
Mechanisms of inward transmembrane proton translocation

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Supplementary Table 1. Summary on the structures obtained in the course of the study.

Description	Resolution, Å	pH	Na in crystallization conditions (yes/no)	Temperature, K
Ground state	1.7	8.2	yes	100
L state	1.6	8.2	yes	100
M state	1.7	8.2	yes	100
Ground state	2.2	7.0	yes	100
M state	2.3	7.0	yes	100
Ground state	1.8	5.2	yes	100
M state	1.9	5.2	yes	100
Ground state	1.7	7.6	no	100
M state	1.98	7.6	no	100
Ground state	2.1	8.2	yes	295
250-750-μs snapshot (L state)	2.3	8.2	yes	295
7.5-15-ms snapshot (M state)	2.2	8.2	yes	295

Supplementary Table 2. Calculated X-ray doses of the *Bc*XeR datasets.

Dataset	Flux, ph/s	Energy, keV	Beam size, x/y, um/um	Maximum exposure time, s	Average dose, MGy
Ground state (pH 8.2, Na)	6.93x10 ¹¹	12.7	88/36 [#]	27	1.1
L state (pH 8.2, Na)	6.93x10 ¹¹	12.7	88/36 [#]	27	1.1
M state (pH 8.2, Na)	1.41x10 ¹²	12.7	117/90 [#]	36	0.9
Ground state (pH 7.0, Na)	1.67x10 ¹²	12.7	105/118 [#]	36	0.9
M state (pH 7.0, Na)	1.91x10 ¹²	12.7	132/108 [#]	36	0.9
Ground state (pH 5.2, Na)	1.91x10 ¹²	12.7	132/108 [#]	36	0.9
M state (pH 5.2, Na)	9.29x10 ¹¹	12.7	89/78 [#]	36	0.9
Ground state (pH 7.6, no Na)	5.89x10 ¹⁰	12.7	50/50*	180	0.8
M state (pH 7.6, no Na)	7.28x10 ¹²	12.7	178/264 [#]	27	0.9
250-750-μs snapshot (L state) / Dark state	2.0x10 ¹³	12.7	2/7*	0.001	0.40
7.5-15-ms snapshot (M state) / Dark state	2.0x10 ¹²	12.7	2/7*	0.005	0.27

[#] Top hat beam

* Full width at half maximum of the Gaussian beam

Supplementary Note 1. Proton transfer components in *BcXeR*.

Time-resolved studies of the membrane potential generation by rhodopsin reconstituted into liposomes could provide essential information on charge movement in the protein. It has been successfully used for analysis of *HsBR*, bacterial light-driven outward proton pump ESR, heliorhodopsin 48C12¹⁻³. Here, we studied the kinetics of the generation of transmembrane potential by *BcXeR*.

First, the kinetics of the generation of the membrane potential by *BcXeR* in response to the laser flash is directed in the opposite direction to that shown for *HsBR*, which corresponds to the pumping of protons not inside the proteoliposomes, but outside. This additionally shows that *BcXeR* acts as an inward proton pump. In the kinetics, 3 components of the potential increase originating from the active light-induced proton transfer through the proteoliposome membrane by *BcXeR* are resolved (15 μ s, 3.8 ms and 98 ms). The 4th negative component of the membrane discharge (250 ms) is caused by the passive leakage of ions through the membrane.

The most notable components for potential generation are those with characteristic times of 3.8 and 98 ms. Taking into account time-resolved spectroscopy data on *BcXeR*, these components could be assigned to the rise and decay of the M state, respectively. Thus, the major steps of proton translocation are associated with the M state. Likely, the 3.8 ms component corresponds to the internal proton relocation from the RSB to the PTG. Consequently, the 98 ms component corresponds to the proton release from the PTG to the cytoplasm and also to the RSB reprotonation from the extracellular bulk.

The 98 ms phase apparently reflects electrogenic events during the breakdown of the intermediate M and the return of the protein to its initial state. The ratio of phase amplitudes of 3.8 ms and 98 ms is: 1: 0.83. That is, the apparent amplitude of the slow phase in *BcXeR* is much smaller than that in *HsBR*, but it should be taken into account that the late dynamics of the *BcXeR* photocycle are relatively slow, therefore their amplitude is affected by the passive discharge of the membrane and therefore processes slower than the discharge of the membrane simply do not manifest themselves.

Notably, the amplitude of the 15 μ s component of the membrane potential generation by *BcXeR* is 1/3 of that of the 3.8 ms phase. The 15 μ s component is optically silent; however, it might correspond to the formation of the L state or to spectrally-silent transitions within the L state. We hypothesize that it can be explained by the RSB-associated proton relocation, or by the change in the dipole moment of the RSB, or by the movement of charged amino acids. However, these hypotheses should be checked experimentally.

Supplementary Note 2. Mutational analysis of *BcXeR*.

To validate the proposed mechanism of light-driven inward proton pumping by *BcXeR* and further probe the roles of the proton wires for *XeRs* functioning, we performed bioinformatic analysis of the conservativity of the key amino acid residues within the clade and also conducted functional tests of the *BcXeR* mutants. The results show that the most critical region for the inward proton pumping is the one between the RSB and R70. Alterations there (Y9A/F, S45A/T, Y49A/F/H, R70A/G/W/Y, D73N/E, S77A, P203A/D/S mutants) result in the loss of proton-pumping activity in most mutants of *BcXeR*. Amino acid residues comprising the RSB region are highly-conserved within archaeal and bacterial *XeRs*, and also ASR-like sensors, which highlights the importance of the region (Extended Data Fig. 5).

Interestingly, this is not the case of the residues surrounding the concavity at the extracellular side of the protein propagating from the bulk to R70. Namely, substitutions of the D118 and E195 (D118N, E195A, E195Q mutants) had no effect on the function of *BcXeR* (Extended Data Fig. 5). The residues are also less conserved within the clade than those of the RSB region (Extended Data Fig. 5). This result allows us to suggest that the proton translocation proceeds via exclusively water-mediated proton wire in the cavity. Thus, none of the internal residues in *BcXeR* might serve as a proton donor to the RSB, which is also supported by the studies of another bacterial *XeR*, *PoXeR*⁴. This hypothesis correlates well with the results of the time-resolved investigations of the transmembrane electric potential generation by *BcXeR*. Indeed, the signal component corresponding to the proton uptake by the protein is smaller than that corresponding to the proton translocation from the RSB to the PTG. This tentatively indicates that the distance of the proton movement through the extracellular side of the protein is shorter than that through the cytoplasmic half. In the case of direct reprotonation of the RSB from the water-filled concavity this distance is $\sim 11\text{\AA}$, which is less than the distance between the RSB and the PTG in the L state ($\sim 14\text{\AA}$). This additionally supports our suggestion of the direct RSB reprotonation from the concavity with the decay of the M state.

The organization of the cytoplasmic part of the protein is essential for the *BcXeR* functioning. Namely, the changes in the central hydrophobic gate formed mostly by L81 or reduction of the volume of the polar cavity between K207 and the PRG (L81C and A84F variants) lead to the notable drop of proton-pumping activity. At the same time, the alterations of the residues surrounding the cavity not decreasing its size (S211A/G, F210A/Y) do not affect much the pumping properties of *BcXeR* (Extended Data Fig. 5). This again evidences that the water-mediated proton wire serves as a pathway for proton translocation through the intracellular part of the rhodopsin.

The PTG and hydrogen bond network in this region is also critical for the inward proton pumping. Several alterations of the PTG (D214N, H161N mutants) result in a complete loss of the proton-pumping activity of

BcXeR, while the pumping of E34Q and D214E variants is notably reduced. The region is highly-conserved within bacterial XeRs, highlighting its importance for the proton translocation (Extended Data Fig. 5). The only exception is the H161 residue, which is less than a half of the members of the clade; therefore, it might be a specific feature providing stabilization to the PTG in *BcXeR*.

Another important region for inward proton pumping by *BcXeR* was found near the W173 residue. We identified the unusual dynamics of W173 throughout the photocycle, which we also suggest to be one of the determinants of unidirectional proton transport by the rhodopsin. Substitution of the W173 by less bulky phenylalanine (W173F variant) decreased the pumping activity of *BcXeR*; however, it was preserved at moderately high level. A surprising finding was that the D107Q mutant showed almost no proton pumping activity. Moreover, D107 is completely conserved within bacterial XeRs, but is absent in ASR-like sensors, which have glutamine at the corresponding position. Importantly, the same conservativity feature was found in the L169 residue, which is completely conserved within bacterial XeRs, but absent in ASR-like sensors. D107 and L169 are located far from the putative proton translocation pathway; however, we showed that D107 stabilizes the W173 conformation, while the L169 relocation allows for enough space for W173 in the M state of the *BcXeR* photocycle. Thus, we suggest that D107 and likely L169 are critical for the stabilization of the M state and likely for preventing the proton backflow to the RSB from the cytoplasmic side of the protein during the M-to-ground state transition. An intriguing question is whether the ASR-like sensors could be converted into effective inward proton pumps by introducing mutations in the corresponding region. Further studies of these proteins are required for answering this question.

Supplementary Note 3. Comparison of different PTG conformations.

To retrieve the protonation state of the PTG in different states of the *BcXcR* photocycle we first solved the structures of *BcXcR* in the ground and cryotrapped M states at pH 5.2, 7.0, and 8.2 in the presence of sodium in the crystallization conditions (Supplementary Table 1).

The PTG was expected to be deprotonated in the ground state at least at pH 8.2 by analogy with the closely-related inward proton pump *PoXcR*⁴. The deprotonation of the group is clearly indicated by the presence of the sodium ion bound to D214 and E34. At pH 7.0 the sodium ion in the PTG is preserved, indicating the deprotonated form of the PTG also at neutral pH. At pH 5.2, in a major fraction (~60%) of proteins within a crystal the sodium ion is dissociated and E34 is reoriented towards the cytoplasm. In the resting fraction (~40%) the conformation of the PTG is the same to that found at pH 7.0 and 8.2 (Extended Data Fig. 11). We suggest that acidification led to the partial protonation of the PTG at pH 5.2, while the resting deprotonated form of the PTG corresponds to the fraction without sodium ions. Thus, since the fraction of protein molecules corresponding to the protonated PTG is only slightly higher than 50% at pH 5.2, we assume that the pK_a of the PTG in the ground state is likely close to 5.2 in *BcXcR* crystals.

Surprisingly, the M state was formed at the same high occupancy (higher than 80%) at all tested pH values (Extended Data Fig. 10). The structure of the M state is also the same at all pH values (Extended Data Fig. 10). At pH 7.0 and 8.2 the similarity of the M states is not surprising since the ground states are also identical and all processes in the protein should occur analogously at these pH values. However, at pH 5.2, where a major fraction of the protein molecules in the ground state has the protonated PTG, the formation of the M state at the same level and with an identical structure is surprising. We suggest that at low pH values the proton released from the RSB could be transported directly to the cytoplasm while the PTG remains protonated at every step of the photocycle. The proton transfer in this case might proceed via the same H-bond proton wire to that shown in the L state at the cytoplasmic part of *BcXcR*, since the PTG is exposed directly to the cytoplasm. We cannot exclude also that at low pH values the proton might be released to the cytoplasm using a relay mechanism, i.e. it is released from the PTG and simultaneously returned to the group from the RSB with the formation of the M state. It should be noted that the conformation of the PTG in the sodium-free fraction in the ground and M states is identical. Thus, we suggest that the sodium-free conformation found in the M state of *BcXcR* at all pH values and also in the fraction of molecules in the ground state of the protein at pH 5.2 reflects the protonated form of the PTG.

Second, we solved the structures of *BcXcR* in the ground and M states at pH 7.6 without sodium in the crystallization conditions (Extended Data Fig. 10). The conformation of the PTG in the ground state in the absence of sodium even at high pH was similar to the sodium-free conformation described above (Extended

Data Fig. 10). Namely, the E34 side chain is oriented towards the cytoplasm and the original position of the sodium is occupied by an additional water molecule. Upon the M state formation under a sodium-free condition there are no structural changes in the PTG visible at 2Å resolution. However, the *BcXeR* structure in the M state in the absence of sodium is identical to that determined in the presence of sodium, indicating a proper formation of the intermediate at high occupancy. The presence of a sodium-free conformation of the PTG in the ground state of *BcXeR* at pH 7.6 suggests that this conformation might also reflect the deprotonated form of the PTG.

The latter hypothesis is also confirmed by the analysis of the *BcXeR* structure determined at room temperature at pH 8.2 in the presence of sodium using serial crystallography. Indeed, at room temperature we found a sodium-free conformation in all ground, L, and M states of the protein (Extended Data Fig. 10). Only a negligible fraction of the protein molecules contained sodium in the ground state of *BcXeR* at room temperature. Since the structures were determined at pH 8.2 in the presence of sodium, we assume that the sodium-free conformation of PTG might in fact reflect both the protonated and deprotonated forms of the PTG, which thus cannot be structurally distinguished at least at 2Å resolution. Nevertheless, we suggest that sodium binding in the PTG unequivocally identifies and stabilizes the deprotonated form of the group. The stabilization (and the corresponding sodium binding) is more pronounced at cryo temperatures, where the flexibility of the group is lowered compared to that at room temperature. Since *BcXeR* remains functional in the absence of sodium (Fig. 1A) and also we demonstrate similar M state formation by the protein irrespectively of sodium presence, we assume that sodium binding is not obligatory for the proper functioning of the XeR; however, we suggest that it might facilitate inward proton transport by stabilizing the deprotonated PTG in the ground and L states of the protein photocycle. We hope that further investigations of sodium binding in bacterial XeRs will shed light on its role in light-driven inward proton translocation.

Supplementary Note 4. Comparison of the ground state structures of *HsBR* and *BcXeR*.

The retinal binding pocket of *BcXeR* is similar to that of *HsBR* and includes many aromatic residues, such as W74, W173, Y176, W180 (analogs of W86, W182, Y185, and W189 in *HsBR*, respectively) (Extended Data Fig. 6A,B). Similar to *HsBR*, a hydrophobic gap of 8 Å formed by I41 and L81 (analogs of V49 and L93 in *HsBR*) separates the RSB from the rest of the internal cytoplasmic part of the protein¹² (Fig. 1F; Extended Data Fig. 6C,D). A π -bulge of the helix G is present in *BcXeR* near S206 resembling that found in *HsBR* near the corresponding residue A215 (Extended Data Fig. 6E). The protonated RSB is oriented towards the extracellular side and is H-bonded to water molecule Wat401 (analog of Wat402 in *HsBR*) (Fig. 1G).

At the same time, *BcXeR* has a unique and unusual organization of all other regions. In contrast to *HsBR*⁵, the RSB region of *BcXeR* contains only one water molecule (Wat401), which mediates a characteristic for XeRs H-bond network involving also S45, Y9, Y49, W74, D73, and S77 (Fig. 1G; Supplementary Fig. 1A). These residues are critical for proton translocation in *BcXeR* and are conserved within XeRs (Extended Data Fig. 5; Supplementary Note 2). D73 (analog of primary proton acceptor D85 in *HsBR*) is deprotonated as unequivocally indicated by its H-bonding to S45 and S77 (Fig. 1G; Supplementary Fig. 1A). Uniquely, the α -helical architecture of the extracellular half of the helix G of *BcXeR* is additionally disturbed at F199 induced by the proline residue P203 (analog of D212 in *HsBR*) (Supplementary Fig. 1E). As a result, the RSB region is isolated from the rest of the extracellular part of *BcXeR* (Fig. 1G; Supplementary Fig. 1A).

Closer to the extracellular bulk, a small cavity is found between Y49, H6, and R70 (analog of R82 in *HsBR*) (Fig. 1G). Unlike *HsBR*, where R82 is pointed towards the RSB in the ground state⁵, R70 is oriented towards the extracellular side (Extended Data Fig. 6C,D; Supplementary Fig. 1B,D). The interaction between H6 and R70 is mediated via a water molecule Wat402, found in two alternating conformations in the ground state of *BcXeR* (Fig. 1G; Extended Data Fig. 7C). Next, *BcXeR* has a large cavity protruding from the extracellular bulk to R70 and filled with numerous water molecules (Fig. 1F, G). These water molecules mediate a H-bond network between the amino acid residues surrounding the cavity such as R70, D118, E192, and E195 (Fig. 1G). According to our mutational analysis, R70 is essential for inward proton pumping, unlike D118, E192, and E195 (Extended Data Fig. 5; Supplementary Note 2). Thus, there is a quasi-continuous H-bond network propagating from the extracellular bulk to the RSB of *BcXeR*, which is only disrupted near Y49 (Fig. 1G).

Supplementary Note 5. Molecular mechanism of inward proton pumping.

The data presented in this work allowed us to propose a proton wire molecular mechanism of the light-driven inward proton pumping by bacterial XeRs (Fig. 4; Supplementary Video 2). Already in the ground state, an almost continuous H-bond proton wire largely mediated by internal water molecules propagates through the protein predominantly through the internal large polar cavities. This wire is interrupted by two gates: the first 8Å-long hydrophobic gate is located at the cytoplasmic side of *BcXeR* near the RSB, while the second is localized at the extracellular side near Y49. Upon the absorption of a photon, retinal isomerizes from an all-*trans* to a 13-*cis* configuration and the protonated RSB is relocated from the extracellular to the cytoplasmic side of the protein. The hydrophobic gate opens and a new water-mediated H-bond proton wire section at the place of the gate is formed in the L state connecting the RSB directly to the PTG. The gate near Y49 remains closed at this step. Upon the L-to-M state transition the RSB proton is translocated to the PTG through the preformed in the L state linear H-bond proton wire at the cytoplasmic part of the protein. After this internal proton transfer, the hydrophobic gate near the RSB is closed, preventing the backflow of the proton. The deprotonated RSB is relocated closer to the extracellular side but remains in the intermediate position. Meanwhile, the gate near Y49 at the extracellular side of the protein is weakened in the M state. Last but not least, the M state decays directly to the ground state with the retinal slowly thermally reisomerizing back to the all-*trans* configuration. The RSB is reprotonated from the water-filled concavity at the extracellular side of the protein via the continuous H-bond chain. At the same moment, the proton is released to the cytoplasm from the PTG. This ends the photocycle and the protein returns to its ground state.

Supplementary Note 6. Comparison of archaeal and bacterial XeRs.

The first structure of bacterial XeR showed notable differences between the archaeal and bacterial inward proton pumps, also suggesting differences in their molecular mechanisms of proton translocation. Nevertheless, some features of the light-driven inward proton pumping are preserved in all XeRs.

For instance, the organization of the RSB region is the same in both archaeal and bacterial proteins, also reflected in their akin spectroscopic properties (Extended Data Fig. 2). This might explain similar photocycle kinetics at the early stages of the photocycle (Extended Data Fig. 2). Indeed, we suggest that the primary photoreactions and the processes leading to the RSB reprotonation towards the cytoplasmic part proceed similarly in archaeal and bacterial XeRs. Namely, these include all-*trans* to 13-*cis* isomerization of the retinal followed by the RSB relocation to the cytoplasmic side of the proteins. Amino acid residues comprising the RSB region are highly conserved within both archaeal and bacterial XeRs, with only minor differences between the clades (Extended Data Fig. 5). This additionally supports the fundamental importance of the region for light-driven inward proton pumping and suggests similar structural rearrangements in the RSB region during the photocycle in all XeRs.

We also assume that the flip of the side chain of the hydrophobic residue, corresponding to the L81 in *BcXeR* (substituted by isoleucine in archaeal XeRs) and the subsequent opening of the hydrophobic gate at the cytoplasmic side of XeRs likely occur in both types of inward pumps, providing the formation of the H-bond proton wire between the RSB and the proton acceptor (D214 and D220 in *BcXeR* and *NsXeR*, respectively). However, such proton wires might be slightly different in archaeal and bacterial XeRs. For instance, there is a completely-conserved histidine residue (H48 in *NsXeR*) in archaeal rhodopsins located closer to the RSB (~10Å) and forming a salt bridge with a proton acceptor (D220 in *NsXeR*) (Extended Data Fig. 5). This histidine is located on the way of the putative H-bond proton wire in archaeal XeRs, predicted by the analogy with that shown for *BcXeR*. Likely, the presence of histidine shortens the proton wire at the cytoplasmic part and thus facilitates a proton release from the RSB in *NsXeR*. This hypothesis is clearly supported by the functional tests of the A38H mutant of *BcXeR*, which showed enhanced proton pumping activity compared to the wild-type protein (Extended Data Fig. 5).

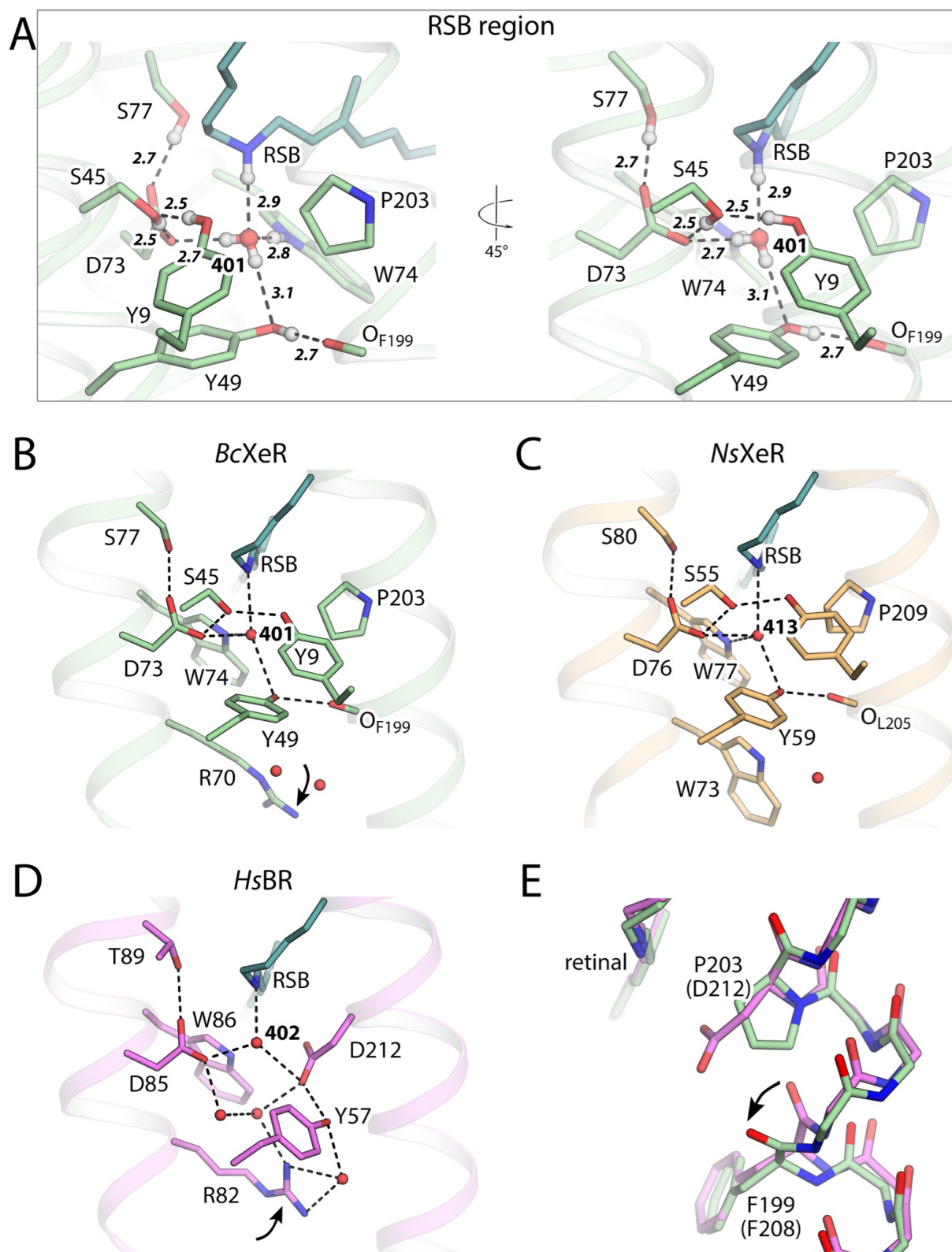
At the same moment, the processes associated with the RSB reprotonation from the extracellular side and also the proton release to the cytoplasm are likely different in bacterial and archaeal XeRs. Indeed, the organization of the extracellular part of *NsXeR* and *BcXeR* is dramatically different (Extended Data Fig. 5). Namely, this region in archaeal proteins is tighter and less polar than in bacterial ones. For instance, in *NsXeR*, there is only an H-bond wire propagating from the W73 (analog of R70 in *BcXeR*) towards the extracellular bulk in the region between helices A and G, which is completely different from bacterial XeRs. This might mean

principally different mechanisms of the RSB reprotonation in two types of IPPs. We speculate that in archaeal XeRs larger conformational changes are required to provide a pathway for the RSB reprotonation from the extracellular side, which is also reflected by the presence of a spectrally distinct M_2 state shown for *NsXeR*. Moreover, it was recently shown that the M_2 state of *NsXeR* corresponds to the protein conformation more accessible to the extracellular space.

The release of the protons from the primary proton acceptor (D214 and D220 in *BcXeR* and *NsXeR*, respectively) to the cytoplasm also proceeds in different ways in archaeal and bacterial XeRs. While in *BcXeR*, D214 and the entire PTG are exposed to the intracellular space, in *NsXeR* the D220 residue is buried inside the protein and more isolated from the inside of the cell. This might mean that some additional conformational changes occur in archaeal XeRs for proton release from the proteins. It might also be one of the reasons for the slightly different kinetics of the two types of XeRs at the late stages of their photocycles.

Thus, we suggest that both archaeal and bacterial XeRs utilize H-bond proton wires and their remodulation during their photocycles for unidirectional proton transport. The internal proton translocation from the RSB to the primary acceptor is likely similar in all XeRs; however, the RSB reprotonation/proton uptake and proton release to the cytoplasm proceed differently in archaeal and bacterial XeRs.

Supplementary Figures



Supplementary Fig. 1 | RSB region in proton-pumping microbial rhodopsins. **A.** Detailed view of the RSB region of *BcXeR*. The protons in the region could be unequivocally assigned and were manually added in PyMOL for presentation only. Protons are shown as white spheres. **B.** The RSB and R70 region of *BcXeR*. **C.** The RSB and W73 region of *NsXeR*. **D.** The RSB and R82 region of *HsBR*. The retinal cofactor is colored teal. Hydrogen bonds are shown with black dashed lines. Water molecules are shown with red spheres and the key molecules are signed with black bold numbers. Black arrows indicate the location of homologous arginines in *BcXeR* (oriented towards the extracellular space) and in *HsBR* (oriented towards the RSB region). **E.** A unique disturbance of helix G at F199 of *BcXeR* (F208 of *HsBR*) caused by P203 (D212 in *HsBR*).

BcXeR and *HsBR* are colored green and violet, respectively. The disturbance in *BcXeR* compared to *HsBR* is shown with a black arrow.

Supplementary References

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