

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 1/5 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).

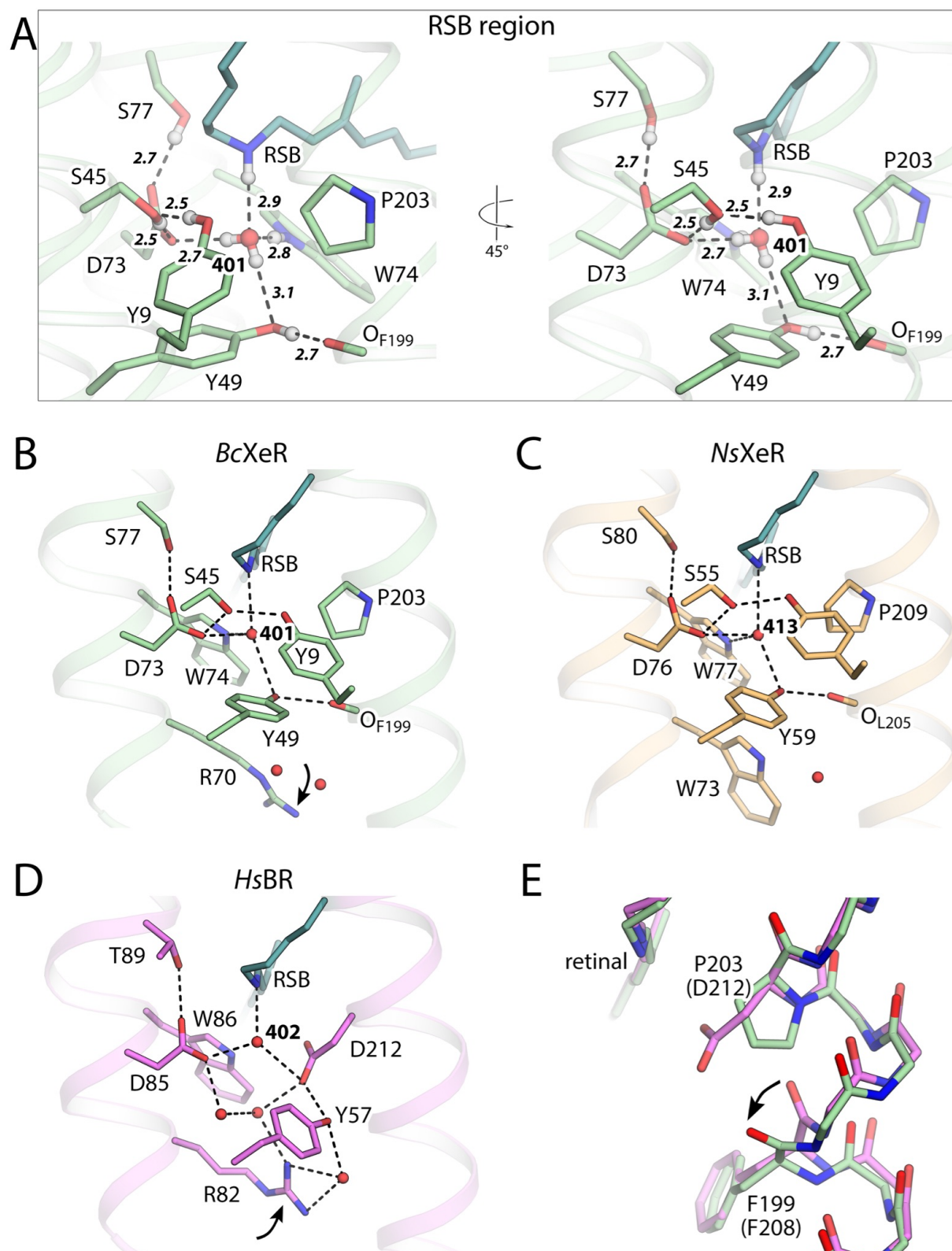
At the same moment, the processes associated with the RSB reprotonation from the extracellular side and also the proton release to the cytoplasm are likely different in bacterial and archaeal XeRs. Indeed, the organization of the extracellular part of *NsXeR* and *BcXeR* is dramatically different (Extended 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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Software and code

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Data collection MxCube2 was used for diffraction data collection

Data analysis XDS package (BUILT=20210205) for traditional crystallography data processing; CrystFEL (v 0.9.1) was used for time-resolved data processing; CCP4i program suite (v7.1.018) for structure solving; PHENIX program suite (v1.19.2-4158) for structure refinement; PyMol (v1.8.4.0) for structure visualization; Microsoft Excel (v16.58) for spectra visualization; Coot (v0.9.6); hmmer (v3.3.2) for bioinformatic analysis; iTol v6 online server for phylogenetic tree generation; Wolfram Mathematica (v11) for bioinformatic analysis; Jupyter Notebook (v5.2.2) for bioinformatic analysis; Python (v3.6.9) for bioinformatic analysis; refmac5 (v5.8.0267) for structure refinement; RADDOS (v4.0.1020) was used for calculation of the radiation dose per crystal.

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- A description of any restrictions on data availability
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Atomic models built using X-ray crystallography data have been deposited in the RCSB Protein Data Bank with PDB codes 7ZMY (for the ground state at pH 8.2 at 100K), 7ZN3 (for the L state at pH 8.2 at 100K), 7ZN0 (for the M state at pH 8.2 at 100K), 7ZN8 (for the ground state at pH 7.0 at 100K), 7ZN9 (for the M state at pH 7.0 at 100K), 7ZNA (for the ground state at pH 5.2 at 100K), 7ZNB (for the M state at pH 5.2 at 100K), 7ZNC (for the ground state at pH 7.6 at 100K in the absence of sodium), 7ZND (for the M state at pH 7.6 at 100K in the absence of sodium), 7ZNE (for the ground state at pH 8.2 at 293K with 7.5-ms-long exposure), 7ZNI (for the 7.5-15-ms snapshot at pH 8.2 at 293K), 7ZNG (for the ground state at pH 8.2 at 293K with 500- μ s-long exposure), and 7ZNH (for the 250-750- μ s snapshot at pH 8.2 at 293K). Extrapolated structure factors and phases for the intermediate states of BcXer are available from Zenodo (10.5281/zenodo.7612803). Publicly available in RCSB Protein Data Bank structures of NsXer (PDB ID: 6EYU) and HsBR (PDB IDs: 5ZIL, 7ZOD, 7ZOE, 6RPH) were used for analysis.

Human research participants

Policy information about [studies involving human research participants and Sex and Gender in Research](#).

Reporting on sex and gender

n/a

Population characteristics

n/a

Recruitment

n/a

Ethics oversight

n/a

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

☒ Life sciences

☐ Behavioural & social sciences

☐ Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see [nature.com/documents/nr-reporting-summary-flat.pdf](https://www.nature.com/documents/nr-reporting-summary-flat.pdf)

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size

We did not use statistical methods to predetermine sample size. In order to guarantee reproducibility of the X-ray data the cryotrapping procedures were repeated 3-5 times with independent crystals on different days. The determined coordinates appeared identical; therefore larger sample size were not required.

Data exclusions

No data were excluded.

Replication

The cryotrapping procedures were repeated no less than 5 times for each of the intermediate states. At least two datasets were collected for each functional state of the BcXer photocycle and only the highest-resolution/highest quality data is presented in the manuscript. All attempts were successful and the resulting structures of all functional states of BcXer of all different attempts coincide. The functional tests of protein activity in E.coli cell suspension were repeated 3 times for each condition and showed similar results. Spectroscopy data (including the time-resolved data) were measured 2 times independently on different days and showed identical protein kinetics.

Randomization

The samples were not allocated into different groups, therefore the randomization was not required.

Blinding

No blinding was applied. Data collection and data processing and analysis were not done blinded, because the observed effects were apparent in the experiments and therefore revealed the conditions.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

n/a	Involved in the study
☒	☐ Antibodies
☒	☐ Eukaryotic cell lines
☒	☐ Palaeontology and archaeology
☒	☐ Animals and other organisms
☒	☐ Clinical data
☒	☐ Dual use research of concern

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☒	☐ Flow cytometry
☒	☐ MRI-based neuroimaging

Peer Review Information

Journal: Structural & Molecular Biology

Manuscript Title: Mechanisms of inward transmembrane proton translocation

Corresponding author name(s): Valentin Gordeliy

Reviewer Comments & Decisions:

Decision Letter, initial version:
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Message: 7th Oct 2022

Dear Dr. Gordeliy,

Thank you again for submitting your manuscript "Mechanisms of inward transmembrane proton translocation". I apologize for the delay in responding, which resulted from the difficulty in obtaining suitable referee reports. Nevertheless, we now have comments (below) from the 3 reviewers who evaluated your paper. In light of those reports, we remain interested in your study and would like to see your response to the comments of the referees, in the form of a revised manuscript.

You will see that referee 2 raised major concerns about the discrepancy between the TR and cryo L-state in terms of sodium versus water binding and the overall support for the proposed binding. The referee proposed a more detailed comparison between the time resolved structures and corresponding cryo-trapped structures. Referee 3 raised major concerns about the structural settings and time resolution. Please be sure to address/respond to all concerns of the referees in full in a point-by-point response and highlight all changes in the revised manuscript text file. If you have comments that are intended for editors only, please include those in a separate cover letter.

Referee 3 noted the lack of PDZ and MTZ files. We would like to run the files past the referee so please send these files by E-mail as soon as possible.

We are committed to providing a fair and constructive peer-review process. Do not hesitate to contact us if there are specific requests from the reviewers that you believe are technically impossible or unlikely to yield a meaningful outcome.

We expect to see your revised manuscript within 6 weeks. If you cannot send it within this time, please contact us to discuss an extension; we would still consider your revision, provided that no similar work has been accepted for publication at NSMB or published elsewhere.

As you already know, we put great emphasis on ensuring that the methods and statistics reported in our papers are correct and accurate. As such, if there are any changes that should be reported, please submit an updated version of the Reporting Summary along with your revision.

Please follow the links below to download these files:

Reporting Summary:

<https://www.nature.com/documents/nr-reporting-summary.pdf>

Please note that the form is a dynamic 'smart pdf' and must therefore be downloaded and completed in Adobe Reader.

When submitting the revised version of your manuscript, please pay close attention to our [href="https://www.nature.com/nature-portfolio/editorial-policies/image-integrity">Digital Image Integrity Guidelines.](https://www.nature.com/nature-portfolio/editorial-policies/image-integrity) and to the following points below:

- that unprocessed scans are clearly labelled and match the gels and western blots presented in figures.
- that control panels for gels and western blots are appropriately described as loading on sample processing controls
- all images in the paper are checked for duplication of panels and for splicing of gel lanes.

Finally, please ensure that you retain unprocessed data and metadata files after publication, ideally archiving data in perpetuity, as these may be requested during the peer review and production process or after publication if any issues arise.

If there are additional or modified structures presented in the final revision, please submit the corresponding PDB validation reports.

Please note that all key data shown in the main figures as cropped gels or blots should be presented in uncropped form, with molecular weight markers. These data can be aggregated into a single supplementary figure item. While these data can be displayed in a relatively informal style, they must refer back to the relevant figures. These data should be submitted with the final revision, as source data, prior to acceptance, but you may want to start putting it together at this point.

SOURCE DATA: we urge authors to provide, in tabular form, the data underlying the graphical representations used in figures. This is to further increase transparency in data reporting, as detailed in this editorial (<http://www.nature.com/nsmb/journal/v22/n10/full/nsmb.3110.html>). Spreadsheets can be submitted in excel format. Only one (1) file per figure is permitted; thus, for multi-paneled figures, the source data for each panel should be clearly labeled in the Excel file; alternately the data can be provided as multiple, clearly labeled sheets in an Excel file. When submitting files, the title field should indicate which figure the source data pertains to. We encourage our authors to provide source data at the revision stage, so that they are part of the peer-review process.

Data availability: this journal strongly supports public availability of data. All data used in accepted papers should be available via a public data repository, or alternatively, as Supplementary Information. If data can only be shared on request, please explain why in your Data Availability Statement, and also in the correspondence with your editor. Please note that for some data types, deposition in a public repository is mandatory - more information on our data deposition policies and available repositories can be found below: <https://www.nature.com/nature-research/editorial-policies/reporting-standards#availability-of-data>

We require deposition of coordinates (and, in the case of crystal structures, structure factors) into the Protein Data Bank with the designation of immediate release upon publication (HPUB). Electron microscopy-derived density maps and coordinate data must be deposited in EMDB and released upon publication. Deposition and immediate release of NMR chemical shift assignments are highly encouraged. Deposition of deep sequencing and microarray data is mandatory, and the datasets must be released prior to or upon publication. To avoid delays in publication, dataset accession numbers must be supplied with the final accepted manuscript and appropriate release dates must be indicated at the galley proof stage.

While we encourage the use of color in preparing figures, please note that this will incur a charge to partially defray the cost of printing. Information about color charges can be found at <http://www.nature.com/nsmb/authors/submit/index.html#costs>

Nature Structural & Molecular Biology is committed to improving transparency in authorship. As part of our efforts in this direction, we are now requesting that all authors identified as 'corresponding author' on published papers create and link their Open Researcher and Contributor Identifier (ORCID) with their account on the Manuscript Tracking System (MTS), prior to acceptance. This applies to primary research papers only. ORCID helps the scientific community achieve unambiguous attribution of all scholarly contributions. You can create and link your ORCID from the home page of the MTS by clicking on 'Modify my Springer Nature account'. For more information please visit <http://www.springernature.com/orcid>.

Please use the link below to submit your revised manuscript and related files:

[Redacted]

Note: This URL links to your confidential home page and associated information about manuscripts you may have submitted, or that you are reviewing for us. If you wish to forward this email to co-authors, please delete the link to your homepage.

We look forward to seeing the revised manuscript and thank you for the opportunity to review your work.

Sincerely,

Carolina Perdigoto, PhD
Chief Editor
Nature Structural & Molecular Biology
orcid.org/0000-0002-5783-7106

Reviewers' Comments:

Reviewer #1:

Remarks to the Author:

Microbial rhodopsin is a superfamily of a membrane protein using retinal pigment to capture visible light and uses its energy to transport various ions across the cell membrane. While the mechanism of outward proton pumping microbial rhodopsin, like bacteriorhodopsin, was revealed in great detail, the knowledge for inward proton pumping microbial rhodopsin, which was discovered recently, is quite small, especially due to the lack of structural information of the photointermediates. In this work, the authors studied the structural change of a new inward proton pumping rhodopsin, BcXeR, during the photocycle by two different methods: cryo-trapping and time-resolved serial crystallography. The quality of the obtained structures of the L and M intermediates is quite high, allowing them to precisely observe the structural change along with the proton transporting process, which is very unique to this protein and distinct from that of outward proton pump. The assignments of the photointermediates and proton transfer steps were also confirmed by time-resolved spectroscopy and time-resolved electrometry, respectively, further substantiating their model created based on the structural insights. Since their results and findings are outstanding and reveal the mechanism of inward proton pumping by rhodopsins in unprecedented detail, I am confident that this article will attract the attention of broad readers and potentially deserve to be published in this journal. I raise, however, several scientific concerns as below, which need to be addressed by the author.

Major points

Line 341-342

"We suggest that the proton translocation through the gated proton wire in the extracellular side of the protein is the time-limiting step of the inward proton transport process"

If the proton transfer from the extracellular bulk phase through the proton wire is the time-limiting step, the rate of reprotonation of the retinal (= the decay of the M) should be proportional to the concentration of proton in the solvent. This is not the case, however, for another XR (PoXeR, Inoue et al. Nat. Commun. 2016) and another process (e.g. the thermal isomerization of the retinal) was proposed to be the time-limiting step of the M decay. In order to clarify which is the time-limiting process, proton uptake or another structural change, the pH dependence of the M decay should be measured.

Minor points

Line 205-207

"The L state structures determined with both methods are similar and demonstrate the same structural rearrangements associated with the intermediate state formation"

Why the residues having exhibited structural change shown in Fig 2B are different between cryotrapping and TR (e.g. F210, S206, L136 were not shown in TR) despite the high similarity of the two L-intermediate structures determined by different methods? Figure 2B looks to demonstrate the structural change becomes smaller for TR than for

cryotrapping

Line 365-368

"Moreover, the region near W173 is conserved within bacterial XeRs, but differs from that in ASR-like sensors which lack the proton-pumping activity²². Importantly, this is a noteworthy difference between the internal organization of bacterial XeRs and ASR-like sensors (Extended Data Fig. 7)."

It is unclear which part of protein they are discussing in this part. Is it the difference between D and Q at 107th position, which is shown in the alignment in Extended Data Fig. 7? A more specific explanation would be helpful to understand what they want to propose.

Figure 4

Since the flipping of W173, L136, L169, and possibly the transient hydrogen bonding of D107 are very unique to inward proton pump, I would like to propose including them in the model of Fig. 4.

Extended Data Fig. 1, legend

Italicize "*Anabaena*" and "*E. coli*"

Supplementary Information, Line 41-45

"Notably, the amplitude of the 15 μ s component of the membrane potential generation by BcXeR..." and "...15 μ s component corresponds to the RSB and the RSB-associated proton relocation towards the cytoplasmic side following the retinal isomerization shown in the L state."

How much is the time resolution of the measurement? Can the 15 μ s component be exclusively assigned to the proton relocation towards the cytoplasmic side on the L intermediate? Is there no possibility that this component is derived from a change in the dipole moment of the Schiff base following the isomerization of the retinal or the change in the orientation of amino acid side chain(s)?

Supplementary Information, Line 93

"D107 and L169 are located far from the putative proton translocation pathway..."

The position of L169 is not clear in Extended Data Fig. 7A.

Reviewer #2:

Remarks to the Author:

The manuscript reports high-resolution structures of inward proton pumping rhodopsin. Novel structures of intermediate L and M states were cryo-trapped and extensively discussed. Authors also report time resolved crystallography data recorded at a synchrotron source, including submillisecond time delay which an impressive achievement. Importantly, the manuscript provides an exciting insight into water rearrangement in the L

state accompanied by Trp rotation. It gives basis to explain the early proton transport pathway. The water chain formed in the L state (cryo-trapped structure) and explaining the likely proton transfer pathway is a beautiful result addressing one of the important questions in rhodopsin field!

Half of the results are referring to structures that bind sodium or not bind sodium.

However, sadly, there is no supporting evidence mentioned or discussion in the manuscript for assigning the sodium ion binding in dark and L state. Furthermore, when sodium is not bound the position is occupied by water molecule according to authors (M state). However, again there is also no mention of supporting evidence for assigning water molecule in the same position (where was sodium in the resting and L states) while calling it a sodium-free structure. Then authors refer to this sodium-free structure repeatedly, even the sodium disappearance being called a signature. At the same time completely disregarding the reasons for distinguishing between sodium and water, which display identical electron densities in X-ray data owing to the same number of electrons. In case the authors meant to propose hypotheses in different parts of the manuscript that are still lacking support then the manuscript should be adjusted accordingly to reflect that these are yet unsupported hypotheses. Similarly, the discussion regarding the PRG or the extracellular gate perhaps needs to be downplayed as a hypothesis unless can be better supported.

Equally important issue is with the impressive submillisecond time resolution for L state, which however seems to give only very poor Fo-Fo maps and then discussion of TR data (especially for L state) is almost completely missing. This being a great achievement needs to be supported by data and discussion or not claimed by the authors. To address it, for TR L state data show the extrapolated maps of the evolving features to provide support and rescue the poor impression from the very weak TR L state Fo-Fo maps presented. Compare the agreement between the TR and cryo-trapped data (in terms of electron density maps and models). The discussed new features are supported (and highlighted in figures) by electron densities coming from the cryotrapped data and not from TR data. If these features are not present in the electron densities derived from the TR data (especially for the L state) then what was the reason for the authors to conclude that the TR experiment at the submillisecond resolution (L state) was at all successful? Please include corresponding reasoning and discussion in the main text of the manuscript and show supporting data in the extended figures. Overall, the reported time-resolved structures and the corresponding cryo-trapped structures on similar crystals could provide an opportunity for a very interesting comparison, which is here only very brief.

This reviewer is excited about the described novel insights into the inward proton pumping rhodopsin and the development in time-resolved crystallography at a synchrotron.

However, several additional extended figures showing electron densities and explanations (as mentioned in the points below) are required to be able to judge if some of the authors' claims/TR intermediates are supported by the data. The above and the following issues need to be resolved before the manuscript can be recommended for publication:

1) Lines 52-61 Discussion is interesting but optional and not needed for the manuscript unless referred in results with specific optogenetic engineering improvements towards use in the optogenetics. Especially if the reference 6 (line 51) already refers to the potential advantages of the IPPs.

2) General comment: The reviewer misses discussion or reasoning or advantage of using 7.5 ms time resolution for the slower dynamics while for the fastest timepoint it was possible to achieve 500 μ s temporal resolution (at least according to the authors). It is not to undermine in any way the slower TR data. A short mention in the text if it was due to some technical challenges or otherwise, will address this reviewer's curiosity and likely this of the readers.

3) Lines 218-219 "we assume that these past-M state transitions are also structurally-silent (at least at 2.2 Å resolution)." Please clarify in the text or rephrase what is meant by the "structurally silent". Does it refer to suspicion that there is in fact no structural changes large enough to discern at 2.2 Å resolution, or rather that the protein is out-of-sync at the late time delays and the populations of each rearrangement (even if all characterized by the spectroscopic M state) might be very low and thus not detectable in the crystallographic data? In the latter case enriching/trapping could still provide insights in the future. Otherwise, if past-M state transitions are spectrally and structurally silent then perhaps they do not exist at all?

4) Fig2B: a) In the L state why there is water modelled in the FoFo positive density in the cryotrapped structure but not in the corresponding positive density in the TR L state structure?

b) Why is this map so unusually weak? There is some correspondence of the L state TR Fo-Fo peaks to the cryotrapped L state but by itself it does not convince that we can obtain useful 500 μs resolution data from the synchrotron (for the first time). So importantly, does the 2Fo-Fc or 2Fextrapol-Fc maps support the L state TR structural changes and in particular these that are present only in the L state? Show in extended figures. The determined active L state population is relatively high even for the TR data so it should not be the reason for poor FoFo map.

5) Extended Data Figure 4 panel E. Shows beautifully the appearance of the three critical water molecules in the L state. Beautiful result! It is not explicitly said in the figure caption but I assume that the density corresponds to the cryo-trapped data (only mentioned in methods; add in the figure caption that it is cryo-trapped data). Since it is a major result, please prepare Extended figure comparing electron densities this region to the TR L state and to the following M states to see the evolution of these 3 new water molecules. Importantly, seeing the TR L state waters in this region will let us judge if the very weak sub-millisecond data set provided useful information or was simply too weak/noisy to be useful for anything (now in Methods I find that the water molecules are not present in TR data; find some other L state unique features to justify that the microsecond TR experiment was successful/useful and show electron densities in an extended figure).

6) Extended Data Fig. 4 Specify if the densities in panels come from cryo-trapped dataset or TR (please specify in all figures throughout manuscript the source of presented data, TR vs. cryo-trapped). If all are cryo-trapped, then prepare also the same panels using the TR data to judge its quality.

7) Extended Data Fig. 8 Address the missing reference to structures (articles or at least PDB codes) which are used for the figures, otherwise it is hard to guess what is being compared in the HsBR.

8) Line 257-258 "Notably, the same structural rearrangements and transiently-formed linear H-bond chain were found in the L state of HsBR" Did the authors mean that the L state water chain in this region is similar to the late photocycle in HsBR? Not L state of HsBR but late state when the RSB is being recharged. Please correct it! In any case very interesting observation and comparison, which provokes numerous other exciting questions.

9) Line 263-264 Any reason that here the Trp flips upon isomerization while in the HsBR it only shifts? Is there less space to the shift or otherwise? Could this be the key to why the protein pumps protons inwards and not outwards?

10) Line 306-307 Mention in the text how/why sodium was assigned and not water. Especially, that presence of sodium leads to discussion on the protonation state of the nearby residue.

11)Line 308-309 "This conformation is present in the ground and L states of BcXer at cryo conditions..." Is this conformation present only in the cryo conditions and not in the TR data of the L state? Elaborate on this in the manuscript.

12)Line 315-316 "The position of the sodium ion is occupied by an additional water molecule..."

So now in the position of sodium is assigned water and it is discussed that sodium disappeared. It needs to be clarified in the manuscript how the authors justify assigning to the density in the same location at different stages sodium or water!

13)Line 323-324 "Nevertheless, the sodium dissociation from the PTG upon the L-to-M state transition is a reliable signature of the proton transfer to the group at this stage of the photocycle" As above, it is unknown to this reviewer the basis on which the sodium and water was distinguished at different stages so it is hard to agree this being a "reliable signature" of anything.

14)Line 327-328 "Thus, the proton released from the RSB is transferred to PTG through the proton wire and stored at the PTG in the M state." Do the authors have any reason for thinking that the proton is stored in PTG in the M state? Is so, please include the reasoning in the manuscript otherwise remove the statement. Especially considering that the "The sodium-free conformation of PTG remains unmodified suggesting that this conformation reflects both deprotonated and protonated forms, which, however, cannot be structurally distinguished, at least at 2 Å resolution"(Line 319-321) . So the proton could well be already transferred to the bulk solvent at this stage instead of being stored at the PTG. As before, the reasons for assigning "sodium-free conformation are also missing". So, now discussion builds upon layers of unsupported statements or at least omitting explanation of the supporting reasoning.

15)Line 332-334 "Namely, while one water molecule Wat402 is found in a double conformation in the ground and L states, indicating its weak interaction with Y49 in only half of the protein molecules, two separate water molecules are well-resolved in the M state" Prepare extended figure supporting that these two water electron densities can only be explained by 0.5 occupancies in the resting and L state and that in the M state can be explained only by 1.0 occupancies. One way could be by showing Fo-Fc densities after a cycle of refinement with 0.5 and 1.0 occupancies for the two waters and for all these data sets. In addition, refer to refinement B factors of these water molecules in extended figure caption.

16)342 – 346 "Although the development of the enhanced Xer-based optogenetic tools is beyond the scope of this work, our data clearly show that the extracellular gate should be optimized in order to accelerate the proton translocation by the rhodopsin; moreover, our primary mutational analysis also suggests that single point mutations might not be sufficient for this (Fig. S7D)."

a) Firstly, because authors do not address development of optogenetic tool then the extensive introduction on the potential of this protein towards optogenetics is unnecessary and a simple statement that there might be potential for optogenetics is sufficient, with the reference to the earlier publication.

b) More importantly, authors do not seem to have any basis for claiming presence of extracellular gate based on their structural data as there is no evolution throughout photocycle on the extracellular side and surely none that would allow suspecting closing or opening of any potential gate for proton transport. If authors mean the change in occupancies of two water molecules it still does not seem to be sufficient to justify non-conductive and conductive states at the extracellular side (why would they not be able to conduct proton when at 0.5 occupancy? Might just be 50% slower in the protein sample?). In addition, as before, authors need to support the change in occupancy of waters with their data e.g., in the form of electron densities in extended figure (see comment to lines

332-334).

17)Line 429-430: "in particular, we propose that F1F0-ATP synthase is using a similar mechanism to drive ATP synthesis." Propose based on what ? Is there any reason to think that the mechanism maybe similar? If so, include here otherwise remove.

18)Line 431-432: "Finally, this work extends the time range of X-ray synchrotron time-resolved crystallography to hundreds of microseconds, which is by an order of magnitude shorter than reported earlier¹⁶." While it would be exciting, unfortunately, the poor Fo-Fo difference map does not show that the L intermediate was successfully captured in the microseconds. Perhaps authors are able to support this claim by showing extrapolated density maps and comparing the new features captured in the cryo-trapped states with TR data (such discussion seems to be avoided throughout the manuscript).

19)Line 533-534: "were obtained with a protein concentration of 20 mg/ml and 0.8 M Na/K-Pi pH 8.2 for the sodium-containing and 1.2 M (NH₄)₃PO₄ pH 7.6 for the sodium-free conditions as precipitant solutions, respectively. " Clarify which conditions were used for which structures. Was the claim of sodium moving away from its binding site in the M state based on sodium-free crystallization conditions?? I hope not but, in any case, add this information to the text.

20)Line 548-549 Expand on "Then, the mesophase with crystals was transferred to 100- μ l Hamilton syringes also using centrifugation." How exactly was the mesophase transferred from tubes to syringes to syringes using the centrifugation. Handling of the viscous mesophase is always problematic and without detailed description it may not be possible to reproduce.

21)Line 628 -631: "After several iterations of manual and automated refinement, some peaks at the Fo-Fc difference electron density maps corresponding to a small amount of the L state appeared near residues W173, L136, and S206; therefore, these residues were fitted with triple conformation in the final model of the M state, where conformations C and D correspond to the M and L states, respectively."

Prepare extended figures to show if the double and triple conformations are justified by 2Fo-Fc electron densities. If they are not, then prepare extended figures with 2Fextrapol-Fc to show if the conformations are justified by the electron density. In addition to raw data please deposit extrapolated amplitudes so that community can easily reproduce the maps for analysis.

22)Line 709-711: "The additional water molecules appearing in the L state but not seen in the time-resolved data were removed from the starting model. The final occupancy of the L state in the case of time-resolved serial crystallography data was 20%." Lack of information on some of the most interesting changes in the L state in TR data likely indicates that the TR experiment was not successful. Perhaps at least some of the other features characteristic for only L state are well justified by electron 2Fo-Fc or 2Fextrapol-Fc (considering how weak is the Fo-Fo map)? If so, prepare extended figures to present it. Include the corresponding information and discussion in the main text of the manuscript.

23)Regarding the processing it is not clear to the reviewer if all atom positions and B-factors were refined in re mac after combining dark and active intermediate models against raw data in cryo-trapped and TR data. Please provide details in the Methods section.

Reviewer #3:

Remarks to the Author:

The manuscript by Kovlev et al describes a detailed structural characterization of a light-driven bacterial inward proton pump. The system is very interesting, also in view of optogenetic applications. They authors use both cryocrystallographic and serial data collection approaches to characterize important reaction intermediates of the protein following light activation. The manuscript is interesting and in view of its comprehensive characterization of an important protein well suited for NSMB. However, before publication can be recommended, a number of technical issues need to be addressed.

The manuscript claims to push the "horizons of time-resolved x-ray crystallography at synchrotron sources to an unprecedented microsecond range...". First, as written this statement is wrong, the time-resolution for time-resolved crystallography at synchrotrons is 150 ps, see for example Schotte et al, Science 2003 and has been pushed to ≤ 100 ps. Second, since the diffraction data of the evolving reaction are integrated during the X-ray exposure, the time resolution is minimally 0.5 ms. Calling this "microsecond time range" seems quite exaggerated, sub-millisecond is appropriate (and still stretching it).

It would be helpful to explain the unusual nomenclature of the "250-750" us snapshots and ideally come up with a better name.

The authors write that diffraction data were collected at 1 kHz. However, they state "A total number of 72,000 detector images were collected for the 250-750 us snapshots." This implies that essentially of these were hits. Thus, the crystal concentration must have been extremely high. It would also mean many multiple hits and also very poorly defined illumination conditions due to shading. Please expand on this issue in the manuscript.

Why were the the subsequent 12 snapshots not analyzed ? The time delay between shots increases by 1 ms after each shot (but see below for the issue of jet speed). Looking at Ext. Data Fig. 2D they would nicely cover the $L \rightarrow M$ intermediate.

The laser flux is very high. What was the rationale? Please explain in the manuscript.

Radiation damage is a great concern for retinal proteins and as shown by Gordeliy and coworkers detrimental for mechanistic interpretation in case of the closely related bR (see e.g. Borshchevskiy et al JMB 2011). It is also an issue in the current work, both for the serial data collection and the cryo data.

Concerning the "250-750" data collection (and similar to the 7.5 ms data)

Combining the displacement of the jet over the X-ray exposure (1.25 micron) with the X-ray spot size (2 micron), the segment of the jet exposed to each X-ray pulse region of the jet is $1.25 + 2$ micron = 3.25 micron FWHM. The exposure distribution is a trapezoidal distribution, 0.75 micron wide at the top and 3.25 micron at the base and so has a FWHM of 2.00 micron. These numbers (2.00 micron FWHM and 3.25 micron FWHM) are to be compared with the 2.5 micron displacement of the jet between X-ray pulses, meaning that a fraction of the sample is always hit by two X-ray pulses. It is only the tails of the trapezoidal distribution that contribute to this double exposure, but nonetheless the contribution is non-zero. The timing was likely set by simply considering the displacement of the jet between X-ray pulses (2.5 micron) with the displacement of the jet during the measurement (1.25 micron) while ignoring the finite width of the X-ray spot. This would be correct for a zero width X-ray beam, but that of course is not the case.

The trapezoidal distribution of X-ray exposure per pulse along the jet means that the X-ray exposure is actually much less than nominally value (X-ray fluence times area of jet illuminated). The peak exposure is down by almost factor of two from that nominal value, and the average exposure by almost another factor of two.

This multiple-delay scheme assumes that the jet speed remains constant to significantly

better than the laser pulse duration (0.5 ms) over the measurement time for one series (25 ms), or <2%! Moreover any variation early in the sequence shifts all the remaining delays. I would classify this scheme on that basis alone as extremely problematic. Since it is well known that HVE jets are subject to variations in jet speed, it is contingent upon the experimenters to explicitly demonstrate the constancy of their jet speed. At the very least the variations in jet speed should be measured and combined with the 500 us pulse width to get an effective delay-time resolution. Variations in jet speed are also problematic in view of X-ray damage.

In view of the radiation sensitivity of retinal proteins it is important to at least state the absorbed dose/dataset (add to the X-ray tables for ALL datasets). Ideally, all numbers required for the calculation should be given in the data collection description (exposure time, flux density, beam profile, number of exposures etc). The issue of radiation damage (or why the authors think it is not an issue) should be discussed. This is particularly important concerning descriptions of the retinal environment/ Schiffbase, including the water structure.

Illumination of macroscopic crystals. Were the crystals rotated during illumination? If not, why not?

The following refers to the refinement of data collected of illuminated macroscopic and microscopic crystals. When adding the coordinates of the intermediate state (derived using extrapolated maps) to the dark coordinates and "automatically refining the against the original data using REFMAC" was the dark state model kept fixed (i.e. not refined)? In our experience, this is the preferred way of doing this, particular in view of the resolution available. Otherwise, overfitting is a real issue. Obviously, overfitted maps will look much nicer and have lower R-factors. Is this the (invalid) rationale of not simply reporting the structures using extrapolated maps? It is well known that the Rfactors will be very high, so this should not be an issue. This issue should be addressed in the manuscript (not just the rebuttal letter).

The occupancy of the intermediates should be indicated in the X-ray table.

Crystallization: "The best crystals were obtained with a protein concentration of 20 mg/ml..." Does this refer to the protein concentration in the mesophase? If so, it should be stated.

I had asked the editor for the PDB and mtz files but since they never materialized I do not want to comment on the structural findings/ descriptions.

Author Rebuttal to Initial comments

Answers to Reviewers' Comments

Reviewer #1

Remarks to the Author:

Microbial rhodopsin is a superfamily of a membrane protein using retinal pigment to capture visible light and uses its energy to transport various ions across the cell membrane. While the mechanism of outward proton pumping microbial rhodopsin, like bacteriorhodopsin, was revealed

in great detail, the knowledge for inward proton pumping microbial rhodopsin, which was discovered recently, is quite small, especially due to the lack of structural information of the photointermediates. In this work, the authors studied the structural change of a new inward proton pumping rhodopsin, BcXeR, during the photocycle by two different methods: cryo-trapping and time-resolved serial crystallography. The quality of the obtained structures of the L and M intermediates is quite high, allowing them to precisely observe the structural change along with the proton transporting process, which is very unique to this protein and distinct from that of outward proton pump. The assignments of the photointermediates and proton transfer steps were also confirmed by time-resolved spectroscopy and time-resolved electrometry, respectively, further substantiating their model created based on the structural insights. Since their results and findings are outstanding and reveal the mechanism of inward proton pumping by rhodopsins in unprecedented detail, I am confident that this article will attract the attention of broad readers and potentially deserve to be published in this journal. I raise, however, several scientific concerns as below, which need to be addressed by the author.

We thank the Reviewer for high evaluation of our work. We have addressed the Reviewer's comments as described below.

Major points

Line 341-342

“We suggest that the proton translocation through the gated proton wire in the extracellular side of the protein is the time-limiting step of the inward proton transport process”

If the proton transfer from the extracellular bulk phase through the proton wire is the time-limiting step, the rate of reprotonation of the retinal (= the decay of the M) should be proportional to the concentration of proton in the solvent. This is not the case, however, for another XR (PoXeR, Inoue et al. Nat. Commun. 2016) and another process (e.g. the thermal isomerization of the retinal) was proposed to be the time-limiting step of the M decay. In order to clarify which is the time-limiting process, proton uptake or another structural change, the pH dependence of the M decay should be measured.

We thank the Reviewer for this valuable comment. We should clarify that our original sentence “We suggest that the proton translocation through the gated proton wire in the extracellular side of the protein is the time-limiting step of the inward proton transport process” is misleading. What we originally meant was that NOT that the proton transfer along the wire BUT that the transient formation of the complete proton wire connecting the RSB to the extracellular bulk in the course of the M-to-ground state transition itself is the time-limiting step. So, first of all, we have re-written this sentence in the revised version of the manuscript as follows: “Therefore, we suggest that the proton translocation from the extracellular side to the RSB requires formation of a continuous proton wire, which, in turn, is the time-limiting step of the inward proton transport process.”

Second, to probe our hypothesis that it is not the process itself of the proton across the extracellular part of the protein that is the time-limiting step of the inward proton transfer, we performed time-resolved spectroscopy experiments to measure the pH dependence of the M state decay in BcXeR. Our data showed that the rate of the decay of the M state is not proportional to the proton concentration in the surrounding media (Extended Data Fig. 2H), which is similar to the homologous XeR reported earlier (PoXeR). This result is in line with our hypothesis. To indicate the results of the additional experiments we have modified the text as follows: “However, spectroscopic data shows that the reprotonation of the RSB of BcXeR proceeds slowly and in pH-independent manner (Fig. 1B; Extended Data Fig. 2H).”

Minor points

Line 205-207

“The L state structures determined with both methods are similar and demonstrate the same structural rearrangements associated with the intermediate state formation”

Why the residues having exhibited structural change shown in Fig 2B are different between cryotrapping and TR (e.g. F210, S206, L136 were not shown in TR) despite the high similarity of the two L-intermediate structures determined by different methods? Figure 2B looks to demonstrate the structural change becomes smaller for TR than for cryotrapping.

We thank the Reviewer for this comment. Indeed, the difference electron density maps obtained using TR crystallography are weaker. There are several reasons for that. First, the occupancy of the L state is almost twice as low in the case of TR data (20% vs 35% for the cryotrapping data). Second, the resolution of the data is also lower (2.3Å vs 1.6Å for the cryotrapping data). Thus, it is not surprising that only the most pronounced structural changes corresponding to the L state were evidenced in the TR experiments. These include the flips of L81, W173, and K207 together with the RSB shift, but do not include the regions of F210, S206, and L136, for which the structural changes were smaller compared to other regions even in the case of the cryotrapping data. We have added a small explanation on this issue to the Fig. 2 legend as follows: “In the latter case the difference maps appear weaker due to the lower resolution and lower occupancy of the intermediate.”

Line 365-368

“Moreover, the region near W173 is conserved within bacterial XeRs, but differs from that in ASR-like sensors which lack the proton-pumping activity²². Importantly, this is a noteworthy difference between the internal organization of bacterial XeRs and ASR-like sensors (Extended Data Fig. 7).”

It is unclear which part of protein they are discussing in this part. Is it the difference between D and Q at 107th position, which is shown in the alignment in Extended Data Fig. 7? A more specific explanation would be helpful to understand what they want to propose.

We agree with the Reviewer that this sentence is unclear. We meant aspartic acid at the 107th position in BcXer. We have modified the sentence to clearly define the region as follows: “Moreover, D107 is conserved within bacterial Xers, but is substituted by glutamine in ASR-like sensors which lack the proton-pumping activity”.

Figure 4

Since the flipping of W173, L136, L169, and possibly the transient hydrogen bonding of D107 are very unique to inward proton pump, I would like to propose including them in the model of Fig. 4.

We added the mentioned amino acid residues to Fig. 4.

Extended Data Fig. 1, legend

Italicize “Anabaena” and “E. coli”

It is done.

Supplementary Information, Line 41-45

“Notably, the amplitude of the 15 μ s component of the membrane potential generation by BcXer...” and “...15 μ s component corresponds to the RSB and the RSB-associated proton relocation towards the cytoplasmic side following the retinal isomerization shown in the L state.” How much is the time resolution of the measurement? Can the 15 μ s component be exclusively assigned to the proton relocation towards the cytoplasmic side on the L intermediate? Is there no possibility that this component is derived from a change in the dipole moment of the Schiff base following the isomerization of the retinal or the change in the orientation of amino acid side chain(s)?

The time resolution of the measurement is 100 ns. This 15 μ s component could not be exclusively assigned to the only one event. The part of the paragraph in lines 41-45 was a suggestion, or a hypothesis. We agree with the Reviewer that the statement could be misleading for the reader. Therefore, we rephrase this paragraph as follows:

“Notably, the amplitude of the 15 μ s component of the membrane potential generation by BcXer is $\frac{1}{5}$ 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, 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 Information, Line 93

“D107 and L169 are located far from the putative proton translocation pathway...”

The position of L169 is not clear in Extended Data Fig. 7A.

To address the comment of the Reviewer we added the L169 residue to Extended Fig. 7A.

Reviewer #2

Remarks to the Author:

The manuscript reports high-resolution structures of inward proton pumping rhodopsin. Novel structures of intermediate L and M states were cryo-trapped and extensively discussed. Authors also report time resolved crystallography data recorded at a synchrotron source, including submillisecond time delay which an impressive achievement. Importantly, the manuscript provides an exciting insight into water rearrangement in the L state accompanied by Trp rotation. It gives basis to explain the early proton transport pathway. The water chain formed in the L state (cryo-trapped structure) and explaining the likely proton transfer pathway is a beautiful result addressing one of the important questions in rhodopsin field!

We thank the Reviewer for the high evaluation of our work.

Half of the results are referring to structures that bind sodium or not bind sodium. However, sadly, there is no supporting evidence mentioned or discussion in the manuscript for assigning the sodium ion binding in dark and L state. Furthermore, when sodium is not bound the position is occupied by water molecule according to authors (M state). However, again there is also no mention of supporting evidence for assigning water molecule in the same position (where was sodium in the resting and L states) while calling it a sodium-free structure. Then authors refer to this sodium-free structure repeatedly, even the sodium disappearance being called a signature. At the same time completely disregarding the reasons for distinguishing between sodium and water, which display identical electron densities in X-ray data owing to the same number of electrons. In case the authors meant to propose hypotheses in different parts of the manuscript that are still lacking support then the manuscript should be adjusted accordingly to reflect that these are yet unsupported hypotheses. Similarly, the discussion regarding the PRG or the extracellular gate perhaps needs to be downplayed as a hypothesis unless can be better supported.

We thank the Reviewer for this comment. Indeed, sodium ion and water are univocally distinguished in our structures by their binding geometry. We added the description to the revised version of the manuscript as indicated in our reply to Referee's comment 10) below.

- Equally important issue is with the impressive submillisecond time resolution for L state, which however seems to give only very poor Fo-Fo maps and then discussion of TR data (especially for L state) is almost completely missing. This being a great achievement needs to be supported by data and discussion or not claimed by the authors.

a) We have chosen to present the time-resolved data on the L state via Fo-Fo difference maps for consistency with the presentation of all structures discussed in the manuscript. We respectfully disagree with the referees' judgement on this map as "very poor". The

contrast in the Fo-Fo map of the time-resolved L state is as expected for a given amplitude and extent of structural alteration versus ground state, hits occupancy, data resolution and signal-to-noise ratio. This could be compared, for instance, to Fig. 5D of (Nango et al. 2016) or Fig. 3E,F of (Skopintsev et al. 2020). We are grateful for the suggestion of the Reviewer and have included other data representations (see below).

- To address it, for TR L state data show the extrapolated maps of the evolving features to provide support and rescue the poor impression from the very weak TR L state Fo-Fo maps presented.

b) We have included multiple sections of the extrapolated map of the L state based on TR data in Extended Data Fig 9.

- Compare the agreement between the TR and cryo-trapped data (in terms of electron density maps and models).

c) The R.M.S.D. of the refined coordinates of L- substructures in the cryotrapped and time-resolved data is 0.231 Å. The R.M.S.D. of the refined coordinates of M- substructures in the cryotrapped and time-resolved data is 0.2 Å. In all cases, the Fo-Fc maps show no unexplained densities above 3.0σ . This information has been included in the «Structures of BcXeR in the L and M intermediate states» section of the revised version of the manuscript.

- The discussed new features are supported (and highlighted in figures) by electron densities coming from the cryotrapped data and not from TR data. If these features are not present in the electron densities derived from the TR data (especially for the L state) then what was the reason for the authors to conclude that the TR experiment at the submillisecond resolution (L state) was at all successful? Please include corresponding reasoning and discussion in the main text of the manuscript and show supporting data in the extended figures.

d) We thank the Reviewer for making this point. We added a representation of a number of features of the L state clearly supported by the TR experiments in Extended Data Fig. 9. These include flips of L81, W173, L136, and retinal isomerization, and are fully consistent with the cryotrapped data. An important feature not resolved in the TR data is the three water molecules in the region between the RSB and S211 (Wat506, Wat507, and Wat508). This is discussed in the revised version of the manuscript. Taking cumulatively the points a-d) allows us to conclude that the TR experiment was successful.

- Overall, the reported time-resolved structures and the corresponding cryo-trapped structures on similar crystals could provide an opportunity for a very interesting comparison, which is here only very brief.

This reviewer is excited about the described novel insights into the inward proton pumping rhodopsin and the development in time-resolved crystallography at a synchrotron.

- However, several additional extended figures showing electron densities and explanations (as mentioned in the points below) are required to be able to judge if some of the authors' claims/TR intermediates are supported by the data. The above and the following issues need to be resolved before the manuscript can be recommended for publication:

As indicated above (a-d), we are grateful to the Reviewer for the comments, which clearly helped us to improve the overall manuscript presentation by including the required data.

1) Lines 52-61 Discussion is interesting but optional and not needed for the manuscript unless referred in results with specific optogenetic engineering improvements towards use in the optogenetics. Especially if the reference 6 (line 51) already refers to the potential advantages of the IPPs.

We agree with the Reviewer and shortened this part in the revised version of the manuscript.

2) General comment: The reviewer misses discussion or reasoning or advantage of using 7.5 ms time resolution for the slower dynamics while for the fastest timepoint it was possible to achieve 500 μ s temporal resolution (at least according to the authors). It is not to undermine in any way the slower TR data. A short mention in the text if it was due to some technical challenges or otherwise, will address this reviewer's curiosity and likely this of the readers.

7.5 ms is the shortest exposure period of an EIGER2 X CdTe detector in a standard 16M mode. The use of the 16M detector vs its 4M mode (as in a 0.5 ms time-resolved experiment) allows for a large sample-detector distance and a reduced background. The higher resolution of the 7.5 ms data vs the 0.5 ms data at a lower dose is due to the use of the 16M mode. Thus, the 7.5 ms time resolution was chosen to sample the time interval of 0 to 250 ms. The size of the laser spot (180 μ m), the laser pulse duration (7.5 ms), the repetition rate of laser pulses (4 Hz), the jet speed (0.5 mm/s) and X-ray flux (2×10^{12} ph/s) were set to match this time interval. Increasing the frame rate would require a proportional increase in the jet speed and hence in the sample consumption (by an order of magnitude), and a decrease in the duration of the laser pulse leading to a lower M state occupancy.

We thank the Reviewer for this remark. In the Methods section of the revised manuscript, we have explained the reason for the use of the 7.5 ms time resolution as indicated above.

3) Lines 218-219 "we assume that these past-M state transitions are also structurally-silent (at least at 2.2 Å resolution)." Please clarify in the text or rephrase what is meant by the "structurally silent". Does it refer to suspicion that there is in fact no structural changes large enough to discern at 2.2

Å resolution, or rather that the protein is out-of-sync at the late time delays and the populations of each rearrangement (even if all characterized by the spectroscopic M state) might be very low and thus not detectable in the crystallographic data? In the latter case enriching/trapping could still provide insights in the future. Otherwise, if past-M state transitions are spectrally and structurally silent then perhaps they do not exist at all?

We thank the Reviewer for this very important question. When naming the past-M transitions “structurally-silent” we mentioned that there are no structural changes which can be determined at a 2.2 Å resolution. We have re-written the sentence in the revised version of the manuscript to avoid the confusion of the reader as follows: “Since our time-resolved crystallography data cover a 250 ms time range corresponding to the spectrally-silent past-M state transitions, but do not display any possible associated structural rearrangements, we assume that these past-M state transitions are also structurally-silent (or at least undeterminable at a 2.2 Å resolution).”

We should mention that one cannot exclude that the protein is out-of-sync at the late time delays, as proposed by the Reviewer; however, it is unlikely, since the occupancy of the intermediate state in our time-resolved crystallography data is high (43% even for the latest time point), as is the occupancy in the cryotrapped intermediate (nearly 100%) and these data do not demonstrate any rearrangements other than protein transition back to the ground state.

Regarding the last comment of the Reviewer, we agree that the past-M transitions might simply not exist. However, this is still a hypothesis which requires further investigations, which are beyond the scope of our manuscript.

4) Fig2B: a) In the L state why there is water modelled in the FoFo positive density in the cryotrapped structure but not in the corresponding positive density in the TR L state structure?

As mentioned above, the TR data did not show clear electron density supporting the waters Wat506, Wat507, and Wat508 (Fig. 3A). The water molecule in question is Wat506 (Fig. 3A). This is not entirely surprising - a water molecule with no more than 20% occupancy may not be confidently visible at 2.3 Å resolution. Our discussion on the critical role of these water molecules is based on the high-resolution, high-occupancy data obtained by cryotrapping.

b) Why is this map so unusually weak? There is some correspondence of the L state TR Fo-Fo peaks to the cryotrapped L state but by itself it does not convince that we can obtain useful 500 μs resolution data from the synchrotron (for the first time). So importantly, does the 2Fo-Fc or 2Fextrapol-Fc maps support the L state TR structural changes and in particular these that are present only in the L state? Show in extended figures. The determined active L state population is relatively high even for the TR data so it should not be the reason for poor FoFo map.

First of all, we thank the Reviewer for this comment, which helped us to improve the corresponding part of the manuscript.

As mentioned above, we believe the Fo-Fo maps of the L state presented in Fig. 2B at 3.0σ level are not unusually weak but are rather similar to what has been observed in previous works at a moderate occupancy (20%) and the given resolution ($2.3 \text{ \AA}$).

In Extended Data Fig. 9 we show the $2F_{\text{extrapol}}-F_c$ maps of the TR L state clearly confirming the pronounced structural rearrangements, such as the flips of W173, L81, and L136 as well as the kink of K207 carbonyl oxygen and the isomerization of the retinal cofactor.

These findings clearly support the usefulness of the $500 \text{ \mu s}$ resolution data from a synchrotron (for the first time).

5)Extended Data Figure 4 panel E. Shows beautifully the appearance of the three critical water molecules in the L state. Beautiful result! It is not explicitly said in the figure caption but I assume that the density corresponds to the cryo-trapped data (only mentioned in methods; add in the figure caption that it is cryo-trapped data). Since it is a major result, please prepare Extended figure comparing electron densities this region to the TR L state and to the following M states to see the evolution of these 3 new water molecules. Importantly, seeing the TR L state waters in this region will let us judge if the very weak sub-millisecond data set provided useful information or was simply too weak/noisy to be useful for anything (now in Methods I find that the water molecules are not present in TR data; find some other L state unique features to justify that the microsecond TR experiment was successful/useful and show electron densities in an extended figure).

We thank the Reviewer for this comment. The new Extended Data Figure 9 is showing the extrapolated electron density map sections around the water molecules in question, as well as other L state unique features, as indicated above.

6)Extended Data Fig. 4 Specify if the densities in panels come from cryo-trapped dataset or TR (please specify in all figures throughout manuscript the source of presented data, TR vs. cryo-trapped). If all are cryo-trapped, then prepare also the same panels using the TR data to judge its quality.

We have specified that the maps presented in Extended Data Fig. 4 come from the data obtained at 100K in the revised version of the manuscript. Following the suggestion of the Reviewer, we added Extended Data Fig. 9 showing the electron density maps corresponding to the time-resolved crystallography data at room temperature.

7)Extended Data Fig. 8 Address the missing reference to structures (articles or at least PDB codes) which are used for the figures, otherwise it is hard to guess what is being compared in the HsBR.

We thank the Reviewer for this comment. We have added the references and the PDB codes in the Extended Data Fig. 8 (Extended Data Fig. 10 in the revised manuscript) legend for the used HsBR structures.

8)Line 257-258 “Notably, the same structural rearrangements and transiently-formed linear H-bond chain were found in the L state of HsBR” Did the authors mean that the L state water chain in this region is similar to the late photocycle in HsBR? Not L state of HsBR but late state when the RSB is being recharged. Please correct it! In any case very interesting observation and comparison, which provokes numerous other exciting questions.

Thank you for this comment. In our manuscript we have mentioned the water chain observed in HsBR in the L state as reported in our recent work (V. Borshchevskiy et al. 2022). This allowed us to directly compare the L state structures of HsBR and BcXeR demonstrating that, similarly for both proteins, the Schiff base is H-bonded to the carboxylic residues at the cytoplasmic side of the proteins via a water chain consisting of 3 water molecules. A similar water chain was found in the N state of HsBR for the reprotonation of the Schiff base from D96. Following your comment, we strengthen this point in the revised manuscript as follows: “Direct comparison of the L state structures of HsBR and BcXeR demonstrates that, similarly for both proteins, the RSB is H-bonded to the carboxylic residues at the cytoplasmic side of the proteins via a water chain consisting of three water molecules. A similar water chain was found in the N state of HsBR for the reprotonation of the RSB from D96 (Weinert et al. 2019).”

9)Line 263-264 Any reason that here the Trp flips upon isomerization while in the HsBR it only shifts? Is there less space to the shift or otherwise? Could this be the key to why the protein pumps protons inwards and not outwards?

This is a very interesting question. In BcXeR the Trp can be rotated since the place for this large-scale movement is provided by the flip of L169. In HsBR, there is a Thr (T178) at the corresponding position and it is stabilized by an H-bond to a water molecule. Thus, we may speculate that in HsBR the flip of W182 is blocked by T178. However, further investigations are needed to probe this hypothesis. We have added this short discussion to the revised version of the manuscript.

10)Line 306-307 Mention in the text how/why sodium was assigned and not water. Especially, that presence of sodium leads to discussion on the protonation state of the nearby residue.

We thank the Reviewer for pointing out this issue. Indeed, the explanation of the sodium ion assignment was missed in the original version of our manuscript. Our high-resolution data allowed us to determine the distances between the sodium and the coordinating oxygens, which are 2.2, 2.3, 2.4, 2.4, and 2.5 Å. The distance and the number of

coordinating oxygens (5) are both typical for sodium ions (Kuppuraj, Dudev, and Lim 2009) but not for water molecules. All these facts allowed us to fit the density with a sodium ion but not with a water molecule. We have added this explanation to the revised manuscript as follows: “The sodium ion was assigned to the electron density by the analysis of its coordination. In BcXeR, the ion is coordinated by five oxygens with a mean Na-O distance of 2.4 Å, which is expected for this type of ions (Kuppuraj, Dudev, and Lim 2009) (Extended Data Fig. 11)”.

We have also added the Na-O distances into Extended Data Fig. 11.

11)Line 308-309 “This conformation is present in the ground and L states of BcXeR at cryo conditions...” Is this conformation present only in the cryo conditions and not in the TR data of the L state? Elaborate on this in the manuscript.

Yes, the sodium ion was only fitted in the high-resolution structures obtained at cryogenic temperatures. The sodium was not identified in the structures at room temperature. We modified the mentioned sentence as follows to clearly state this in the manuscript: “This conformation is present in the ground and L states of BcXeR at cryo conditions but could not be found at room temperature (Supplementary Note 3, Fig. 3A).”

12)Line 315-316 “The position of the sodium ion is occupied by an additional water molecule...” So now in the position of sodium is assigned water and it is discussed that sodium disappeared. It needs to be clarified in the manuscript how the authors justify assigning to the density in the same location at different stages sodium or water!

We would like to point out that the position of the sodium ion in the PTG in the ground and L states and the water molecule in the M state are not identical. Of course, in the absence of sodium the conformation of the PTG is changed and additional water molecule is found near the original position of the sodium. We agree with the Reviewer that in the original version of our manuscript the sentence is misleading. We re-wrote it as follows: “An additional water molecule was found between the E34 and D214 residues. This water molecule is located within 0.8 Å from the position of the sodium ion in the sodium-bound conformation; however, due to the flip of E34 it is only coordinated with four oxygens with the mean O-O distance of 2.8 Å, characteristic to a standard H-bond (Szalewicz 2003).”

13)Line 323-324 “Nevertheless, the sodium dissociation from the PTG upon the L-to-M state transition is a reliable signature of the proton transfer to the group at this stage of the photocycle” As above, it is unknown to this reviewer the basis on which the sodium and water was distinguished at different stages so it is hard to agree this being a “reliable signature” of anything.

We hope that the above-presented explanation of the sodium and/or water molecule assignment (also reflected in the revised version of our manuscript) supported our

hypothesis on the sodium release being a reliable signature of the PTG protonation in the M state. However, we also agree with the Reviewer that, although our structural data provides hints on the protonation state of the PTG, it does not allow to univocally distinguish between the protonated and deprotonated forms of the PTG. Therefore, we have re-written the paragraph in lines 323-328 as follows:

“Nevertheless, the sodium dissociation from the PTG upon the L-to-M state transition might be a signature of the proton transfer to the group at this stage of the photocycle (Fig. 3A).”

14)Line 327-328 “Thus, the proton released from the RSB is transferred to PTG through the proton wire and stored at the PTG in the M state.” Do the authors have any reason for thinking that the proton is stored in PTG in the M state? Is so, please include the reasoning in the manuscript otherwise remove the statement. Especially considering that the “The sodium-free conformation of PTG remains unmodified suggesting that this conformation reflects both deprotonated and protonated forms, which, however, cannot be structurally distinguished, at least at 2 Å resolution”(Line 319-321) . So the proton could well be already transferred to the bulk solvent at this stage instead of being stored at the PTG. As before, the reasons for assigning “sodium-free conformation are also missing”. So, now discussion builds upon layers of unsupported statements or at least omitting explanation of the supporting reasoning.

We thank the Reviewer for this valuable comment. We agree that the original version of our manuscript was lacking the experimental proof of the proton storage at the PTG in the M state. We performed additional time-resolved spectroscopy analysis of the M state formation depending on the pH of the surrounding media, strongly supporting the presence of an internal proton acceptor from the RSB during the formation of the M state. We have added the description of the results and a corresponding discussion to the last paragraph of the “Structural organization of BcXeR in the ground state” section of the main text of the revised manuscript as follows: “First, it works as a proton acceptor from the RSB upon the formation of the M state. It is supported by our analysis of the pH dependence of the M state formation (Extended Data Fig. 2H). We showed that the rise of the M state does not depend on pH in the range between 6 and 9.5 meaning that there should be an internal proton acceptor from the RSB. Moreover, the rise of the M state is delayed at pH lower than 5, where the E34-D214 pair becomes protonated according to our structural data (Supplementary Note 3). Consequently, in the M state, the region of the E34-D214 pair is a transient proton storage place. Lastly, it also acts as a proton release group during the M-to-ground state transition.”

We cannot completely exclude the possibility of direct proton transfer to the cytoplasm; therefore, we have modified the original text pointed out by the Reviewer as follows: “Thus, the proton released from the RSB is transferred through the proton wire and is likely stored at the PTG in the M state. The direct proton transfer to the cytoplasm from the RSB with

the formation of the M state cannot be completely excluded but is unlikely, according to our time-resolved spectroscopy experiments (Extended Data Fig. 2H). ”

15)Line 332-334 “Namely, while one water molecule Wat402 is found in a double conformation in the ground and L states, indicating its weak interaction with Y49 in only half of the protein molecules, two separate water molecules are well-resolved in the M state” Prepare extended figure supporting that these two water electron densities can only be explained by 0.5 occupancies in the resting and L state and that in the M state can be explained only by 1.0 occupancies. One way could be by showing Fo-Fc densities after a cycle of refinement with 0.5 and 1.0 occupancies for the two waters and for all these data sets. In addition, refer to refinement B factors of these water molecules in extended figure caption.

We thank the Reviewer for this suggestion. In the original version of our manuscript the supporting information on the Wat402 conformations and their evolution was missing. We would like to clarify, that the double conformation of Wat402 in the ground state was assigned since (1) the distance between the alternative positions is very short for O-O distance between two water molecules (2.2 Å) and (2) fitting of the electron densities with a double conformation (50% occupancy of each conformation) resulted in B-factors (30 Å²) comparable to that of the nearby residues (also 30 Å² of R70 and H6). Thus, two water molecules hardly fit the electron densities of the ground state of BcXeR and, in contrast, are well fitted by the double conformation of Wat402.

We have prepared and inserted into the revised version of the manuscript an Extended Data Fig. 8 as suggested by the Reviewer showing the electron densities around one and two water molecules in the ground, L, and M states, respectively. We are also referring to this figure in the revised manuscript while discussing the region of Wat402 and its possible involvement in the proton translocation.

16)342 – 346 “Although the development of the enhanced XeR-based optogenetic tools is beyond the scope of this work, our data clearly show that the extracellular gate should be optimized in order to accelerate the proton translocation by the rhodopsin; moreover, our primary mutational analysis also suggests that single point mutations might not be sufficient for this (Fig. S7D).”

a) Firstly, because authors do not address development of optogenetic tool then the extensive introduction on the potential of this protein towards optogenetics is unnecessary and a simple statement that there might be potential for optogenetics is sufficient, with the reference to the earlier publication.

b) More importantly, authors do not seem to have any basis for claiming presence of extracellular gate based on their structural data as there is no evolution throughout photocycle on the extracellular side and surely none that would allow suspecting closing or opening of any potential gate for proton transport. If authors mean the change in occupancies of two water molecules it still

does not seem to be sufficient to justify non-conductive and conductive states at the extracellular side (why would they not be able to conduct proton when at 0.5 occupancy? Might just be 50% slower in the protein sample?). In addition, as before, authors need to support the change in occupancy of waters with their data e.g., in the form of electron densities in extended figure (see comment to lines 332-334).

We agree with both points of the Reviewer. The sentence in lines 342-346 of our original manuscript is not yet an experimentally supported hypothesis; therefore, we have decided to remove it from the manuscript.

17)Line 429-430: “in particular, we propose that F1F0-ATP synthase is using a similar mechanism to drive ATP synthesis.” Propose based on what ? Is there any reason to think that the mechanism maybe similar? If so, include here otherwise remove.

We thank the Reviewer for this correction. Indeed, this statement is too strong and we have modified it to: “... in particular, we speculate that F1F0-ATP synthase may use a similar mechanism of inward proton translocation to drive ATP synthesis. Unfortunately, there are no structural data elucidating all H-bonds (and water molecules) in the putative proton pathway in F1F0-ATP synthase”

18)Line 431-432: “Finally, this work extends the time range of X-ray synchrotron time-resolved crystallography to hundreds of microseconds, which is by an order of magnitude shorter than reported earlier¹⁶.” While it would be exciting, unfortunately, the poor Fo-Fo difference map does not show that the L intermediate was successfully captured in the microseconds. Perhaps authors are able to support this claim by showing extrapolated density maps and comparing the new features captured in the cryo-trapped states with TR data (such discussion seems to be avoided throughout the manuscript).

We thank the Reviewer for this comment, which we have addressed above by providing a different map representation.

19)Line 533-534: “were obtained with a protein concentration of 20 mg/ml and 0.8 M Na/K-Pi pH 8.2 for the sodium-containing and 1.2 M (NH₄)₃PO₄ pH 7.6 for the sodium-free conditions as precipitant solutions, respectively.” Clarify which conditions were used for which structures. Was the claim of sodium moving away from its binding site in the M state based on sodium-free crystallization conditions?? I hope not but, in any case, add this information to the text.

In line with the comment of the Reviewer comment we have re-written this part of the Methods section to be clearer to the Reader as follows: “The crystals for the determination of the BcXcR structures in the ground and M states at pH 5.2, 7.0, and 8.2 and in the L state at pH 8.2 as well as for the time-resolved crystallography were obtained using 0.8 M

Na/K-Pi pH 8.2 as a precipitant. The crystals for the determination of the BcXeR structures in the ground and M state at pH 7.6 in the absence of sodium were obtained using 1.2 M (NH₄)₃PO₄ pH 7.6 as a precipitant.”

The sodium absence in the M state was identified by the analysis of the electron densities as well as the distances and the coordination of the placed water molecules. The structures determined in the absence of sodium were used as additional evidence of the sodium-free conformations of BcXeR in the ground and M states. We have added this information to the revised manuscript as identified in our replies to the previous questions.

20)Line 548-549 Expand on “Then, the mesophase with crystals was transferred to 100- μ l Hamilton syringes also using centrifugation.” How exactly was the mesophase transferred from tubes to syringes to syringes using the centrifugation. Handling of the viscous mesophase is always problematic and without detailed description it may not be possible to reproduce.

We have added the description of the procedure of the mesophase with crystals transfer from the PCR tube to a syringe in the revised version of the manuscript as follows: “Then, the bottom part of the PCR tube with the mesophase was cut and inserted upside down into the sealed 200 μ l pipette tip. The tip was sealed at the very end using the gas burner. The tip with the inserted PCR tube part were then centrifuged at 4 krpm and 22°C for 2 minutes using a 1.5 ml eppendorf tube as an adaptor for the centrifuge rotor. After the centrifugation, the mesophase was found in the sealed tip. The empty PCR tube part was removed and the tip was covered from the wide end with Parafilm to avoid dehydration of the crystals. The plunger was removed from an empty clean 100 μ l Hamilton syringe and the syringe was sealed from the side of the needle using Parafilm. The tip with mesophase was cut from the narrow end and inserted into the back side of the prepared Hamilton syringe. The syringe with the inserted tip were fixed in the 50 ml falcon tube; for that, the syringe was wrapped into the paper towel to fit tightly into the falcon tube. Then, the tube with the syringe were centrifuged at 4 krpm and 22°C for 3 minutes to transfer the mesophase from the tip into the syringe. After the centrifugation, the tip was removed, the syringe was coupled to another clean 100 μ l Hamilton syringe and the plunger was slowly inserted into the first syringe. Thus, the phase was slowly transferred into the second syringe. In the case of excessive precipitant solution and/or air, the system was uncoupled for their removal and then coupled back to homogenize the mesophase with crystals.”

21)Line 628 -631: “After several iterations of manual and automated refinement, some peaks at the Fo-Fc difference electron density maps corresponding to a small amount of the L state appeared near residues W173, L136, and S206; therefore, these residues were fitted with triple conformation in the final model of the M state, where conformations C and D correspond to the M and L states, respectively.”

Prepare extended figures to show if the double and triple conformations are justified by 2Fo-Fc electron densities. If they are not, then prepare extended figures with 2Fexrapol-Fc to show if the

conformations are justified by the electron density. In addition to raw data please deposit extrapolated amplitudes so that community can easily reproduce the maps for analysis.

We thank the Reviewer for pointing out this issue. We have corrected this section in the revised version of the manuscript. Indeed, in the M state structure we found double, but NO triple conformation. The conformations should be named A and B, and NOT C and D as originally stated. We have corrected these misprints in the revised manuscript. We have also prepared the additional Extended Data Fig. 8A showing the 2Fo-Fc electron densities around the L136, W173, and S206 residues fitted in double conformations and the Extended Data Fig. 8B showing the difference Fo-Fc electron density maps built with 1 cycle of refinement omitting the conformation B in the model. Both maps identify the presence of the second conformation of the residues.

22)Line 709-711: “The additional water molecules appearing in the L state but not seen in the time-resolved data were removed from the starting model. The final occupancy of the L state in the case of time-resolved serial crystallography data was 20%.” Lack of information on some of the most interesting changes in the L state in TR data likely indicates that the TR experiment was not successful. Perhaps at least some of the other features characteristic for only L state are well justified by electron 2Fo-Fc or 2Fextrapol-Fc (considering how weak is the Fo-Fo map)? If so, prepare extended figures to present it. Include the corresponding information and discussion in the main text of the manuscript.

As described above in the replies to the previous questions, we have added the Extended Data Fig. 9 showing electron density maps corresponding to the time-resolved data. They identify key structural rearrangements associated with the L state, showing that the time-resolved experiment was successful and providing valuable data, although not allowing the determination of the mentioned water molecules, identified in the cryo-trapped intermediate due to insufficient structural resolution. We have added a brief comparison of the time-resolved and cryo-trapping data to the main text of the revised manuscript as described in the replies to the previous questions.

23)Regarding the processing it is not clear to the reviewer if all atom positions and B-factors were refined in refmac after combining dark and active intermediate models against raw data in cryo-trapped and TR data. Please provide details in the Methods section.

We have added further explanations of the structure refinement procedures throughout the Methods section of the revised manuscript. The changes are highlighted in yellow.

Reviewer #3

Remarks to the Author:

The manuscript by Kovlev et al describes a detailed structural characterization of a light-driven bacterial inward proton pump. The system is very interesting, also in view of optogenetic applications. They authors use both cryocrystallographic and serial data collection approaches to characterize important reaction intermediates of the protein following light activation. The manuscript is interesting and in view of its comprehensive characterization of an important protein well suited for NSMB. However, before publication can be recommended, a number of technical issues need to be addressed.

We thank the Reviewer for the high evaluation of our work. We have addressed the listed technical issues in the revised version of our manuscript.

The manuscript claims to push the “horizons of time-resolved x-ray crystallography at synchrotron sources to an unprecedented microsecond range...”. First, as written this statement is wrong, the time-resolution for time-resolved crystallography at synchrotrons is 150 ps, see for example Schotte et al, Science 2003 and has been pushed to ≤ 100 ps. Second, since the diffraction data of the evolving reaction are integrated during the X-ray exposure, the time resolution is minimally 0.5 ms. Calling this “microsecond time range” seems quite exaggerated, sub-millisecond is appropriate (and still stretching it).

We agree with the Reviewer and have corrected the statement in the revised version of the manuscript as follows: “We demonstrate the use of serial time-resolved crystallography at synchrotron source with sub-millisecond resolution for studies of a rhodopsin opening principally new applications.”

It would be helpful to explain the unusual nomenclature of the “250-750” us snapshots and ideally come up with a better name.

We have added an explanation to the nomenclature of the 250-750-us snapshot in the revised version of the manuscript as follows: “First, since the L state is accumulated in the BcXeR crystals in sub-ms time scales (Fig. 1C), we obtained a structural snapshot of the protein with 500- μ s-long X-ray exposure at 2.3 Å resolution with a 250 μ s delay to the trigger of a 532-nm laser flash using time-resolved serial crystallography (Fig. 2A). Hereafter, we refer to it as a 250-750- μ s snapshot.”

The authors write that diffraction data were collected at 1 kHz. However, they state “A total number of 72,000 detector images were collected for the 250-750 us snapshots.” This implies that essentially of these were hits. Thus, the crystal concentration must have been extremely high. It

would also mean many multiple hits and also very poorly defined illumination conditions due to shading. Please expand on this issue in the manuscript.

We agree with the Reviewer that multiple hits could be the origin of the poor illumination conditions. As communicated in the Methods section of the original manuscript, among 72,000 detector images collected for the 250-750-us snapshot, 17,527 images were hits (24.3% hit rate). Among these 17,527 images 20,908 crystals were identified, which means that no more than 3381 images, or 19%, contain multiple diffraction patterns. Thus, shading was minimal and unlikely affected the data.

Following the suggestion of the Reviewer we have discussed the poor illumination conditions as a result of the high crystal density in the revised version of the manuscript as follows: "This means that approx. 19% of the images contained multiple diffraction patterns; thus, the crystal density was close to (slightly higher than) optimal, which could lower the quality of the laser illumination in only a small fraction of hits."

Why were the the subsequent 12 snapshots not analyzed ? The time delay between shots increases by 1 ms after each shot (but see below for the issue of jet speed). Looking at Ext. Data Fig. 2D they would nicely cover the L $\rightleftharpoons$ M intermediate.

We thank the Reviewer for this question. Fig. 2A shows that the time range covered in our fast time-resolved crystallography experiment would cover L-to-M state transition. However, only the first snapshot of the series contains notably higher fraction of the L state compared to that of the M state. The following snapshots would contain a mixture of the states, which cannot be resolved at a 2.3Å resolution. As expected, our data for the subsequent snapshots provided difference electron maps, which at this point we could not interpret.

Another point is that the late snapshots of the series (but not the first one) can be affected by the variation in the jet speed, resulting in the arrival of non-illuminated crystals into the X-ray beam before the end of the series.

As a result, only the first snapshot corresponding to the 250-750 us time period following the trigger of the laser pulse provided reliable structural information on the L state of BcXeR.

The laser flux is very high. What was the rationale? Please explain in the manuscript.

In our experiments we have adjusted the laser power to be at a high level that is not yet affecting the diffraction quality of the crystals. This was done to maximize the occupancy of the intermediate states. We have added the calculations and a discussion on the laser illumination conditions and have explained in the revised manuscript why the experiments were performed at a such laser flux (Serial crystallography data collection and treatment subsection of the Methods section of the revised manuscript).

Radiation damage is a great concern for retinal proteins and as shown by Gordeliy and coworkers detrimental for mechanistic interpretation in case of the closely related bR (see e.g. Borshchevskiy et al JMB 2011). It is also an issue in the current work, both for the serial data collection and the cryo data.

We thank the Reviewer for this valuable comment. Indeed, X-ray-induced radiation damage is a critical issue when studying proteins and in particular microbial rhodopsins. Therefore, all our data were collected with low X-ray doses. The typical average doses were about 1 MGy in the case of the single-crystal data collection, 270 kGy in the case of the 7.5 ms exposure time-resolved experiment and 400 kGy in the case of the 500 us exposure time-resolved experiment. According to (V. I. Borshchevskiy et al. 2011) such doses would account for not more than 15-20% decarboxylation of HsBR Asp85. The exact doses are provided in the revised version of the manuscript as requested by the Reviewer in the following questions.

Concerning the “250-750” data collection (and similar to the 7.5 ms data)

Combining the displacement of the jet over the X-ray exposure (1.25 micron) with the X-ray spot size (2 micron), the segment of the jet exposed to each X-ray pulse region of the jet is $1.25 + 2$ micron = 3.25 micron FWHM. The exposure distribution is a trapezoidal distribution, 0.75 micron wide at the top and 3.25 micron at the base and so has a FWHM of 2.00 micron. These numbers (2.00 micron FWHM and 3.25 micron FWHM) are to be compared with the 2.5 micron displacement of the jet between X-ray pulses, meaning that a fraction of the sample is always hit by two X-ray pulses. It is only the tails of the trapezoidal distribution that contribute to this double exposure, but nonetheless the contribution is non-zero. The timing was likely set by simply considering the displacement of the jet between X-ray pulses (2.5 micron) with the displacement of the jet during the measurement (1.25 micron) while ignoring the finite width of the X-ray spot. This would be correct for a zero width X-ray beam, but that of course is not the case.

The trapezoidal distribution of X-ray exposure per pulse along the jet means that the X-ray exposure is actually much less than nominally value (X-ray fluence times area of jet illuminated). The peak exposure is down by almost factor of two from that nominal value, and the average exposure by almost another factor of two.

We see that the corresponding part of the manuscript was somehow misleading for the Reviewer. Let us describe better the experimental setup we used for the time-resolved crystallography experiments. In our experiments we had no separate X-ray pulses; X-rays are delivered quasi-continuously (the PETRAIII bunch separation is in the nanosecond range), in shutterless mode and the frames are collected by triggering the X-ray detector. The notion of “double exposure” is not applicable here.

With all due respect, we fail to understand what the Referee implies by a “nominal value” of X-ray exposure - X-ray fluence (photon/mm²/sec) times illuminated area gives the flux

(photons/second) not the exposure. Consequently, we could not follow the Referee's notion of "peak" and "average" exposure. The dose estimates which we have provided in the revised Methods section take into account the finite beam dimensions, the jet velocity, the detector integration times as well as the variation in the dose due to the propagation of the jet through the beam before and during the diffraction pattern acquisition.

This multiple-delay scheme assumes that the jet speed remains constant to significantly better than the laser pulse duration (0.5 ms) over the measurement time for one series (25 ms), or <2%! Moreover any variation early in the sequence shifts all the remaining delays. I would classify this scheme on that basis alone as extremely problematic. Since it is well known that HVE jets are subject to variations in jet speed, it is contingent upon the experimenters to explicitly demonstrate the constancy of their jet speed. At the very least the variations in jet speed should be measured and combined with the 500 us pulse width to get an effective delay-time resolution. Variations in jet speed are also problematic in view of X-ray damage.

Once again, we think that there is a misunderstanding of the experimental setup we used for the time-resolved experiments. We apologize for not being sufficiently clear on that. We agree that the experiment schema mentioned by the Reviewer above would be extremely unreliable. In our experiments there was no offset between the laser and the X-ray interaction points. The time delay between the laser flash and the detector frames was controlled electronically as a delay between the triggers of the laser and detector. The laser beam was illuminating a large area of the jet. The time delay is not at all affected by the variation in the jet velocity.

For the later frames in the series, slow down of the jet could potentially lead to a situation when the laser-exposed area of the jet would not fully pass and a double laser exposure occurs. This would occur if the jet would slow down by a factor of 1.4 in the slow 7.5-ms experiment, and by more than a factor of 4 in the 500-us experiment - i.e. the jet would practically stop. During both experiments the microscope image of the jet was monitored visually, and such dramatic variations were excluded.

Variation in the jet speed would inversely proportionally reduce or increase the dose instantaneously but would not affect the average dose. Given the very low dose in our TR experiments even unrealistically large variations would unlikely affect the conclusions.

In view of the radiation sensitivity of retinal proteins it is important to at least state the absorbed dose/dataset (add to the X-ray tables for ALL datasets).

We have calculated and added the X-ray dose corresponding to each dataset to the Supplementary Table 4 of the revised manuscript.

Ideally, all numbers required for the calculation should be given in the data collection description (exposure time, flux density, beam profile, number of exposures etc).

This information has been added to the Supplementary Table 4 in the revised manuscript.

The issue of radiation damage (or why the authors think it is not an issue) should be discussed. This is particularly important concerning descriptions of the retinal environment/ Schiffbase, including the water structure.

We have added a special chapter with a discussion on the radiation damage issue in the Methods section of the revised manuscript.

Illumination of macroscopic crystals. Were the crystals rotated during illumination? If not, why not?

We thank the Reviewer for pointing out the missing information in the Methods section. We have added the information regarding the rotation of the macroscopic crystals during illumination. During the L state accumulation, the crystal was rotated 3 times by 90 degrees to illuminate all sides of the crystal in order to increase the fraction of the trapped intermediate. During the M state accumulation, the crystal was not rotated since the illumination was performed with the crystal in the loop at room temperature for a short time (2 s) to avoid a dehydration of the crystal. Thus, for the better accumulation of the M state in the macroscopic crystal of BcXeR it was oriented with the large face orthogonal to the laser beam. We have added this information to the Methods section of the revised manuscript.

The following refers to the refinement of data collected of illuminated macroscopic and microscopic crystals. When adding the coordinates of the intermediate state (derived using extrapolated maps) to the dark coordinates and “automatically refining the against the original data using REFMAC” was the dark state model kept fixed (i.e. not refined)? In our experience, this is the preferred way of doing this, particular in view of the resolution available. Otherwise, overfitting is a real issue. Obviously, overfitted maps will look much nicer and have lower R-factors. Is this the (invalid) rationale of not simply reporting the structures using extrapolated maps? It is well known that the Rfactors will be very high, so this should not be an issue. This issue should be addressed in the manuscript (not just the rebuttal letter).

In fact, the atom positions for the ground state were fixed when refining the mixed model against the original data to avoid overfitting. We have added this essential information in the revised version of the manuscript. We report only non-manipulated data, which in our case contains structural information on the mixture of the ground and intermediate states. All extrapolated maps can be calculated from the reported and deposited data; on the contrary, no original data can be calculated from the extrapolated structure factors. Thus, reporting of the extrapolated structure factors would be misleading to the Reader.

Moreover, we have added the new Extended Data Fig. 9 to demonstrate that the key regions of the extrapolated 2Fo-Fc electron density maps are in agreement with the obtained models.

The occupancy of the intermediates should be indicated in the X-ray table.

We have added the occupancies for the intermediates to the X-ray tables of the revised manuscript.

Crystallization: “The best crystals were obtained with a protein concentration of 20 mg/ml...”
Does this refer to the protein concentration in the mesophase? If so, it should be stated.

This refers to the protein concentration in the water part of the mesophase. We have clarified this in the revised version of the manuscript.

I had asked the editor for the PDB and mtz files but since they never materialized I do not want to comment on the structural findings/ descriptions.

We apologize for this inconvenience. The PDB and MTZ files were only requested from us at the point of receiving the reviews. We have provided the files immediately. We hope that the files were received by the Reviewer and that the Reviewer will be able to analyze the data.

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Decision Letter, first revision:

Message: Our ref: NSMB-A46508A

17th Jan 2023

Dear Dr. Gordeliy,

Thank you for submitting your revised manuscript "Mechanisms of inward transmembrane proton translocation" (NSMB-A46508A). It has now been seen by the original referees and their comments are below. The reviewers find that the paper has improved in revision, and therefore we'll be happy in principle to publish it in Nature Structural & Molecular Biology, pending minor revisions to address the referees' final requests, such as specifying obtaining L and M intermediates, crystal concentration, and extrapolated structure factors. Please note that we might need to consult reviewer #3 before formal acceptance. At the same time, we will ask you to address the editorial guidelines.

We are now performing detailed checks on your paper and will send you a checklist detailing our editorial and formatting requirements in about a week. Please do not upload the final materials and make any revisions until you receive this additional information from us.

Thank you again for your interest in Nature Structural & Molecular Biology Please do not hesitate to contact me if you have any questions.

Sincerely,

Katarzyna Ciazynska
(she/her)
Associate Editor
Nature Structural & Molecular Biology
<https://orcid.org/0000-0002-9899-2428>

Reviewer #1 (Remarks to the Author):

The authors appropriately addressed to the reviewer's comments and revised the description in their manuscript, which made their work suitable for publication in Nat. Struct. Mol. Biol. This reviewer recommends accepting for the publication.

Reviewer #2 (Remarks to the Author):

My concerns were addressed and resolved fully during the revision. I find the presented results exciting and suitable for NSMB. Therefore, I can recommend the current version of the manuscript for publication.

Reviewer #3 (Remarks to the Author):

The manuscript is very much improved and I now support publication. Nevertheless, there are a few things that should be addressed.

Discussion about the W173 rotation.

I do not think the argument involving the flip of Leu169 and the H-bond of Thr178(bR) and a water is the critical point. The Thr is small enough to accommodate a potential Trp flip in bR.

Interestingly there is a complementary substitution of Thr/Leu in bR and XeR: The one just mentioned (bR:Thr178; XeR:L169) AND (bR:Leu152; XeR:Thr143). In my view the latter is the crucial one, the CD1 methyl group of Leu152 would be 2 Ang from CH2 of Trp173 in the rotated orientation. I suggest to modify the argument.

Illumination conditions.

This is not the way to do such an experiment, but it is too late now. Not affecting the diffraction properties is important but by no means sufficient to generate a mechanistically well-defined intermediate. Both the L and M intermediate were obtained under multiphoton excitation conditions and this should be stated for both, not just the M intermediate.

Loss by scattering from the jet and light absorption by the mesophase is typically ~ 10-20%. Using a turbid medium increases scattering and prevents any quantification of how many photons are impinging on the crystals. While some photons are scattered out of the jet, others are scattered within the jet, reaching crystals upstream (see for example <https://journals.iucr.org/m/issues/2021/03/00/zf5015/>). It is thus by no means certain that the effective number of photons impinging on the crystal probed by X-rays is reduced due to scattering.

Crystal concentration.

It is too high. Why not simply say what it was without adding any value to it such as stating that it was close to ideal (which it was not). It does not take anything away from the manuscript and does not mislead future users.

Line 749: ...delete "; thus, the crystal ... to .. of hits" line 751

Electron density maps of time-resolved data.

There is no density for the rotated position of Trp. The B-factors are relatively high which suggests that the occupancy is actually less than 0.3. I would strongly suggest to deposit the extrapolated structure factor amplitudes for the time-resolved data, there are a number of data bases that allow this. I understand the reason of the authors to prefer to deposit the original data and I think it is a good one. However, people will look at the maps using pdb redo, wonder, and maybe complain.

line 213: 0.231 Ang -> 0.2 Ang

Main:

line73 ... to capture the structural .. THROUGHOUT the photocycle.

Not really true since K is missing. I would suggest DURING

Line 78: One does not propose a movie.

propose a mechanism or propose a structural mechanism / structure-based mechanism

Author Rebuttal, first revision:**Answers to Reviewers' Comments****Reviewer #3**

Remarks to the Author:

The manuscript is very much improved and I now support publication. Nevertheless, there are a few things that should be addressed.

Discussion about the W173 rotation.

I do not think the argument involving the flip of Leu169 and the H-bond of Thr178(bR) and a water is the critical point. The Thr is small enough to accommodate a potential Trp flip in bR. Interestingly there is a complementary substitution of Thr/Leu in bR and XeR: The one just mentioned (bR:Thr178; XeR:L169) AND (bR:Leu152; XeR:Thr143). In my view the latter is the crucial one, the CD1 methyl group of Leu152 would be 2 Ang from CH2 of Trp173 in the rotated orientation. I suggest to modify the argument.

We agree and thank the Reviewer for this comment. Indeed, the Leu152 (bR) to Thr143 (BcXeR) substitution is more likely to prevent the Trp flip in the M state of bR. We have modified the corresponding part of the Manuscript as follows: "We suggest that, in BcXeR, W173 can be rotated since there is sufficient place near the T143 residue for this large-scale movement. The reorientation of W173 is also provided by the flip of L169. In HsBR, T143 is substituted by a larger residue L152, which would cause a steric conflict with W182 in the rotated orientation. Moreover, there is a T178 residue at the position of L169 stabilized by an H-bond to a water molecule. Thus, we may speculate that in HsBR the flip of W182 is blocked by the presence of the L152 and T178 residues."

Illumination conditions.

This is not the way to do such an experiment, but it is too late now. Not affecting the diffraction properties is important but by no means sufficient to generate a mechanistically well-defined intermediate. Both the L and M intermediate were obtained under multiphoton excitation conditions and this should be stated for both, not just the M intermediate. Loss by scattering from the jet and light absorption by the mesophase is typically ~ 10-20%. Using a turbid medium increases scattering and prevents any quantification of how many photons are impinging on the crystals. While some photons are scattered out of the jet, others are scattered within the jet, reaching crystals upstream (see for example <https://journals.iucr.org/m/issues/2021/03/00/zf5015/>). It is thus by no means certain that the effective number of photons impinging on the crystal probed by X-rays is reduced due to scattering.

We agree with the Reviewer's comment and modified the manuscript to state clearly that the data for the L state were also collected in the multiphoton excitation conditions.

Crystal concentration.

It is too high. Why not simply say what it was without adding any value to it such as stating that it was close to ideal (which it was not). It does not take anything away from the manuscript and does not mislead future users. Line 749: ...delete "; thus, the crystal ... to .. of hits" line 751

We agree with the Reviewer's comment and removed the second part of the sentence in line 749.

Electron density maps of time-resolved data.

There is no density for the rotated position of Trp. The B-factors are relatively high which suggests that the occupancy is actually less than 0.3. I would strongly suggest to deposit the extrapolated structure factor amplitudes for the time-resolved data, there are a number of data bases that allow this. I understand the reason of the authors to prefer to deposit the original data and I think it is a good one. However, people will look at the maps using pdb redo, wonder, and maybe complain.

We agree with the Reviewer that it is useful for the reader to deposit also the extrapolated structure factor amplitudes. Therefore, we have deposited the extrapolated structure factor amplitudes for the time-resolved L and M states as well as for the cryotrapped L state to Zenodo database (10.5281/zenodo.7612803). This information is also included into Data Availability Statement.

line 213: 0.231 Ang -> 0.2 Ang

It is modified.

Main:

line73 ... to capture the structural .. THROUGHOUT the photocycle.

Not really true since K is missing. I would suggest DURING

We agree with the Reviewer and modified the sentence as suggested.

Line 78: One does not propose a movie.

propose a mechanism or propose a structural mechanism / structure-based mechanism

We modified the word "movie" to the word "mechanism" in the revised manuscript.

Final Decision Letter:**Message** 15th May 2023

:

Dear Dr. Gordeliy,

We are now happy to accept your revised paper "Mechanisms of inward transmembrane proton translocation" for publication as an Article in Nature Structural & Molecular Biology.

Acceptance is conditional on the manuscript's not being published elsewhere and on there being no announcement of this work to the newspapers, magazines, radio or television until the publication date in Nature Structural & Molecular Biology.

Over the next few weeks, your paper will be copyedited to ensure that it conforms to Nature Structural & Molecular Biology style. Once your paper is typeset, you will receive an email with a link to choose the appropriate publishing options for your paper and our Author Services team will be in touch regarding any additional information that may be required.

After the grant of rights is completed, you will receive a link to your electronic proof via email with a request to make any corrections within 48 hours. If, when you receive your proof, you cannot meet this deadline, please inform us at rjsproduction@springernature.com immediately.

You will not receive your proofs until the publishing agreement has been received through our system.

Due to the importance of these deadlines, we ask that you please let us know now whether you will be difficult to contact over the next month. If this is the case, we ask you provide us with the contact information (email, phone and fax) of someone who will be able to check the proofs on your behalf, and who will be available to address any last-minute problems.

To assist our authors in disseminating their research to the broader community, our SharedIt initiative provides all co-authors with the ability to generate a unique shareable link that will allow anyone (with or without a subscription) to read the published article. Recipients of the link with a subscription will also be able to download and print the PDF.

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Note the policy of the journal on data deposition:
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