations at the pore entrance. The barriers experienced by cations in this pore arrangement are ~1.5-2.0 kcal/mol, lower than those observed in Pore I, but symmetrically located at the two pore mouths. A pronounced minimum is found at the center of the FE profile corresponding to the disposal of two rings of Asp76 and Asp146 which generate a negatively charged chamber with a diameter of almost 12 Å. Consistently, a FE barrier of 5 kcal/mol is obtained at the cavity center for the permeation of chloride (**Fig. 6**). The hydration profile of the anion significantly varies along the cavity. In particular, the particle sheds almost 2 water molecules in correspondence of interactions between the ion and the positively charged sidechains of the residues Lys65, Arg31 and Arg158 and where the channel reveals major constrictions. Conversely, it preserves its hydration sphere in the central region of the cavity where the unfavorable contact with the negatively charged residues takes place (**Fig. 7B**) and the maximum in the FE profile is observed (**Fig. 6**). This evidence confirms the driving role of electrostatic interactions in the formation of the FE barriers to anionic permeation.

In conclusion, the two Pore models suggest opposite behaviors for the Cldn4-based TJ functionality, hence resulting mutually exclusive. Remarkably, the Pore I configuration provides the first atom-detailed model recapitulating the observed anion-selectivity of Cldn4 based TJs [23–25,27]. In contrast, the Pore II model forms a barrier to both cations and anions[28,32,95–97]. The physiological role of Cldn4 as channel or barrier remains elusive[23–28,30,32,33,95–98], and the factors modulating the expression of its functionality have not been fully clarified yet[30]. The capability to arrange in strands is also questioned due to the dependence on the cell expression system[26,30–32,98], although experimental data supporting it have been reported[99] . This notwithstanding, our FE profiles for Pore I

configuration show higher barriers to the passage of the cations than Pore II. According to these considerations, we propose Pore I as the putative configuration to recapitulate the physiological properties of anion-selective Cldn4-based TJs. Further experimental characterization of Cldn4 physiology will ultimately provide the necessary knowledge to discriminate between different predicted configurations of Cldn4-based TJs. Additional structural studies are also required to refine the peculiarities of the two architectures discussed in this work. In particular, the atom-detailed characterization of Pore II model is currently less complete than Pore I model, and fewer experimental data supporting the former arrangement are available to date[45]. Testing these models for different Cldns in terms of the reproduction of tissue-specific TJ physiology will provide a fundamental contribution to their validation.

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Author contributions

A. Berselli contributed to design the research, performed the simulations, analyzed the data, participated to the discussion for the data interpretation and wrote the manuscript. G. Alberini shared his know-how for the simulations set-up, supervised the data production and analysis, participated to the discussion and wrote the manuscript. L. Maragliano and F. Benfenati supervised the entire research, discussed the results and revised the manuscript.

Competing interests

The authors declare no competing interests.

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