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Mechanisms of inward transmembrane proton translocation

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21 **3. Source Data**

Parent Figure or Table	Filename Whole original file name including extension. i.e.: <i>Smith_SourceData_Fig1.xls</i> , or <i>Smith_Unmodified Gels_Fig1.pdf</i>	Data description i.e.: Unprocessed western Blots and/or gels, Statistical Source Data, etc.
Source Data Fig. 1	Fig1.xlsx	Experimental Source Data
Source Data Fig. 2	Fig2.xlsx	Experimental Source Data
Source Data Extended Data Fig./Table 1	Ext1.xlsx	Experimental Source Data
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24 **Title: Mechanisms of inward transmembrane proton translocation**

25

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49 **Abstract**

50

51 Proton transport is indispensable for cell life. It is believed that molecular mechanisms of proton movement
52 through different types of proton-conducting molecules have general universal features. However, elucidation
53 of such mechanisms is a challenge. It requires true-atomic-resolution structures of all key proton conducting
54 states. Here we present a comprehensive function-structure study of a light-driven bacterial inward proton
55 pump xenorhodopsin from *Bacillus coahuilensis* in all major proton conducting states. The structures reveal
56 that proton translocation is based on proton wires regulated by internal gates. The wires serve as both
57 selectivity filters and translocation pathways for protons. The cumulative results suggest a general concept of
58 proton translocation. We demonstrate the use of serial time-resolved crystallography at a synchrotron source
59 with sub-millisecond resolution for rhodopsin studies opening the door for principally new applications. The
60 results might also be of interest for optogenetics since xenorhodopsins are the only alternative tools to fire
61 neurons.

62

63 **Main text**

64

65 Ion gradients across cell membranes are crucial for life and are maintained by ion transporters. Proton
66 transporters are in the focus of studies of molecular mechanisms of ion transport. In many microorganisms
67 from all domains of life proton gradients are maintained by membrane hepta- α -helical light-driven proteins -
68 microbial rhodopsins (MRs) comprising a retinal as a chromophore¹. The most studied proton-transporting
69 MRs are light-driven outward pumps serving as a reference for the understanding of proton transfer and
70 storage in proteins².

71 Moreover, MRs found their principal application in optogenetics, which revolutionized neuroscience and
72 offered novel biomedical approaches; recently it was used for partial vision recovery in humans³. Mostly
73 cation channelrhodopsins (CCRs) are employed for optical activation of cells. Starting in 2016, the first
74 inwardly-directed proton pumps (IPPs) xenorhodopsins (XeRs) were found and characterized⁴⁻⁶. IPPs are the
75 only alternative optogenetic instruments to CCRs affording a set of competitive advantages⁶. However, the
76 optimization of XeRs (and IPPs in general) is essential for optogenetics due to their slow kinetics resulting in
77 insufficient photocurrents for routine usage^{4,7}. High-resolution insights into the mechanisms of XeRs
78 functioning are imperative for their rational optimization.

79 Unfortunately, the molecular mechanism of inward proton translocation remains unclear. It is believed that
80 upon light illumination the retinal Schiff base (RSB) of XeR donates its proton to the internal proton acceptor
81 located in the cytoplasmic part of the protein^{4,6}. This transfer coincides with the formation of the strongly
82 blue-shifted M intermediate, which then decays with the protein returning back to its ground state
83 accompanied by a proton release to the cytoplasm and a proton uptake from the extracellular bulk⁴⁻⁶. However,
84 there is no certain information on the internal proton transfer and also on the determinants of inwardly-directed
85 proton translocation.

86 In this work, we found a bacterial xenorhodopsin from *Bacillus coahuilensis* (*BcXeR*), which is an ideal target
87 to reveal the molecular mechanism of inward proton translocation. We used time-resolved serial
88 crystallography at the P14 beamline of the PETRAIII synchrotron source with time resolution up to 500 μ s to
89 capture the structural rearrangements in *BcXeR* during the photocycle. Moreover, X-ray data on the
90 cryotrapped intermediates allowed us to build more detailed models of the ground, L, and M states at 1.6-1.7
91 Å resolution to visualize water-mediated proton wires inside *BcXeR* acting as pathways for unidirectional
92 proton translocation. We validated our structural findings by functional and spectroscopic studies of the
93 protein and its mutant forms. Based on the described data we propose a molecular mechanism of the light-
94 driven inward proton translocation also serving as a unique reference for understanding of the proton transport
95 in general.

96

97 ***BcXeR* is a light-driven inward proton pump**

99 The gene encoding *BcXeR* was identified bioinformatically in GenBank (ID: WP_059282687) using Hidden
100 Markov Model algorithms (see Methods for details). The protein is a member of the bacterial XeR clade and
101 could be easily expressed in *E.coli* with high yield (Extended Data Fig. 1). The same gene was previously
102 predicted to encode a sensory rhodopsin⁸. However, *BcXeR*-expressing *E.coli* cell suspension showed strong
103 alkalization upon illumination in different unbuffered salt solutions, abolished by the addition of the
104 protonophore to the cell suspension (Fig. 1A). Therefore, *BcXeR* acts as an inward light-driven proton pump.
105 Spectroscopic properties of the *BcXeR* showed that the maximum absorption wavelength of *BcXeR* in purified
106 solubilized form is 558 nm and remains stable in a wide range of pH values, similar to that found for other
107 XeRs⁴⁻⁶ (Extended Data Fig. 2A).

108

109 The photocycle kinetics of *BcXeR* was determined using time-resolved visible light absorption spectroscopy
110 (Fig. 1B). The model of the photocycle was validated by investigations of temperature dependences and an
111 Arrhenius plot (Extended Data Fig. 2B). Similar to other XeRs, *BcXeR* demonstrates a fast formation of the
112 K state, followed by the rise of the early L state in a microsecond (Fig. 1B, Extended Data Fig. 2). Within
113 approx. 20 ms the strongly blue-shifted M state characteristic to the deprotonated RSB is formed^{4-6,9} (Fig.
114 1B). The formation of the M state coincides in time with the first step of proton transfer within *BcXeR*, as
115 evidenced by the generation of the membrane electric potential in the lipid vesicles with the rhodopsin in
116 response to a ns green light flash (Extended Data Fig. 2C; Supplementary Note 1). Then, the M state
117 biexponentially decays directly to the ground state with half-times of 70 ms and 0.8 s. The M decay coincides
118 in time with the second notable proton transfer stage identified by a further change of the membrane electric
119 potential inside the proteoliposomes containing *BcXeR* upon laser illumination (Extended Data Fig. 2C;
120 Supplementary Note 1).

121

122 Next, we crystallized *BcXeR* using *in meso* approach and solved its ground state structure at 1.7 Å resolution¹⁰
123 (Table 1; see Methods for details). The photocycle of the crystallized protein is similar to that in solution,
124 indicating that the protein remains functional in crystals (Fig. 1C, D; Extended Data Fig. 2C,D). The L state
125 in crystals is at its highest occupancy at 30 μs and persists up to 1 ms (Fig. 1C). The M state is at its maximum
126 occupancy at 20 ms and decays to the ground state in seconds (Fig. 1D). *BcXeR* forms trimers in crystals
127 similar to those of other trimeric MRs, such as bacteriorhodopsin from *Halobacterium salinarum* (*HsBR*) and
128 a XeR from *Nanosalina* (*NsXeR*)^{6,11,12} (Extended Data Fig. 3). The protein protomer has seven transmembrane
129 α-helices (A to G) (Fig. 1E; Extended Data Fig. 3). The BC loop at the extracellular part of the protein forms
130 a 9 Å long β-sheet (Extended Data Fig. 3). The cofactor retinal is in its all-*trans* configuration in the ground
131 state and is covalently bound to K207 of helix G via the protonated RSB (Fig. 1E; Extended Data Fig. 4A).

132

133 The cytoplasmic part of *BcXeR* contains a large cavity between S211 and the pair of carboxylic residues E34
134 and D214, directly exposed to the cytoplasm (Fig. 1F). The cavity is separated from the RSB by an 8 Å

135 hydrophobic gap and is filled with water molecules mediating an H-bond network between the carbonyl
136 oxygen of K207 to the E34-D214 pair (Fig. 1F). D214 is stabilized by T88. The role of these residues was
137 studied in detail for other bacterial XeRs using functional tests of their mutant forms and also FTIR studies^{4,5}.
138 Our mutational analysis of *BcXeR* showed consistent results (Extended Data Fig. 5; Supplementary Note 2).
139 Indeed, the data suggest multiple roles of this region in XeRs functioning throughout a single photocycle.
140 First, it works as a proton acceptor from the RSB upon the formation of the M state. This is supported by our
141 analysis of the pH dependence of the M state formation (Extended Data Fig. 2H). We showed that the rise of
142 the M state does not depend on pH in the range between 6 and 9.5 meaning that there should be an internal
143 proton acceptor from the RSB. Moreover, the rise of the M state is delayed at pH lower than 5, where the E34-
144 D214 pair becomes protonated according to our structural data (Supplementary Note 3). Consequently, in the
145 M state, the region of the E34-D214 pair is a transient proton storage place. Lastly, it also acts as a proton
146 release group during the M-to-ground state transition. Thus, the region is a transit point for the proton coming
147 from the RSB before its release to the cytoplasm. Therefore, we denote this cluster as a proton transit group
148 (PTG) of *BcXeR* by an analogy with the proton release group (PRG) of *HsBR* formed by E194-E204 pair and
149 surrounding polar amino acid residues (Fig. 1F; Extended Data Fig. 6C,D)^{11,13}.

150

151 The RSB region is critical for proton translocation in *BcXeR* and is conserved within XeRs (Extended Data
152 Fig. 5; Supplementary Note 2). At the extracellular side of *BcXeR*, there is a quasi-continuous H-bond network
153 propagating from the bulk to the RSB of *BcXeR*, which is only disrupted near Y49 (Fig. 1G; Extended Data
154 Fig. 7C). The central residue in this network, R70, is oriented towards a large cavity, which leads to the
155 extracellular bulk and is filled with numerous water molecules (Fig. 1G). According to our mutational
156 analysis, R70 is essential for inward proton pumping, unlike D118, E192, and E195, which surround the cavity
157 (Extended Data Fig. 5; Supplementary Note 2). More details on the structure of *BcXeR* in the ground state is
158 given in Supplementary Note 4.

159

160 **Structures of *BcXeR* in the L and M intermediate states**

161

162 To follow the early structural rearrangements in *BcXeR* associated with the L state formation we used two
163 complementary approaches. First, since the L state is accumulated in the *BcXeR* crystals in sub-ms time scales
164 (Fig. 1C), we obtained a structural snapshot of the protein with a 500- μ s-long X-ray exposure at 2.3 Å
165 resolution with a 250 μ s delay to the trigger of a 532-nm laser flash using time-resolved serial crystallography
166 (Fig. 2A; Table 2). Hereafter, we refer to it as a 250-750- μ s snapshot. Thus, we obtained time-resolved
167 structural data on *BcXeR* at a synchrotron source with a time resolution of 500 μ s, which is one order of
168 magnitude higher than that shown in the studies on MRs^{14,15} (Fig. 2B). Second, we determined the structure
169 of the cryotrapped L state in a single *BcXeR* crystal at 1.6 Å resolution (Table 1). For that, the cryocooled
170 crystal was warmed up to 170 K and illuminated for 6 min with a 633-nm laser; then, the crystal was cooled
171 down to 100 K and the X-ray diffraction data were collected (see Methods for details). Similar procedures

172 have already been successfully used for the cryotrapping and structure determination of the L states of *HsBR*
173 and the light-driven chloride pump halorhodopsin^{16,17}. The microspectrophotometry of the *BcXeR* crystal
174 before and after the procedure indicated the accumulation and trapping of solely the L state (Fig. 2C). The
175 cryotrapping of the L state resulted in almost twice as high occupancy of the intermediate in crystals (35%
176 and 20% obtained with cryotrapping and time-resolved crystallography, respectively) as well as a much higher
177 resolution (1.6 Å vs. 2.3 Å). Nevertheless, both structures demonstrate similar structural rearrangements
178 associated with the intermediate state formation, such as the flips of L81, W173, L136 and retinal
179 isomerization (Fig. 2B; Extended Data Fig. 4, 8B,C,F). The R.M.S.D. of the refined coordinates of the L-
180 substructures in the cryotrapped and time-resolved data is 0.2 Å. In both cases, the $F_o - F_c$ maps shows no
181 unexplained density above 3.0σ . However, the structure of the cryotrapped L state demonstrates more essential
182 details such as appearance of key functional water molecules in the region between the RSB and S211
183 (Wat506, Wat507, and Wat508) not observed in the time-resolved L state data (Fig. 2B; Extended Data Fig.
184 4); therefore, hereafter we refer to the cryotrapped L intermediate.

185

186 Next, to visualize the following structural rearrangements accompanying the rise of the M state and a possible
187 subsequent spectrally-silent sub-M state transition during its biexponential decay we first used a time-resolved
188 serial crystallography approach. Namely, we obtained a series of 33 datasets covering in total 250 ms of the
189 *BcXeR* photocycle with a time resolution of 7.5 ms and a spatial resolution of 2.2 Å (Fig. 2A; Table 2;
190 Extended Data Fig. 8D,E,H,I). Structures of the intermediate states of *BcXeR* obtained at all 33 time points
191 are identical as also evidenced by their corresponding $F_o^{\text{light}} - F_o^{\text{dark}}$ difference electron density maps.
192 Nevertheless, we captured the rise of the M state occupancy in the crystals in the range between the 0-7.5 and
193 7.5-15 ms and its gradual decay in the following snapshots (Fig. 2D). Since our time-resolved crystallography
194 data cover a 250 ms time range corresponding to the spectrally-silent past-M state transitions, but do not
195 display any possible associated structural rearrangements, we assume that these past-M state transitions are
196 also structurally-silent (or at least undeterminable at a 2.2 Å resolution).

197

198 Second, following the photocycle data in crystals (Extended Data Fig. 2) clearly indicating that under steady
199 illumination with a 532-nm light the M state is the dominant one, we cryotrapped the M state in a single crystal
200 of *BcXeR* and solved the structure at 1.7 Å resolution (Table 1). In brief, the crystal was illuminated at 293 K
201 for 1 s with a 532-nm laser beam and cryocooled in nitrogen stream with the light still switched on (see
202 Methods for details). Microspectrophotometry of the *BcXeR* crystal confirmed the accumulation and trapping
203 of solely the M state (Fig. 2C). Indeed, the cryotrapped structure contained >70% of the M state with atomic
204 structure in perfect agreement with the time-resolved data (the R.M.S.D. of the refined coordinates of the M-
205 substructures in the cryotrapped and time-resolved data is 0.2 Å) albeit higher intermediate state occupancy
206 and resolution (Fig. 2B; Extended Data Fig. 4, 8).

207 **Evolution of *BcXeR* structure in the course of the photocycle**

208

209 Time-resolved crystallography and cryotrapping of the intermediate states allowed us to follow with high
210 precision the structural rearrangement of *BcXeR* in the course of inward proton pumping. Since the
211 mechanism of proton pumping by MRs was studied in detail mostly for outward proton pump *HsBR*^{13,14,18},
212 also serving as a benchmark for studies of many other MRs, we used it as a reference for the analysis of the
213 *BcXeR* structure and mechanism. We conclude that several aspects of the molecular mechanism of proton
214 translocation in *BcXeR* differs principally from that of *HsBR*.

215

216 However, it is also based on proton wires, similar to *HsBR*¹⁹. We found essential structural rearrangements in
217 the L state of *BcXeR* resembling those shown for the L state of *HsBR*. First, similar to *HsBR*, in *BcXeR* retinal
218 adopts 13-*cis* configuration in the L state and the protonated RSB is relocated to the cytoplasmic side (Fig.
219 2B, Fig. 3A). The π -bulge of the helix G near S206 is enlarged (Fig. 3A; Extended Data Fig. 6E). As clearly
220 seen from the difference electron density maps, the side chain of L81 is distinctly flipped and three water
221 molecules (Wat506, Wat507, and Wat508) emerge between the RSB and S211 (Fig. 3A; Extended Data Fig.
222 4E). They mediate the linear H-bond chain between the RSB and S211 propagating further to the PTG at the
223 cytoplasmic side (Fig. 3A; Extended Data Fig. 4E). Thus, in the L state, the RSB of *BcXeR* is stabilized by
224 the linear water-mediated H-bond chain. Notably, the same structural rearrangements and transiently-formed
225 linear H-bond chain were found in the L state of *HsBR*^{18–20} (Extended Data Fig. 9A). A direct comparison of
226 the L state structures of *HsBR* and *BcXeR* demonstrates that, similarly for both proteins, the RSB is H-bonded
227 to the carboxylic residues at the cytoplasmic side of the proteins via a water chain consisting of three water
228 molecules. A similar water chain was found in the N state of *HsBR* for the reprotonation of the RSB from
229 D96¹⁴.

230

231 At the same time, in contrast to *HsBR*, we found marked rearrangements of the retinal binding pocket of
232 *BcXeR*, such as the 180°-flip of W173 (analog of W182 in *HsBR*) (Fig. 2B; Fig. 3B; Supplementary Video
233 1). In *BcXeR* this extended conformational change occurs in order to avoid steric conflict with the C20 atom
234 of the isomerized retinal (Fig. 3B). To allow the flip, the L136 side chain is reoriented towards the lipid
235 membrane (Fig. 3B). A water molecule (Wat509) appears near Wat501 to maintain the interaction between
236 helices F and G weakened by the W173 reorientation (Fig. 3B). Wat501 is relocated closer to F210 to provide
237 more space for the reorientation of L81 (Fig. 3B). Also unlike in *HsBR*, where extended changes in the
238 extracellular part are observed^{16,19}, the corresponding region of *BcXeR* is preserved in the L state. The PTG
239 also remains unmodified at this stage (Fig. 3A).

240

241 In the M state, both time-resolved and cryotrapping data undoubtedly display the retinal in the 13-*cis*
242 conformation, similar to that in *HsBR* (Fig. 2B; Extended Data Fig. 4B). The water-mediated H-bond chain
243 connecting the RSB to S211 in the L state disappears in the M state, and the L81 side chain returns back to
244 the ground position (Fig. 3A). Thus, the hydrophobic gate between the RSB and the rest of the cytoplasmic

245 part is restored, somewhat similar to that found in *HsBR*^{18,19}. The restoration of the gate in *BcXeR* is essential
246 for preventing proton backflow to the RSB from the cytoplasmic side of the protein.

247 At the same time, there are unique structural features specific to the M state of *BcXeR* but not to *HsBR*. First
248 of all, in *BcXeR* the RSB is oriented towards helix G and is stabilized by an H-bond to the S206 side chain,
249 while in *HsBR* the RSB orients toward helix C and is H-bonded to T89 from a DTD signature motif of archaeal
250 outward proton pumps^{18,19} (Fig. 3A; Extended Data Fig. 9B). The H-bond of the RSB to S206 indicates the
251 deprotonated form of the base in the M state, in line with the strong blue-shift of the absorption spectrum of
252 *BcXeR* at this stage. The helix G is slightly shifted towards the lipid membrane to allow for more space for
253 the 13-*cis* retinal while helix C remains unmodified (Extended Data Fig. 9B). The maximal displacement
254 occurs in the region of P203 (analog of D212 in *HsBR*), which is a unique characteristic residue in XeRs.

255

256 **Proton translocation events in *BcXeR***

257

258 The structural data strongly suggests that the PTG becomes protonated in the M state. Indeed, the analysis of
259 the PTG structure in different states of *BcXeR* showed that there are two principally different conformations
260 of the group (Fig. 3A; Extended Data Fig. 4C,D, 10; Supplementary Note 3). The first is characterized by a
261 sodium ion bound in the PTG coordinated by D214, E34, T88, and carbonyl oxygen of A84. The sodium ion
262 was assigned to the electron density by the analysis of its coordination. In *BcXeR*, the ion is coordinated by
263 five oxygens with a mean Na-O distance of 2.4 Å, which is expected for this type of ions²¹ (Extended Data
264 Fig. 10). In this sodium-bound conformation, the side chain of E34 is oriented towards D214. This
265 conformation is present in the ground and L states of *BcXeR* at cryo conditions but could not be found at room
266 temperature (Fig. 3A; Supplementary Note 3). Sodium binding in the PTG reflects the deprotonated state of
267 the group. Thus, the sodium-bound conformation unequivocally corresponds to the deprotonated PTG.
268 Therefore, in the ground and in the L states the PTG is deprotonated, which also agrees well with FTIR data⁴.

269

270 In the second conformation of the PTG there is no sodium ion and E34 is reoriented towards the cytoplasm
271 (Fig. 3A; Extended Data Fig. 10). An additional water molecule was found between the E34 and D214
272 residues. This water molecule is located within 0.8 Å from the position of the sodium ion in the sodium-bound
273 conformation; however, due to the flip of E34 it is only coordinated with four oxygens with a mean O-O
274 distance of 2.8 Å, characteristic to a normal H-bond²². This sodium-free conformation is found in the M state
275 (Fig. 3A). The protonation state of the PTG in the sodium-free conformation is an intriguing and non-trivial
276 question. We determined the structures of the ground state of *BcXeR* in a wide range of pH values (5.2-8.2,
277 Extended Data Fig. 10; Supplementary Note 3). The sodium-free conformation of PTG remains unmodified
278 suggesting that this conformation reflects both deprotonated and protonated forms, which, however, cannot
279 be structurally distinguished, at least at 2 Å resolution (Supplementary Note 3).

280

281 Nevertheless, the sodium dissociation from the PTG upon the L-to-M state transition might be a signature of
282 the proton transfer to the group at this stage of the photocycle (Fig. 3A). Since the RSB is deprotonated upon
283 the L-to-M state transition and there is a continuous H-bond chain connecting the RSB to PTG in the L state,
284 we assume that the proton is relocated from the RSB towards the cytoplasm via this linear H-bond chain (Fig.
285 3A). Thus, the proton released from the RSB is transferred through the proton wire and is likely stored at the
286 PTG in the M state. The direct proton transfer to the cytoplasm from the RSB with the formation of the M
287 state cannot be excluded but is unlikely, according to our time-resolved spectroscopy experiments (Extended
288 Data Fig. 2H).

289
290 Next, the extracellular side of *BcXeR* remains almost identical to that in the ground and L states (Fig. 3D).
291 The H-bond network is only slightly modified in the region between H6 and R70 (Fig. 3D; Extended Data
292 Fig. 7C,D). Namely, while one water molecule Wat402 is found in a double conformation in the ground and
293 L states, indicating its weak interaction with Y49 in only half of the protein molecules, two separate water
294 molecules are well-resolved in the M state (Fig. 3D; Extended Data Fig. 7C-E). This minor rearrangement
295 provides a stronger connection between Y49 and Wat402 in the M state, resulting in an almost continuous H-
296 bond chain from the RSB to the large water-filled concavity exposed to the extracellular bulk (Fig. 3D). We
297 suggest that this H-bond chain serves as a pathway, a proton wire, for the RSB reprotonation. It should be
298 noted that although the retinal is in a 13-*cis* conformation in the M state, the RSB is located already very close
299 to its position in the ground state and is within an H-bond distance from Wat401 (Fig. 3D). Thus, the RSB is
300 accessible for reprotonation from the extracellular bulk. However, spectroscopic data shows that the
301 reprotonation of the RSB proceeds slowly in *BcXeR* and in a pH-independent manner (Fig. 1B; Extended
302 Data Fig. 2H). Therefore, we suggest that the proton translocation from the extracellular side to the RSB
303 requires the formation of a continuous proton wire, which, in turn, is the time-limiting step of the inward
304 proton transport process.

305

306 **Gating of inward proton translocation in *BcXeR***

307

308 Notably, in the M state of *BcXeR* the W173 side chain is largely flipped to avoid steric conflict with the 13-
309 *cis* retinal and is oriented nearly perpendicular to its initial position (Fig. 2B; Fig. 3C; Supplementary Movie
310 1). L169 is flipped towards helix G to allow the rearrangement of W173. The conformation of W173 in the M
311 state of *BcXeR* is stabilized by its water-mediated H-bonding to D107 (Fig. 3C). In contrast, W182 in *HsBR*
312 (analog of W173 of *BcXeR*) preserves its orientation throughout the photocycle^{14,18,19} (Extended Data Fig.
313 9C). We suggest that, in *BcXeR*, W173 can be rotated since there is sufficient place near the T143 residue for
314 this large-scale movement. The reorientation of W173 is also provided by the flip of L169. In *HsBR*, T143 is
315 substituted by a larger residue L152, which would cause a steric conflict with W182 in the rotated orientation.
316 Moreover, there is a T178 residue at the position of L169 stabilized by an H-bond to a water molecule¹⁹. Thus,
317 we may speculate that in *HsBR* the flip of W182 is blocked by the presence of the L152 and T178 residues.

318 However, further investigations are needed to probe this hypothesis. The pedal-like pushing of the retinal on
319 the W182 in *HsBR* is claimed to trigger a 9 Å tilting of the cytoplasmic parts of E and F helices resulting in
320 the opening of the protein for proton uptake through its cytoplasmic part to the RSB¹⁴ (Extended Data Fig.
321 9C). In contrast, an unusual flexibility of the retinal binding pocket of *BcXeR* and particularly that of W173
322 allows mitigation of the large-scale structural rearrangements caused by retinal isomerization resulting in
323 almost no movements of the transmembrane helices (Extended Data Fig. 9C). This helps to maintain the
324 hydrophobic gate at the cytoplasmic part of *BcXeR* in the closed state after proton translocation from the RSB
325 to the PTG, preventing the backflow of the protons during the M-to-ground state transition. Thus, we suggest
326 that the notable rearrangements of W173 and its surroundings are one of the key determinants of the
327 unidirectional proton transport in bacterial XeRs. Indeed, D107, stabilizing the conformation of W173 in the
328 M state, is shown to be essential for proton pumping in *BcXeR* (Extended Data Fig. 5; Supplementary Note
329 2). Moreover, D107 is conserved within bacterial XeRs, but is substituted by glutamine in ASR-like sensors
330 which lack proton-pumping activity²³. Importantly, this is a noteworthy difference between the internal
331 organization of bacterial XeRs and ASR-like sensors (Extended Data Fig. 5).

332

333 Finally, our time-resolved crystallography data show that the M-to-ground transition proceeds directly without
334 structurally distinguishable intermediate states. This suggests that the following events occur simultaneously:
335 the retinal returns to the all-*trans* configuration; the RSB is reprotonated from the extracellular side; the W173
336 and its surroundings are relocated back to the ground conformations; and the PTG releases the proton to the
337 cytoplasm and re-binds the sodium ion. In contrast to *HsBR*, no large-scale structural rearrangements
338 accompany these events, which is also supported by the absence of the late N- and O-like states in XeRs
339 (Extended Data Fig. 2).

340

341 **Conclusion**

342

343 In this work we used bacterial xenorhodopsin *BcXeR* to study the molecular mechanism of light-driven inward
344 proton translocation (Fig. 4; Supplementary Note 5; Supplementary Video 2). We utilized synchrotron
345 radiation to capture high-resolution snapshots of the early sub-ms and the subsequent ms-time-scale reactions
346 in *BcXeR* using time-resolved serial crystallography. Thus, this work extends the time range of X-ray
347 synchrotron time-resolved crystallography on microbial rhodopsins to hundreds of microseconds, which is by
348 an order of magnitude shorter than reported earlier¹⁴. Our work is a basis for further time-resolved studies of
349 light-sensitive proteins at synchrotrons and X-ray Free-Electron Lasers. Such further structural information
350 on *BcXeR* in the ps-ns time range would be beneficial for the deeper understanding of its primary
351 photoreactions.

352 Together with the recent data on the outward proton pump *HsBR*¹⁹, the present work demonstrates that proton
353 wires are at the core of both outwardly and inwardly directed proton transfer through the membrane serving
354 as pathways within the proteins. In *BcXeR*, only a minor reorganization of the proton wires accompanied by
355 subsequential opening of two internal gates in the course of the protein function is sufficient for inwardly
356 directed proton translocation. Our results suggest that mutations at the extracellular gate of *BcXeR* could
357 accelerate the protein photocycle, as is required for the engineering of a potent IPP-based optogenetic tools⁶.
358 Mutational, bioinformatical, and structural analysis of XeRs suggests that the proton wire mechanism is
359 fundamental and characteristic not only for bacterial, but also for archaeal rhodopsins (Supplementary Note
360 6). We also speculate that proton wires could be universal for other proton transporters. At the same time, we
361 demonstrate that *BcXeR* differs from outward proton pumps and has a specific cavity-gate-cavity-gate-water
362 pore structure resembling that of channelrhodopsin-2 (ChR2)²⁴. However, in contrast to channelrhodopsins,
363 where all gates are opened simultaneously, in XeRs the gates open sequentially, resulting in unidirectional
364 proton translocation.

365

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393

394 **Author Contributions Statement:**

395 K.K., A.A., and F.T. contributed equally and either has the right to list himself first in bibliographic
396 documents. A.A. found a gene bioinformatically, proposed it as a target and performed initial functional tests
397 in *E. coli*; F.T. did cloning, expression, and purification of the protein and its mutants; F.T. did functional
398 studies of the proteins in *E.coli* cell suspension; A.A. supervised the expression and purification; S.S.
399 performed studies of electrogenic behavior of *BcXeR* in proteoliposomes; D.S. and F.T. performed flash-
400 photolysis studies on protein in solution and in crystals; I.C. supervised flash-photolysis experiments; K.K.
401 crystallized the protein; R.A. helped with the crystallization; S.B., R.A., and A.Roy. performed cryotrapping
402 of the intermediates at the *icOS* Lab and recorded the spectra of the states in crystals; K.K. performed

403 cryotrapping of the intermediates in crystals at the P14 beamline; M.A. installed the laser setup at the P14
404 beamline for the cryotrapping and TR studies; K.K. collected and processed the diffraction data, solved and
405 refined the structures; G.B. helped with data collection, structure refinement, and analysis; M.N. provided
406 online monitoring of X-ray diffraction images during TR crystallography data collection at 1 kHz; D.v.S.
407 provided scripts for serial data processing and help with serial data analysis; V.S., N.I., E.B., T.R.S., A.Rog.,
408 F.R.-V., V.B., and G.B. helped with data analysis; A.K. animated the transitions between the intermediates
409 under the supervision of I.G.; A.A. performed bioinformatic analysis with the help of R.R.; F.T., A.A., K.K.,
410 and V.G. analyzed the results and prepared the manuscript with the important contribution of G.B. and with
411 input from all the other authors. V.G. supervised and analyzed the results of experiments and proposed the
412 conception of molecular mechanism of inward proton transport.

413

414 **Competing Interest Statement:**

415 Authors declare no competing interests.

416

Table 1. Data collection and refinement statistics on *BcXeR* at 100 K.

	Ground state, pH 8.2	L state, pH 8.2	M state, pH 8.2	Ground state, pH 7.0	M state, pH 7.0	Ground state, pH 5.2	M state, pH 5.2	Ground state, pH 7.6, no Na	M state, pH 7.6, no Na
PDB ID	<i>7ZMY</i>	<i>7ZN3</i>	<i>7ZN0</i>	<i>7ZN8</i>	<i>7ZN9</i>	<i>7ZNA</i>	<i>7ZNB</i>	<i>7ZNC</i>	<i>7ZND</i>
Data collection									
Space group	P 21 21 21								
Cell dimensions									
<i>a, b, c</i> (Å)	68.24, 109.57, 119.47	68.39, 109.49, 119.39	68.50, 109.50, 119.00	68.60, 109.60, 119.30	68.90, 109.50, 119.40	68.57, 109.59, 119.62	68.50, 109.50, 119.00	68.45, 109.63, 118.46	69.09, 109.36, 119.31
α, β, γ (°)	90, 90, 90	90, 90, 90	90, 90, 90	90, 90, 90	90, 90, 90	90, 90, 90	90, 90, 90	90, 90, 90	90, 90, 90
Resolution (Å)	49.799-1.700 (1.742-1.700)*	49.763-1.600 (1.739-1.600)*	49.738-1.700 (1.813-1.700)*	49.798-2.200 (2.293-2.200)*	49.767-2.300 (2.370-2.300)*	49.817-1.800 (1.916-1.800)*	49.738-1.900 (1.963-1.900)*	44.790-1.700 (1.780-1.700)*	59.792-1.985 (2.168-1.985)*
R_{merge} , %	7.9 (335.3)	7.6 (283.5)	8.3 (346.9)	16.3 (377.5)	17.7 (246.4)	10.4 (337.6)	7.8 (194.8)	9.5 (280.8)	11.4 (163.6)
$I / \sigma I$	15.9 (0.9)	15.9 (1.1)	16.5 (0.8)	10.9 (0.8)	9.6 (1.3)	14.1 (0.8)	17.5 (1.5)	15.0 (0.9)	13.7 (1.3)
Completeness (%)	92.0 (65.4)	92.3 (75.8)	93.9 (82.4)	85.5 (66.6)	96.3 (78.3)	94.5 (88.6)	95.3 (79.6)	96.2 (86.3)	90.2 (77.0)
Redundancy	13.8 (14.1)	13.7 (13.0)	13.0 (13.1)	13.1 (12.0)	13.2 (12.8)	13.1 (13.1)	13.1 (12.2)	13.3 (12.5)	12.0 (7.2)
Refinement									
Resolution (Å)	20-1.7	20-1.6	20-1.7	20-2.2	20-2.3	20-1.8	20-1.9	20-1.7	20-1.98
No. reflections	86,518	78,514	65,890	35,350	36,269	62,007	59,830	78,775	40,266
$R_{\text{work}} / R_{\text{free}}$	17.4/20.3	20.4/21.6	19.9/22.7	19.9/23.8	21.6/25.3	19.6/23.0	19.6/22.7	18.5/20.8	25.0/28.2
Occupancy of the intermediate, %	-	35	100	-	100	-	100	-	100
No. atoms									
Protein	5301	5490	5351	5301	5342	5304	5342	5362	5236
Retinal	60	120	60	60	60	60	60	60	60
Sodium	3	3	-	3	-	3	-	-	-
Lipid	737	599	627	732	626	720	627	807	802
Water	283	246	241	281	243	289	240	375	75
<i>B</i> -factors									
Protein	33	30	34	36	38	31	34	27	32
Retinal	27	28	29	27	32	25	29	20	26
Sodium	26	25	-	36	-	25	-	-	-
Lipid	60	47	58	58	60	58	58	54	60
Water	46	39	44	48	45	45	44	42	33
R.m.s. deviations									
Bond lengths (Å)	0.006	0.014	0.002	0.004	0.002	0.004	0.002	0.003	0.002
Bond angles (°)	1.223	1.649	1.019	1.091	1.019	1.131	1.022	1.078	0.994

*Values in parentheses are for highest-resolution shell.

Table 2. Serial crystallography data collection and refinement statistics on BcXeR at room temperature.

	Dark state, pH 8.2 7.5 ms exposure	7.5-15.0 ms snapshot, pH 8.2	Dark state, pH 8.2 500 μ s exposure	250-750 μ s snapshot, pH 8.2
PDB ID	7ZNE	7ZNI	7ZNG	7ZNH
Data collection				
Space group	P 21 21 21	P 21 21 21	P 21 21 21	P 21 21 21
Cell dimensions				
<i>a</i> , <i>b</i> , <i>c</i> (Å)	71.50, 111.50, 119.30	71.50, 111.50, 119.30	71.91, 111.86, 119.52	71.91, 111.86, 119.52
α , β , γ (°)	90, 90, 90	90, 90, 90	90, 90, 90	90, 90, 90
Indexed patterns	67,912	12,449	17,997	17,526
Indexing rate, %	33.9	10.4	25.0	24.3
Resolution (Å)	45.8-2.1 (2.17-2.10)*	45.8-2.2 (2.28-2.20)*	45.96-2.30 (2.38-2.30)*	45.96-2.30 (2.38-2.30)*
<i>R</i> _{split} , %	6.6 (156.74)	14.3 (198.8)	19.2 (260.1)	19.6 (292.3)
<i>I</i> / σ <i>I</i>	9.0 (0.6)	4.5 (0.5)	4.0 (0.4)	3.9 (0.4)
Completeness (%)	100.0 (100.0)	100.0 (100.0)	100.0 (100.0)	100.0 (100.0)
Redundancy	926.8 (629.9)	177.3 (121.1)	526.2 (360.9)	507.4 (348.2)
CC*, %	99.9 (72.0)	99.4 (42.2)	99.6 (42.5)	99.6 (41.4)
Refinement				
Resolution (Å)	20-2.1	20-2.2	20-2.3	20-2.3
No. reflections	54,544	47,347	41,079	40,880
<i>R</i> _{work} / <i>R</i> _{free}	18.7/21.5	18.5/21.4	21.8/25.1	22.0/24.8
Occupancy of the intermediate, %	-	62	-	20
No. atoms				
Protein	5189	5507	5194	5383
Retinal	60	120	60	120
Lipid	528	566	540	540
Water	75	95	75	75
<i>B</i> -factors				
Protein	55	56	48	49
Retinal	46	50	38	36
Lipid	63	65	66	67
Water	62	63	55	54
R.m.s. deviations				
Bond lengths (Å)	0.002	0.010	0.002	0.006
Bond angles (°)	1.050	1.428	1.040	1.250

*Values in parentheses are for highest-resolution shell.

422

423

424

425

427 **Figure 1 | Functional, spectroscopical, and structural features of *BcXeR*.** **A.** Light-induced pH changes in
428 suspension of *E. coli* expressing *BcXeR* in non-buffered salt solutions (salt molarity is 100 mM each). Solid
429 lines represent experiments in the absence of any protonophore, dashed lines show pH changes in the presence
430 of 30 μM of protonophore carbonyl-cyanide *m*-chlorophenyl-hydrazone (CCCP). The yellow area indicates
431 the period of time when the light was on. **B.** Photocycle scheme of *BcXeR*. **C.** Time-resolved traces of *BcXeR*
432 in solution and in crystals at 480 nm characteristic for the L state. **D.** Time-resolved traces of *BcXeR* in
433 solution and in crystals at 390 nm characteristic for the M state. **E.** Overall side view of the *BcXeR* protomer.
434 **F.** Detailed view of the cytoplasmic side of *BcXeR* in the ground state. The proton transit group (PTG) is
435 highlighted in yellow. The hydrophobic gate between the RSB and S211 is shown with a red arrow. **G.**
436 Detailed view of the extracellular side of *BcXeR*. The gate near Y49 is shown with a red arrow. Cavities were
437 calculated using HOLLOW²⁵ and are shown with a blue surface. Hydrogen bonds are shown with black dashed
438 lines.

439
440 **Figure 2 | Obtaining of *BcXeR* structures in the L and M intermediate states.** **A.** A global fit of flash-
441 photolysis data revealed the presence of three spectrally distinct states of *BcXeR*: K, L, and M. Time regions
442 captured in two time-resolved (TR) experiments are highlighted in red and in yellow for the L and M states,
443 respectively. **B.** Examples of $F_o^{\text{light}} - F_o^{\text{dark}}$ difference electron density maps in the region of retinal obtained
444 with TR and cryotrapping methods. The maps are contoured at the level of 3.5σ . The maps of the L state
445 obtained with TR approach are contoured at 3.0σ . In the latter case the maps appear weaker due to a lower
446 resolution and a lower occupancy of the intermediate. **C.** UV-visible absorption spectra measured *in crystallo*
447 at 100 K of the ground (green solid), the L (red solid), and M (yellow solid) states (insets in the right panel:
448 photos of the same frozen *BcXeR* crystal in the cryoloop before and after the laser illumination, near the
449 corresponding spectra). Spectra of the corresponding states of the *BcXeR* photocycle in solution are shown
450 with dashed lines. **D.** Evolution of the occupancy of the M state determined from the TR crystallography
451 experiments (red bars). Red bars indicate the experimental error of the M state occupancy determination
452 ($\pm 2\%$). The evolution is in line with the difference absorption evolution at 390 nm (yellow line) corresponding
453 to the M state population determined by TR spectroscopy of the *BcXeR* crystals.

454
455 **Figure 3 | Structural rearrangements in *BcXeR* in the L and M states.** **A.** Structural rearrangements at the
456 cytoplasmic part of *BcXeR* in the course of the photocycle. Blue arrows indicate the key conformational
457 changes. The wide light blue arrow indicates the putative pathway for proton translocation. Three water
458 molecules appearing in the L state and forming a transient H-bond chain from the RSB to S211 are highlighted
459 in yellow. The proton at the PTG in the M state is highlighted in light blue. The key hydrogen atoms are shown
460 as white spheres. **B.** Structural rearrangements in the retinal and retinal binding pocket upon the ground-to-L
461 transition. Black arrows indicate the most notable conformational changes between the ground (green) and
462 the L (salmon) states. **C.** Structural rearrangements in the retinal and retinal binding pocket upon the L-to-M
463 transition. Black arrows indicate the most notable conformational changes between the L (salmon) and the M
464 (yellow) states. H-bonds connecting D107 and W173 via water molecule Wat501' are shown with yellow
465 dashed lines. **D.** Extracellular side of *BcXeR* in the ground (green), L (salmon), and M (yellow) states. The
466 continuous H-bond connecting the RSB to the concavity (highlighted with light blue surface) exposed to the
467 extracellular bulk is shown with yellow dashed lines. The blue arrow indicates the additional water molecule
468 Wat403 found only in the M state. The wide light blue arrow indicates the putative pathway for RSB
469 reprotonation.

470
471 **Figure 4 | Proposed mechanism of inward proton transport by *BcXeR*.** The putative scheme of the key
472 structural rearrangements during the *BcXeR* photocycle is shown. Inactive regions of the proton wire
473 propagating through the inside of the protein are shown with yellow thick dashed lines. Active regions of the
474 wire are shown with green thick dashed lines. Positively and negatively charged groups are colored blue and
475 red, respectively. The protein surface is shown in light gray. Water molecules involved in the proton wire are
476 shown as red spheres. Sodium ions are shown as purple spheres. The retinal cofactor is colored cyan. Central
477 hydrophobic and weak extracellular gates are marked and indicated with arrows. The states of the gates are
478 indicated with the color of the arrow and a corresponding description (red - closed; green - opened; yellow -
479 intermediate). Blue arrows indicate putative proton translocation pathways. The lipid membrane is shown in
480 blue.

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535 **Methods**

536

537 **Phylogenetic analysis of rhodopsin genes**

538 To retrieve xenorhodopsins from available sequence data, a two-step procedure based on sequence similarity
539 search and Hidden Markov Models was followed. First, bona fide XeR amino acid sequences were queried
540 against the non-redundant database at NCBI using Diamond v0.9.4.105²⁶ and applying the substitution matrix
541 BLOSUM45 to retrieve distantly related proteins with the E-value set to 1e-5. Next, all genomes whose
542 proteins gave hits with bona fide XeR were downloaded and all encoding rhodopsins (either XeR-related or
543 other types) were identified using HMMER version v3.3.2 (<http://hmmer.org>) and further confirmed by
544 checking for the presence of 7 transmembrane domains using TMHMM²⁷.

545 The obtained set of putative xenorhodopsins was aligned with ClustalO. The phylogenetic tree was
546 constructed in iTol v.6²⁸. Structurally important regions were identified manually according to the obtained
547 high-resolution structure of *BcXeR*, taking into account structure-functional information on *NsXeR*, ASR, and
548 other published xenorhodopsins. The data and figures, showing conserved amino acids in the structurally
549 important regions, were calculated and designed in an in-house Python Jupyter notebook and Wolfram
550 Mathematica.

551

552 **Cloning**

553 *BcXeR* coding DNA was optimized for *E.coli* codons using GeneArt (Thermo Fisher Scientific). Genes were
554 synthesized commercially (Eurofins). For protein expression and measurements of pump activity pET15b
555 plasmid with 6xHis-tag at the C-terminal was used. Site-directed mutagenesis with PCR, phosphorylation and
556 ligation was performed.

557

558 **Protein expression, solubilization, and purification**

559 *E.coli* cells were transformed with pET15b plasmid containing the gene of interest. Transformed cells were
560 grown at 37°C in shaking baffled flasks in an autoinducing medium ZYP-5052²⁹, containing 10 mg/L
561 ampicillin. They were induced at an OD₆₀₀ of 0.6-0.7 with 1 mM isopropyl-β-D-thiogalactopyranoside
562 (IPTG). Subsequently, 10 μM all-*trans*-retinal was added. Incubation continued for 3 hours. The cells were
563 collected by centrifugation at 5000g for 20 min. Collected cells were disrupted in an M-110P Lab
564 Homogenizer (Microfluidics) at 25,000 p.s.i. in a buffer containing 20 mM Tris-HCl, pH 7.5, 5% glycerol,
565 0.5% Triton X-100 (Sigma-Aldrich) and 50 mg/L DNase I (Sigma-Aldrich). The membrane fraction of the
566 cell lysate was isolated by ultracentrifugation at 125,000g for 1 h at 4°C. The pellet was resuspended in a
567 buffer containing 50 mM NaH₂PO₄/Na₂HPO₄, pH 7.5, 0.15 M NaCl and 1% DDM (Anatrace, Affymetrix)
568 and stirred overnight for solubilization. The insoluble fraction was removed by ultracentrifugation at 125,000g
569 for 1h at 4 °C. The supernatant was loaded on an Ni-NTA column (Qiagen), and protein was eluted in a buffer
570 containing 50 mM NaH₂PO₄/Na₂HPO₄, pH 7.5, 0.15 M NaCl, 0.4 M imidazole and 0.05% DDM. The eluate
571 was subjected to size-exclusion chromatography on a 125-ml Superdex 200 PG column (GE Healthcare Life

572 Sciences) in a buffer containing 50 mM NaH₂PO₄/Na₂HPO₄, pH 7.5, 0.15 M NaCl and 0.05% DDM. In the
573 end protein was concentrated to 70 mg/ml for crystallization and -80°C storage.

574

575 **Measurements of pump activity in the *E. coli* cells suspension**

576 The proteins (*BcXeR*, *AaS*R, *PpaSR*) were expressed as described above. The cells were collected by
577 centrifugation at 4000g for 15 min and were washed three times with an unbuffered salt solution, with 30-min
578 intervals between the washes to allow for exchange of the ions inside the cells with the bulk. After that, the
579 cells were resuspended in an unbuffered salt solution and adjusted to an OD₆₀₀ of 8.5. The measurements were
580 performed in 3-ml aliquots of stirred cell suspension kept at ice temperature (0.3-1.0°C). The cells were
581 illuminated using a halogen lamp (Olympus KL2500 LCD), and the light-induced pH changes were monitored
582 with a pH meter (SevenEasy, METTLER TOLEDO). The measurements were repeated under the same
583 conditions after the addition of 30 μM CCCP protonophore.

584

585 **Spectroscopic characterization and time-resolved absorption spectroscopy**

586 The absorption spectra in pH range were obtained with Biotek Synergy HT. The setup for time-resolved
587 absorption spectroscopy was as follows. A brilliant B Nd:YAG laser (Quantel, France) was used for providing
588 pulses of 4 ns duration at a 532 nm wavelength and an energy of about 2 mJ/pulse. Samples were placed
589 between two collimated mechanically coupled monochromators (LSH-150, LOT, Germany). The probing
590 light (Xe-arc lamp, 75 W, Hamamatsu, Japan) passed the first monochromator and the sample and arrived
591 after a second monochromator at a PMT detector (R12829, Hamamatsu, Japan). Two digital oscilloscopes
592 (Keysight DSO-X 4022A) were used to record the traces of transient transmission changes in two overlapping
593 time windows.

594 In this setup we recorded transient absorption changes starting from 700 ns after the laser pulse until the
595 completion of the photocycle, at each wavelength from 330 to 730 nm in 10-nm steps, 25 laser pulses were
596 averaged to improve the signal-to-noise ratio. We analyzed the data sets using the global multiexponential
597 nonlinear least-squares fitting program MEXFIT as reported earlier.

598

599 **Electrometric Time-Resolved Measurements of the Membrane Potential**

600 The generation of the transmembrane electric potential difference $\Delta\Psi$ was observed using a direct
601 electrometric setup with a time resolution of 100 ns as described earlier.^{30,31} Proteoliposomes with protein
602 were fused with the surface of a collodion phospholipid-impregnated film (a measuring membrane) separating
603 two sections of the measuring cell filled with a buffer solution. The potential difference $\Delta\Psi$ across the
604 measuring membrane were detected by Ag⁺/AgCl electrodes immersed in the solution at both sides of
605 membrane. This membrane should be thin enough and possess large electric capacitance (about 5 nF) for
606 detecting fast charge translocation events. Typical resistance of the measuring membrane was 2 to 3 GOhm.
607 A Nd-YAG laser (YG-481, $\lambda = 532$ nm, pulse half-width 12 ns, pulse energy up to 40 mJ; Quantel) was used

608 as a light source of the light pulses. In the process of the light-driven proton transfer, rhodopsin creates $\Delta\Psi$
609 across the vesicle membrane, which is proportionately divided by the measuring membrane.

610

611 **Proteoliposomes preparation**

612 Protein containing proteoliposomes were prepared as described previously³². Soybean asolectin was
613 dissolved (1% w/v in final solution) in chloroform, which was then removed using a rotary evaporator (25°C)
614 and a vacuum pump. Asolectin film was resuspended in 0.1 M NaCl, 2% (w/v) sodium cholate to the final
615 asolectin concentration of 1% (w/v). Suspension was sonicated for 5 min at 4°C and centrifuged. Supernatant
616 was sonicated for 1 min and then solubilized protein was quickly added to the final concentration 0.7 mg/ml
617 which was followed by adding detergent-absorbing beads (Amberlite XAD-2, Sigma-Aldrich). The beads
618 were exchanged with new ones several times (one of them was kept overnight and the others were close to an
619 hour each).

620

621 **Crystallization**

622 The crystals of *BcXeR* were grown with an *in meso* approach¹⁰, similar to that used in our previous works^{33,34}.
623 In particular, the solubilized protein (60 mg/ml) in the crystallization buffer was mixed with premelted at 42°C
624 monoolein (MO, Nu-Chek Prep) in a 3:2 ratio (lipid:protein) to form a lipidic mesophase. The mesophase was
625 homogenized in coupled syringes (Hamilton) by transferring the mesophase from one syringe to another until
626 a homogeneous and gel-like material was formed.

627 For the growing of single crystals of *BcXeR* used for determination of the cryo structures, 150 nl drops of a
628 protein–mesophase mixture were spotted on a 96-well LCP glass sandwich plate (Marienfeld) and overlaid
629 with 400 nL of precipitant solution by means of the NT8 crystallization robot (Formulatrix). The best crystals
630 were obtained with a protein concentration of 20 mg/ml (in the water part of the mesophase). The crystals for
631 the determination of the *BcXeR* structures in the ground and M states at pH 5.2, 7.0, and 8.2 and in the L state
632 at pH 8.2 were obtained using 0.8 M Na/K-Pi pH 8.2 as a precipitant. The crystals for the determination of
633 the *BcXeR* structures in the ground and M state at pH 7.6 in the absence of sodium were obtained using 1.2
634 M (NH₄)₃PO₄ pH 7.6 as a precipitant. The crystals were grown at 22°C and appeared in 2 months. Once the
635 crystals reached their final size, crystallization wells were opened, and drops containing the protein-
636 mesophase mixture were covered with 100 µl of the respective precipitant solution. For the data collection,
637 harvested crystals were incubated for 5 min in the respective precipitant solutions. For obtaining the structures
638 of *BcXeR* at pH 5.2 the crystallization well was covered with a solution of 1.2 M Na/K-Pi pH 5.2. The crystals
639 were soaked for 24 hours with exchanging the buffer three times. Crystals were then harvested using
640 micromounts (Mitegen, USA), flash-cooled and stored in liquid nitrogen.

641 For time-resolved serial crystallography, crystals of *BcXeR* were grown in 200-µl PCR tubes. For that, 0.8-
642 1.6 µl of precipitant solution (3.2 M Na/K-Pi pH 8.2) were dispensed at the bottom of the PCR tube and
643 covered with 12 µl of the mesophase from the Hamilton syringe. The PCR tube was quickly closed and
644 centrifuged for 1 min at 6000g at 22°C to sediment the phase to the bottom of the PCR tube. The PCR tubes

645 were kept at 22°C. The crystals of *BcXeR* appeared within 1 week and reached 30x20x5 μm^3 in size. The
646 crystals from several PCR tubes were collected into a single PCR tube using centrifugation for 2 min at
647 20,800g and 22°C. Then, the bottom part of the PCR tube with the mesophase was cut and inserted upside
648 down into the sealed 200 μl pipette tip. The tip was sealed at the very end using the gas burner. The tip with
649 the inserted PCR tube part was then centrifuged at 6000g and 22°C for 2 minutes using a 1.5 ml tube as an
650 adaptor for the centrifuge rotor. After the centrifugation, the mesophase was found in the sealed tip. The empty
651 PCR tube part was removed and the tip was covered from the wide end with Parafilm to avoid dehydration of
652 the crystals. The plunger was removed from an empty clean 100 μl Hamilton syringe and the syringe was
653 sealed from the side of the needle using Parafilm. The tip with mesophase was cut from the narrow end and
654 inserted into the back side of the prepared Hamilton syringe. The syringe with the inserted tip were fixed in
655 the 50 ml falcon tube; for that, the syringe was wrapped into the paper towel to fit tightly into the falcon tube.
656 Then, the tube with the syringe were centrifuged at 6000g and 22°C for 3 minutes to transfer the mesophase
657 from the tip into the syringe. After the centrifugation, the tip was removed, the syringe was coupled to another
658 clean 100 μl Hamilton syringe and the plunger was slowly inserted into the first syringe. Thus, the phase was
659 slowly transferred into the second syringe. In the case of excessive precipitant solution and/or air, the system
660 was uncoupled for their removal and then coupled back to homogenize the mesophase with crystals. The
661 mesophase was homogenized before the experiment using an LCP-coupler.

662

663 **Accumulation of the intermediate state in *BcXeR* crystals**

664

665 Absorption spectra of *BcXeR* in solution were collected using a UV-2401PC spectrometer (Shimadzu, Japan).
666 The spectroscopic characterization of the L and M states in *BcXeR* crystals was performed at the *icOS* Lab
667 located at the ESRF³⁵. Briefly, UV-visible absorption spectra were measured using as a reference light that of
668 a DH-2000-BAL deuterium-halogen lamp (Ocean Optics, Dunedin, FL) connected to the incoming objective
669 via a 200 μm diameter fiber, resulting in a 50 μm focal spot on the sample, and a QE65 Pro spectrometer
670 (Ocean Optics, Dunedin, FL) connected to the outgoing objective via a 400 μm diameter fiber. The actinic
671 light comes from 532-nm or 633-nm lasers (CNI Laser, Changchun, P.R. China) coupled to a 1000 μm
672 diameter fiber which is connected to the third objective whose optical axis is perpendicular to those of the
673 ingoing and outgoing objectives. All spectra (100 ms acquisition time averaged 20 times) were collected on
674 crystals flash-cooled in liquid nitrogen and kept under a cold nitrogen stream at 100 K.

675 For the accumulation and cryotrapping of the L state, a crystal was warmed to 170 K and put under constant
676 633-nm-laser illumination for 6 min at 170 K. During the illumination the crystal was rotated 3 times by 90
677 degrees to illuminate it from all sides (1.5 minutes for each side). Then the laser was switched off and the
678 crystal was cooled back to 100 K. For the accumulation of the L state the laser power density was 32 W/cm^2
679 at the position of the sample. A UV-visible absorption spectrum was subsequently recorded to show the
680 slightly blue-shifted absorption maximum characteristic of the L state of *BcXeR*. The laser power was adjusted
681 to maximize the fraction of the accumulated L state; the absence of destructive effect of the laser on crystals

682 was justified by the remaining diffraction quality after the cryotrapping procedure as well as by the full return
683 of the protein to the ground state after short test annealing of the crystal to 293 K after cryotrapping of the L
684 state.

685 For the accumulation and cryotrapping of the M state, the crystal was originally kept at 100 K. It was then
686 illuminated by a 532-nm-laser with the nitrogen stream simultaneously blocked for 2 seconds. Due to the short
687 illumination time the crystal was not rotated during the illumination. For better illumination, the crystal was
688 oriented with the largest plane perpendicular to the laser beam. The laser was then switched off once the
689 crystal was back at 100 K. For the accumulation of the M state a laser power density of 10 W/cm² at the
690 position of the sample was used. A UV-visible absorption spectrum was subsequently recorded to show the
691 strongly blue-shifted absorption maximum characteristic of the M state of *BcXeR*. The laser power was
692 adjusted to maximize the fraction of the accumulated M state; the absence of destructive effect of the laser on
693 crystals was justified by the remaining diffraction quality after the cryotrapping procedure as well as by the
694 full return of the protein to the ground state after short test annealing of the crystal to 293 K after cryotrapping
695 of the M state.

696 The mean size of the crystals was 200x100x20 μm³. The plate-like crystals were oriented so that the largest
697 plane (200x100 μm²) was perpendicular to the laser beam. The laser beam was focused to the size of 200x200
698 μm² (1/e²).

699 A similar setup for the accumulation and cryotrapping of the intermediate states in the *BcXeR* crystals was
700 established at the P14 beamline of the PETRAIII synchrotron source (Hamburg, Germany) for X-ray
701 diffraction data collection. Also, the same accumulation procedure was applied to crystals at *icOS* and P14
702 beamline. In the case of the M state the trapping of the strongly blue-shifted M intermediate state characteristic
703 for the deprotonated RSB was directly visible by a notable change of the crystal color in the micro loop
704 before/after the cryotrapping procedure.

705

706 **Single-crystal diffraction data collection and treatment**

707 Single-crystal X-ray diffraction data of all structures except the one of the ground state of *BcXeR* obtained at
708 pH 7.6 in the absence of sodium were collected at the P14 beamline of PETRAIII (Hamburg, Germany) using
709 an EIGER X 16M and EIGER2 X 16M CdTe detectors. The data of the ground state of *BcXeR* at pH 7.6 in
710 the absence of sodium were collected at the ID23-1 beamline of the ESRF (Grenoble, France) using a
711 PILATUS 6M-F detector. The data collection was performed using MxCube2 software. Diffraction images
712 were processed using XDS³⁶. The reflection intensities were scaled and merged using the Staraniso server³⁷.
713 There is no possibility of twinning for the crystals. In all cases, diffraction data from a single crystal were
714 used. The data statistics are presented in Table 1.

715

716 **Structure determination and refinement of the ground state of *BcXeR***

717 Initial phases for the ground state of trimeric *BcXeR* at pH 8.2 with sodium were successfully obtained in the
718 P2₁2₁2₁ space group by molecular replacement using MOLREP³⁸ from the CCP4 program suite³⁹ using the
719 1XIO structure of *Anabaena* sensory rhodopsin⁴⁰ as a search model. The initial models were iteratively refined
720 using REFMAC⁵⁴¹ and Coot⁴². The phases for all other structures of *BcXeR* determined in the course of the
721 study were determined using MOLREP with the phases of the ground state of *BcXeR* at pH 8.2 in the presence
722 of sodium.

723

724 **Structure refinement of the cryotrapped L and M intermediates of *BcXeR***

725 For the determination of the L state structure of *BcXeR* two datasets were collected using the same crystal.
726 First, data were collected from a part of the not-yet illuminated crystal. These data correspond to the ground
727 state of *BcXeR* (we refer to the structure factors corresponding to this dataset as F_o^{dark}). Then, the L state was
728 cryotrapped in the same crystal using the above-described protocol. Then, the second dataset was collected
729 from a laser illuminated but not yet X-ray exposed region of the crystal. These data correspond to the
730 intermediate state (we refer to the structure factors corresponding to this dataset as F_o^{light}). Although the F_o-F_c
731 and F_o^{light}-F_o^{dark} difference electron density maps clearly indicated structural rearrangements associated with
732 the intermediate state formation, the fraction of the L state was not sufficient for direct fitting of the model
733 into 2F_o-F_c electron density maps corresponding to the second dataset. Therefore, to build the initial model of
734 the L state we used extrapolated structure factors, calculated from two isomorphous datasets using linear
735 approximation as follows: $F_o^{\text{extrapolated}} = (1/\alpha) * (F_o^{\text{light}} - (1-\alpha) * F_o^{\text{dark}})$, where α is the occupancy of the L state.
736 The α value was screened while calculating the extrapolated maps from 0.1 to 0.5 with a step of 0.05, and was
737 selected to be 0.3 via an analysis of the electron density maps built using extrapolated structure factors. Then
738 the model of the L state was built manually in Coot using 2F_o^{extrapolated}-F_c electron density maps in accordance
739 with the F_o^{light}-F_o^{dark} difference electron density maps. The positions of residues 81, 136, 173, 205-210 of the
740 protein molecule and several internal water molecules were refined manually compared to the ground state as
741 identified by the strongest peaks at the F_o^{light}-F_o^{dark} difference electron density maps. Then, the new positions
742 of the above-mentioned residues and water molecules were added to the ground state model as a second
743 conformation (named conformation C in the final model of the L state). The combined model was
744 automatically refined against the original data using PHENIX. Only atom positions of the L state were refined;
745 the atom positions of the ground state were fixed. B-factor were also refined for all atoms. Analysis of the
746 resulting model and electron density maps showed consistency of the L state structure with the
747 crystallographic data and the F_o^{light}-F_o^{dark} difference electron density maps. The final occupancy of the L state
748 after refinement was 35%.

749 In the case of the M state at pH 8.2 in the presence of sodium, the occupancy of the intermediate was high as
750 also evidenced by the color of the crystals after the cryotrapping procedure (the crystal was almost completely

751 yellow, corresponding to the M state of *BcXeR*). Therefore, the structure of the M state was directly built
752 using $2F_o-F_c$ electron density maps. After several iterations of manual refinement in Coot and automated
753 refinement using REFMAC5, some peaks at the F_o-F_c difference electron density maps corresponding to a
754 small amount of the L state appeared near residues W173, L136, and S206 (Extended Data Fig. 7B); therefore,
755 these residues were fitted with double conformation in the final model of the M state, where conformations A
756 and B correspond to the M and L states, respectively. In the case of the M states at other pH values and sodium
757 concentrations the structure of the intermediate was refined iteratively in Coot and REFMAC5 using the model
758 of the M state at pH 8.2 in the presence of sodium as a starting model (Supplementary Table 1).

759

760 **Serial crystallography data collection and treatment**

761 Serial crystallography data of *BcXeR* was obtained at RT at the P14 beamline of PETRA III (Hamburg,
762 Germany) using an EIGER2 X 16M CdTe detector and a 2 (vertical) x 7 (horizontal) μm^2 sized beam at 12.7
763 keV (2×10^{12} and 2×10^{13} ph/s in the cases of experiments with 500 μs and 7.5 ms X-ray exposures,
764 respectively). The data collection was performed using MxCube2 software. In the case of the time-resolved
765 crystallography experiments with 7.5 ms X-ray exposures the X-ray beam was attenuated to 10% to reduce
766 the X-ray dose. The crystals were delivered into the X-ray beam in a stream of mesophase provided by high-
767 viscosity LCP injector⁴³. The mesophase containing protein crystals was loaded into the 20- μl -reservoir of the
768 LCP injector without additives directly from the Hamilton syringe. The injector was oriented at 20° from
769 vertical. The extrusion of the mesophase containing the *BcXeR* crystals was performed using capillaries with
770 50 and 100 μm diameters for the collection of the 250-750- μs snapshot and the series of 7.5-ms-long
771 snapshots, respectively. The mesophase stream was stabilized by a helium gas stream. A 50 μm capillary was
772 used in the case of the fast data collection to reduce sample consumption. The linear speed of the injected
773 mesophase was estimated using high-speed camera recordings and was 2.5 and 0.5 mm/s, respectively. The
774 serial crystallography data collection and treatment statistics are given in Table 2. The overall scheme of data
775 collection was similar to that used in earlier time-resolved crystallography studies of *HsBR* at a synchrotron
776 source¹⁴.

777 Two sets of serial crystallography data of the dark state of *BcXeR* were collected as a reference for the time-
778 resolved studies. First, the data of the dark state were collected using a 500- μs -long X-ray data acquisition per
779 image at a frame rate of 1 kHz using the central 4M region of the EIGER2 X 16M CdTe detector. A total
780 number of 72,000 detector images were collected and processed with CrystFEL⁴⁴ (version 0.9.1) without any
781 additional modification. Potential crystal hits were identified with SNR=4, --threshold=7, --highres=2.3 using
782 the peakfinder8 algorithm as implemented in CrystFEL. The second dataset of the dark state was collected
783 using 7.5-ms-long X-ray diffraction data acquisition per image at a frame rate of 133 Hz using the 16M region
784 of the EIGER2 X 16M CdTe detector. A total number of 199,980 detector images were collected and
785 processed with CrystFEL (version 0.9.1) without any additional modification. Potential crystal hits were

786 identified with SNR=5, --threshold=20, --highres=2.1 using peakfinder8 algorithm as implemented in
787 CrystFEL. In both cases, the initial geometry, provided by the beamline data staff, was optimized with
788 detector-shift. Cell unit parameters were iteratively adjusted from the initial parameters taken from single-
789 crystal cryo data. For indexing, indexer XGANDALF⁴⁵ was used with the --multi option enabled. Integration
790 was performed using --int-radius=3,4,5. This yielded datasets with 17,997 and 67,912 indexed images,
791 yielding 21,569 and 80,082 crystals for the datasets with 500- μ s-long and 7.5-ms-long X-ray data acquisition
792 per frame, respectively. Data were merged using partialator with --model=unity, --symmetry=mmm, and
793 number of iterations=1. Finally, 21,533 and 79,892 crystals were used for merging together after partialator
794 rejection.

795 Time-resolved crystallography data on the L state of *BcXeR* (250-750- μ s snapshot) were collected by
796 illuminating the crystals at a 40 Hz repetition rate with a 532 nm pump laser focused to the laser spot directly
797 at the intersection between the crystal stream and the X-ray beam of 60 μ m in diameter ($1/e^2$). Per each laser
798 pulse a series of 25 consecutive snapshots was collected. The length of the laser pulse was 500 μ s. The energy
799 of a single laser pulse was 6.2 μ J with laser power density of 438 W/cm², which is \sim 11 times higher than the
800 power density used in previous time-resolved studies of microbial rhodopsins at a synchrotron source^{14,15}.
801 Nevertheless, due to the short laser pulse these illumination conditions resulted in an uncorrected value of
802 \sim 15-65 photons per retinal depending on crystal orientation (protein concentration in crystals was 21 mM and
803 an extinction coefficient value used was 50,000 M⁻¹cm⁻¹); however, this calculation does not account for the
804 laser beam scattering by the jet and light absorption by the mesophase itself. The latter issue is especially valid
805 in the case of *BcXeR*, since the final mesophase in which the crystals were delivered was not fully transparent
806 probably due to the change of the phase type under crystallization conditions. We assume that the above-
807 mentioned factors reduce the real number of photons per retinal molecule. Nevertheless, the experiment was
808 performed in a multiphoton regime. The laser power was set to its maximum value in order to increase the
809 occupancy of the intermediate state. Analysis of the diffraction patterns collected with and without laser
810 illumination showed that the diffraction quality was not affected by the laser illumination. Data were collected
811 at a frame rate of 1 kHz using the central 4M region of the EIGER2 X 16M CdTe detector. The start of the X-
812 ray diffraction data acquisition for each series was electronically delayed by 250 μ s relative to the optical laser
813 trigger. The overall time of data collection from a sample with a total volume of 20 μ l was about 40 minutes.
814

815 A total number of 72,000 detector images were collected for the 250-750- μ s snapshot and processed with
816 CrystFEL (version 0.9.1) without any additional modification. Potential crystal hits were identified with
817 SNR=4, --threshold=7, --highres=2.3 using the peakfinder8 algorithm as implemented in CrystFEL. For
818 indexing, indexer XGANDALF⁴⁵ was used with the --multi option enabled. Integration was performed using
819 --int-radius=3,4,5. This yielded a dataset with 17,527 indexed images and 20,908 crystals. This means that
820 approx. 19% of the images contained multiple diffraction patterns. Data were merged using partialator with --
821 model=unity, --symmetry=mmm, and number of iterations=1. Finally, 20,866 crystals were used for merging
822 together after partialator rejection.

823 Time-resolved crystallography data of the M state of *BcXeR* (7.5-ms-long snapshot) were collected by
824 illuminating the crystals at a 4 Hz repetition rate with a 532 nm pump laser focused to the laser spot directly
825 at the intersection between the crystal stream and the X-ray beam of 180 μm in diameter ($1/e^2$). Per each laser
826 pulse a series of 33 consecutive snapshots was collected. The laser was triggered simultaneously with the
827 detector. The length of the laser pulse was 7.5 ms. The energy of a single laser pulse was 540 μJ with laser
828 power density of 283 W/cm^2 , which is ~ 7.5 times higher than the power density used in previous time-resolved
829 studies of microbial rhodopsins at a synchrotron source^{14,15}. These illumination conditions resulted in an
830 uncorrected value of ~ 150 -600 photons per retinal depending on crystal orientation (protein concentration in
831 crystals was 21 mM and an extinction coefficient value used was 50,000 $\text{M}^{-1}\text{cm}^{-1}$); however, this calculation
832 does not account for the laser beam scattering by the jet and light absorption by the mesophase itself. The
833 latter issue is especially valid in the case of *BcXeR*, since the final mesophase in which the crystals were
834 delivered was not fully transparent probably due to the change of the phase type under crystallization
835 conditions. We assume that the above-mentioned factors reduce the real number of photons per retinal
836 molecule. Nevertheless, the experiment was performed in a multiphoton regime. The laser power was set to
837 its maximum value in order to increase the occupancy of the intermediate state. Analysis of the diffraction
838 patterns collected with and without laser illumination showed that the diffraction quality was not affected by
839 the laser illumination. Data were collected at a frame rate of 133 Hz using the 16M region of the EIGER2 X
840 16M CdTe detector. Use of the 16M detector vs its 4M mode (as in the 500 μs time-resolved experiment)
841 allows for a large sample-detector distance and a reduced background. The higher resolution of the 7.5 ms
842 data vs the 500 μs data at lower dose is due to the use of the 16M mode. Since 7.5 ms is the shortest exposure
843 period of EIGER2 X CdTe detector in a standard 16M mode, the 7.5 ms time resolution was chosen to sample
844 the time interval of 0 to 250 ms. Moreover, the size of the laser spot, the laser pulse duration, the repetition
845 rate of laser pulses, the jet speed and X-ray flux were set to match this time interval. Increasing the frame rate
846 would require a proportional increase in the jet speed and hence in the sample consumption (by an order of
847 magnitude), and a decrease in the duration of the laser pulse leading to lower M state occupancy. The start of
848 the X-ray diffraction data acquisition for each series was electronically synchronized to the optical laser
849 trigger. The overall time of data collection from a sample with a total volume of 40 μl was about 5 hours.

850

851 A total number of 3,958,702 detector images were collected and processed with CrystFEL (version 0.9.1)
852 without any additional modification. Among the collected images, potential crystal hits were identified with
853 $\text{SNR}=5$, $--\text{threshold}=20$, $--\text{highres}=2.2$ using the peakfinder8 algorithm as implemented in CrystFEL,
854 corresponding to an average hit rate of 10.8%. For indexing, indexer XGANDALF⁴⁵ was used with the $--\text{multi}$
855 option enabled. Integration was performed using $--\text{int-radius}=3,4,5$. This yielded a dataset with 389,438
856 indexed images and 428,729 crystals. Data were scaled and merged together using partialator with $--\text{model}=\text{unity}$,
857 $--\text{symmetry}=\text{mmm}$, and number of iterations=1 and datasets for each time point were splitted
858 by partialator using the $--\text{custom-split}$ option. Finally, 427,677 crystals were used for merging and splitting
859 after partialator rejection. 12,449 crystals were merged for the 7.5-15-ms snapshot.

860

861 **Time-resolved serial crystallography structure refinement**

862

863 The models of the ground state of *BcXeR* at room temperature determined via serial crystallography were
864 built iteratively using the structure of *BcXeR* at 100 K as a starting model using Coot and REFMAC5. The
865 structures of the ground state of *BcXeR* at room temperature in the cases of the 500- μ s-long and the 7.5-ms-
866 long X-ray data acquisition per frame were identical (Extended Data Fig. 8).

867 In the case of the 250-750- μ s snapshot the $F_o^{\text{light}}-F_o^{\text{dark}}$ difference electron density maps built in PHENIX⁴⁶
868 identified structural rearrangement at the residues 81, 173, 136, and 207 similar to those shown in the case of
869 the cryotrapped L intermediate. However, in the case of time-resolved data, the occupancy of the intermediate,
870 the resolution, and the overall quality of the data were lower. Therefore, the model of the L state obtained with
871 the cryotrapping procedure was used as a starting model for the automated refinement in REFMAC5. The
872 additional water molecules appearing in the L state but not seen in the time-resolved data were removed from
873 the starting model. Only atom positions of the L state were refined; the atom positions of the ground state
874 were fixed. B-factor were also refined for all atoms. The final occupancy of the L state in the case of time-
875 resolved serial crystallography data was 20%.

876 In the case of the series of 33 7.5-ms-long snapshots of the *BcXeR* following the laser excitation, the $F_o^{\text{light}}-$
877 F_o^{dark} difference electron density maps built in PHENIX for each time point were similar and identified
878 structural rearrangements almost identical to those shown in the case of the cryotrapped M intermediate at all
879 33 time points. F_o^{dark} corresponds to the dark state of *BcXeR*, F_o^{light} corresponds to each of the 33 datasets
880 collected following the laser excitation in the time-resolved experiments. To build the model of the
881 intermediate state we used extrapolated structure factors, calculated from two isomorphous datasets using
882 linear approximation as follows: $F_o^{\text{extrapolated}} = (1/\alpha)*(F_o^{\text{light}} - (1-\alpha)*F_o^{\text{dark}})$, where α is the occupancy of the
883 intermediate state. For each time point, the α value was screened while calculating the extrapolated maps from
884 0.1 to 0.5 with a step of 0.05, and was selected to be in the range of 0.4-0.5, time-point dependent, via an
885 analysis of the electron density maps built using extrapolated structure factors. Subsequently, the model of the
886 intermediate state was manually built in Coot using electron density maps calculated using extrapolated
887 structure factors in accordance with the $F_o^{\text{light}}-F_o^{\text{dark}}$ difference electron density maps. The manual building
888 was performed for the 7.5-15-ms snapshot since it has the highest α value (0.5) corresponding to the highest
889 occupancy of the intermediate state. After that, the combined model was prepared containing the structures of
890 the ground and the intermediate states as alternative conformations (A and C, respectively). Then, the model
891 was automatically refined in REFMAC5 against each of the 33 datasets. Only atom positions of the M state
892 were refined; the atom positions of the ground state were fixed. B-factor were also refined for all atoms. The
893 analysis of the resulting intermediate state structures and electron difference maps confirmed their identity to
894 that of the M state of *BcXeR* obtained at 100 K. Final occupancies were identified using occupancy refinement
895 performed in REFMAC5. It showed that the occupancy of the M state varies from 62% (at the 7.5-15-ms

896 snapshot) to 43% (at the 240-247.5-ms snapshot). For the occupancy refinement all atom positions and B-
897 factors were fixed. Since all the models were identical and only differ in the occupancy of the intermediate
898 state, here we have only reported the one with the highest occupancy of the state (7.5-15-ms snapshot) (Table
899 2).

900

901 **Analysis of the X-ray radiation damage of the *BcXeR* crystallography data**

902

903 The X-ray-induced radiation damage is a critical issue in studying of microbial rhodopsins as demonstrated
904 earlier for *HsBR*⁴⁷. The most prominent signs of the X-ray radiation damage appear as negative difference
905 densities at the *HsBR* D85 (analog of D76 in *BcXeR*) and water molecules in close proximity of the RSB. The
906 decarboxylation rates were determined⁴⁷ at 12-15% MGy⁻¹.

907 In order to assess possible influence of X-ray-induced radiation damage on the findings presented in this work,
908 we estimated the X-ray dose for each of the reported datasets. In all cases, the X-ray dose rates were calculated
909 using RADDOS (v4.0.1020)⁴⁸. The parameters used for calculation and the resulting doses are given in
910 Supplementary Table 2. In our single-crystal data average doses did not exceed 1.1 MGy, which corresponds
911 to less than 17% expected decarboxylation. Consistently, we have not observed any negative electron densities
912 at carboxylic residues including D76, as well as at water molecules near the RSB and in other regions.

913 In the case of time-resolved experiments the data were collected in the shutterless mode, therefore the total
914 exposure time was estimated as the time within which the jet front traverses the full beam width at half
915 maximum, accounting for the 20° angle of the jet to vertical (1 and 5 ms for the fast and slow time-resolved
916 experiments, respectively). In case of the fast experiment, the traversal time is longer than the detector
917 integration time. Therefore, the average exposure to the beam effectively contributing to diffraction data varies
918 uniformly between 0.5 ms (at any point entering the beam during detector frame integration) and 1.0 ms (at
919 any point exiting the beam). Resulting average exposure time 0.75 ms was used for estimating the average
920 dose (400 kGy). We have not observed any signs of X-ray-induced radiation damage in the electron densities
921 of the serial time-resolved crystallography data. Thus, also a minor fraction of the molecules was damaged by
922 X-rays the effects of this does were not visible at 2.2-2.3 Å resolution.

923 **Data Availability**

924

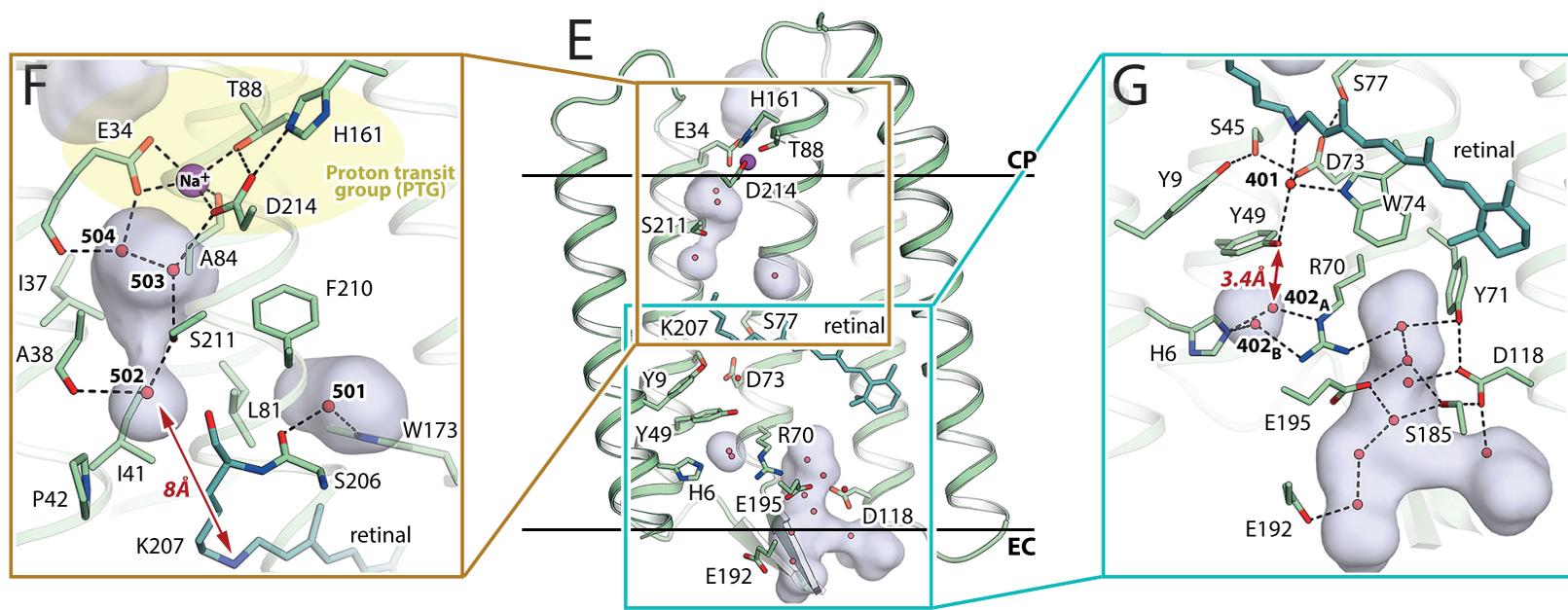
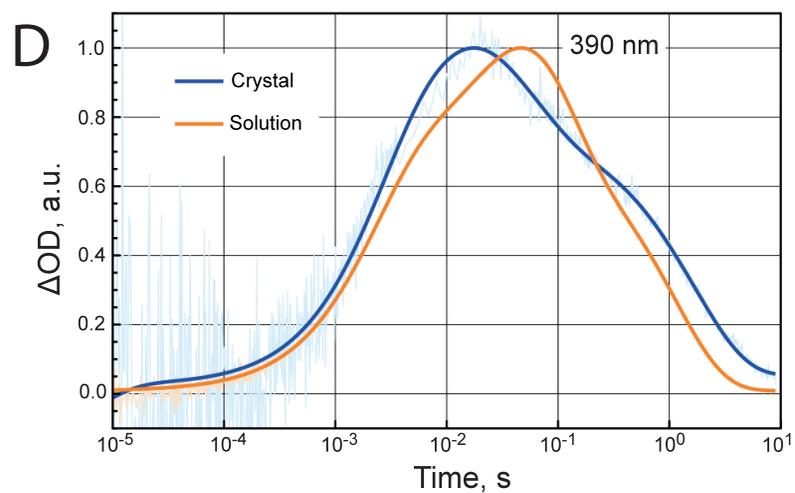
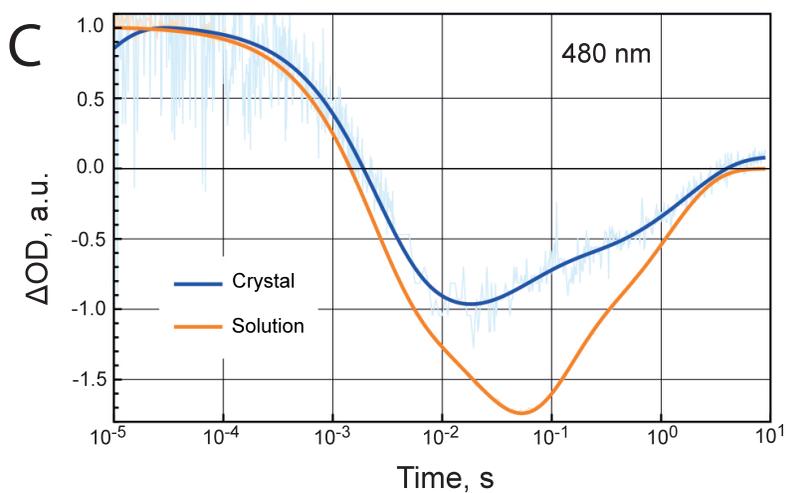
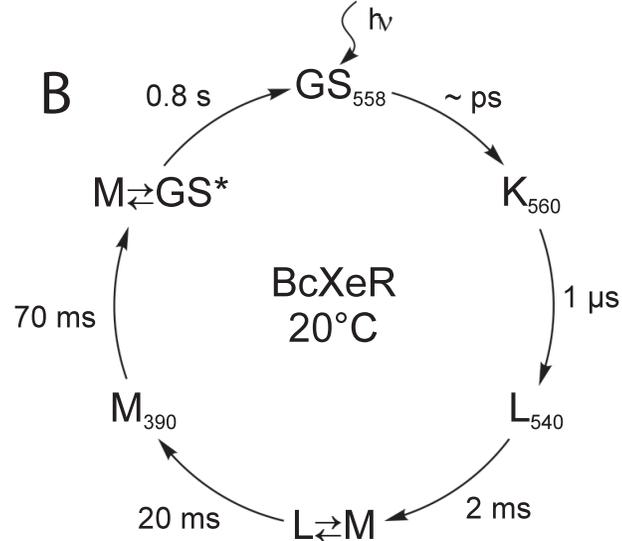
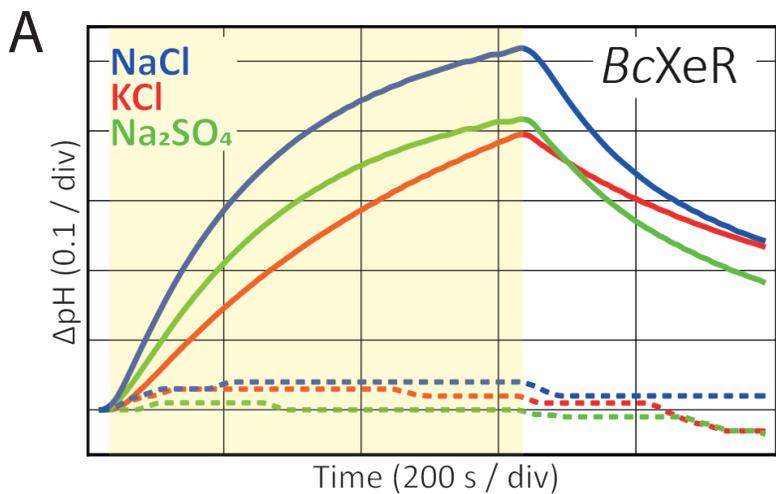
925 Atomic models built using X-ray crystallography data have been deposited in the RCSB Protein Data Bank
926 with PDB codes 7ZMY (for the ground state at pH 8.2 at 100 K), 7ZN3 (for the L state at pH 8.2 at 100 K),
927 7ZN0 (for the M state at pH 8.2 at 100 K), 7ZN8 (for the ground state at pH 7.0 at 100 K), 7ZN9 (for the M
928 state at pH 7.0 at 100 K), 7ZNA (for the ground state at pH 5.2 at 100 K), 7ZNB (for the M state at pH 5.2 at
929 100 K), 7ZNC (for the ground state at pH 7.6 at 100 K in the absence of sodium), 7ZND (for the M state at
930 pH 7.6 at 100 K in the absence of sodium), 7ZNE (for the ground state at pH 8.2 at 293 K with 7.5-ms-long
931 exposure), 7ZNI (for the 7.5-15-ms snapshot at pH 8.2 at 293 K), 7ZNG (for the ground state at pH 8.2 at
932 293 K with 500- μ s-long exposure), and 7ZNH (for the 250-750- μ s snapshot at pH 8.2 at 293 K). Extrapolated
933 structure factors and phases for the intermediate states of *BcXeR* are available from Zenodo
934 (10.5281/zenodo.7612803). Publicly available in RCSB Protein Data Bank structures of *NsXeR* (PDB ID:
935 6EYU) and *HsBR* (PDB IDs: 5ZIL, 7Z0D, 7Z0E, 6RPH) were used for analysis.

936

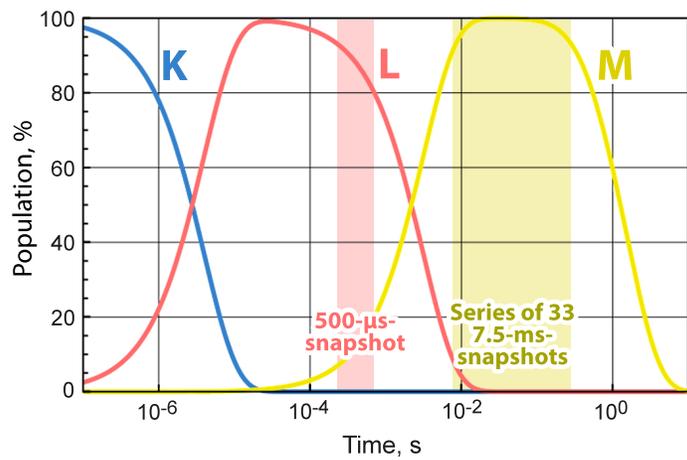
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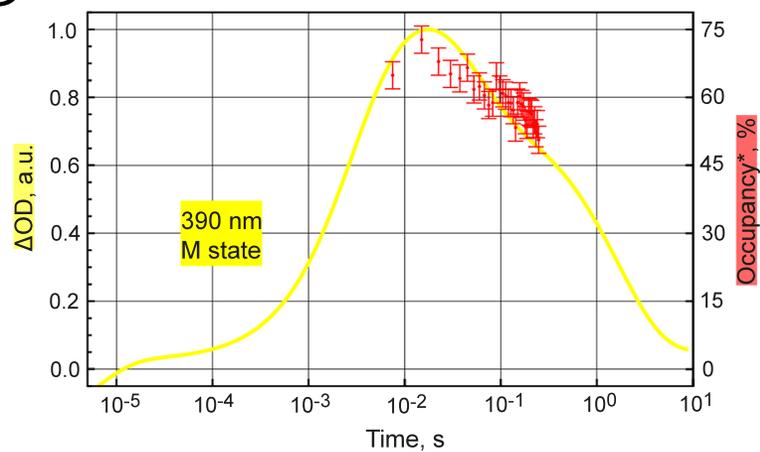
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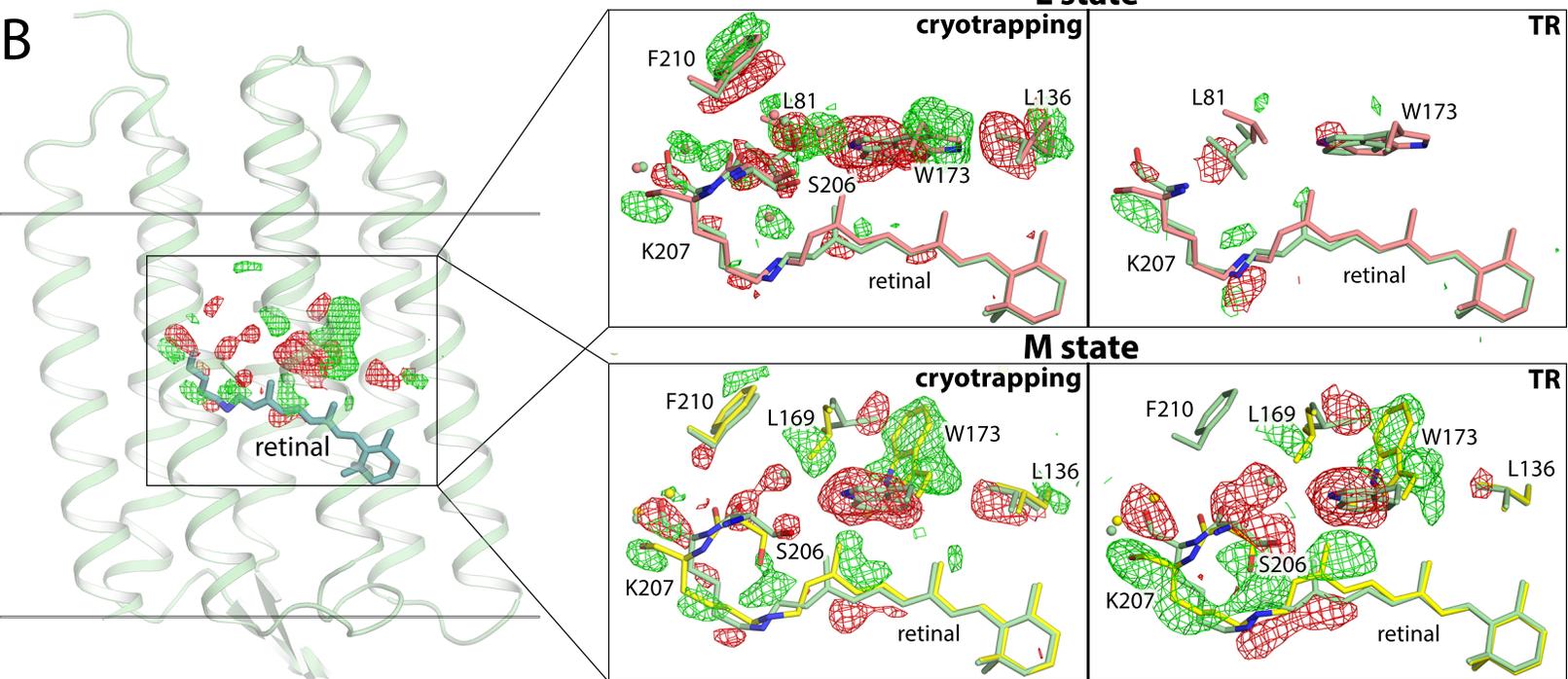
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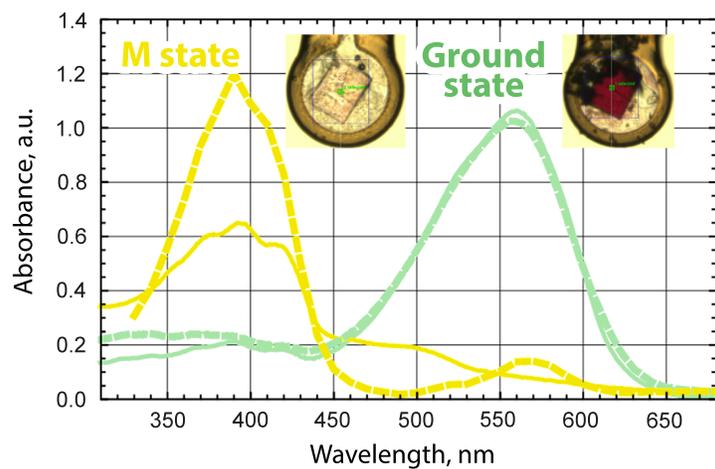
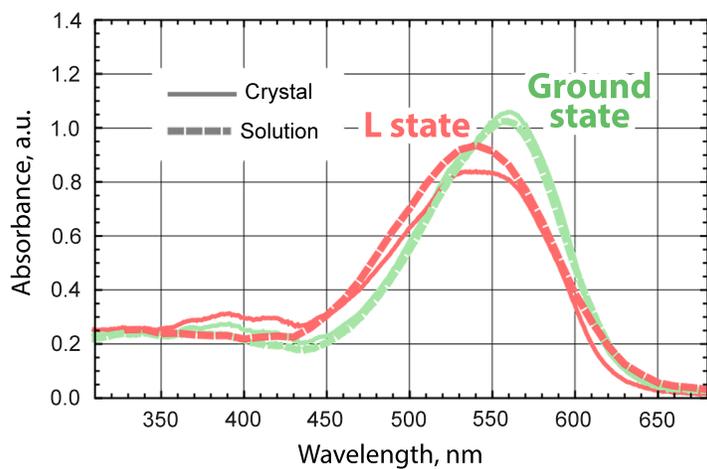
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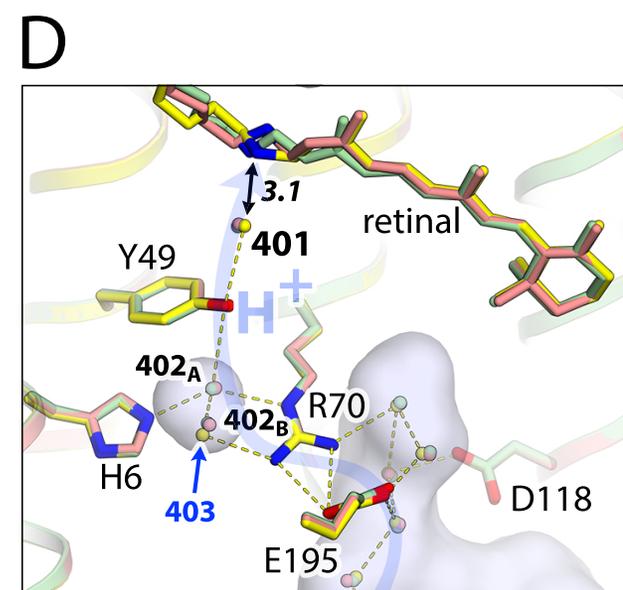
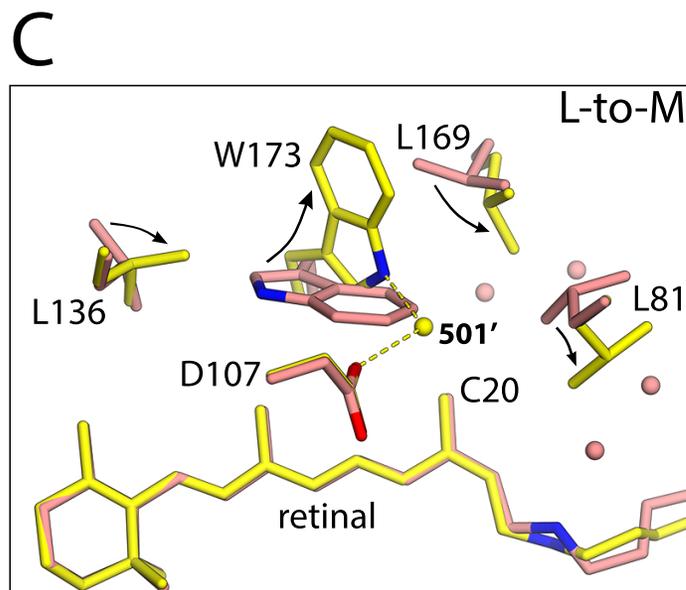
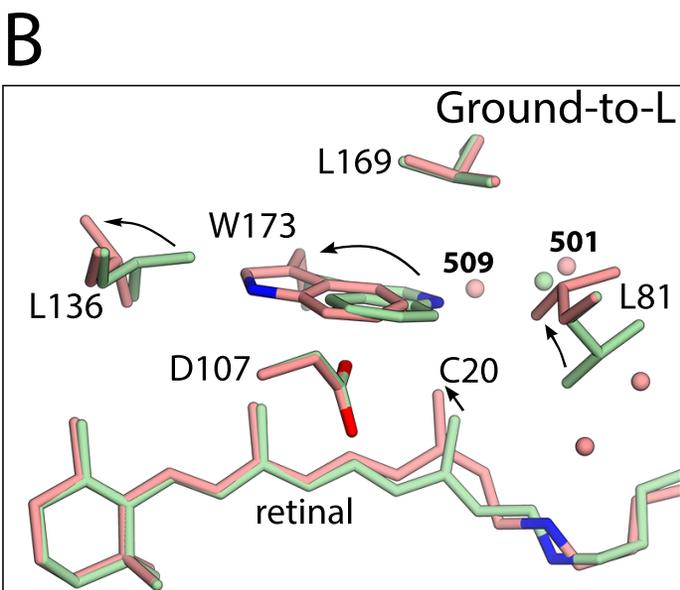
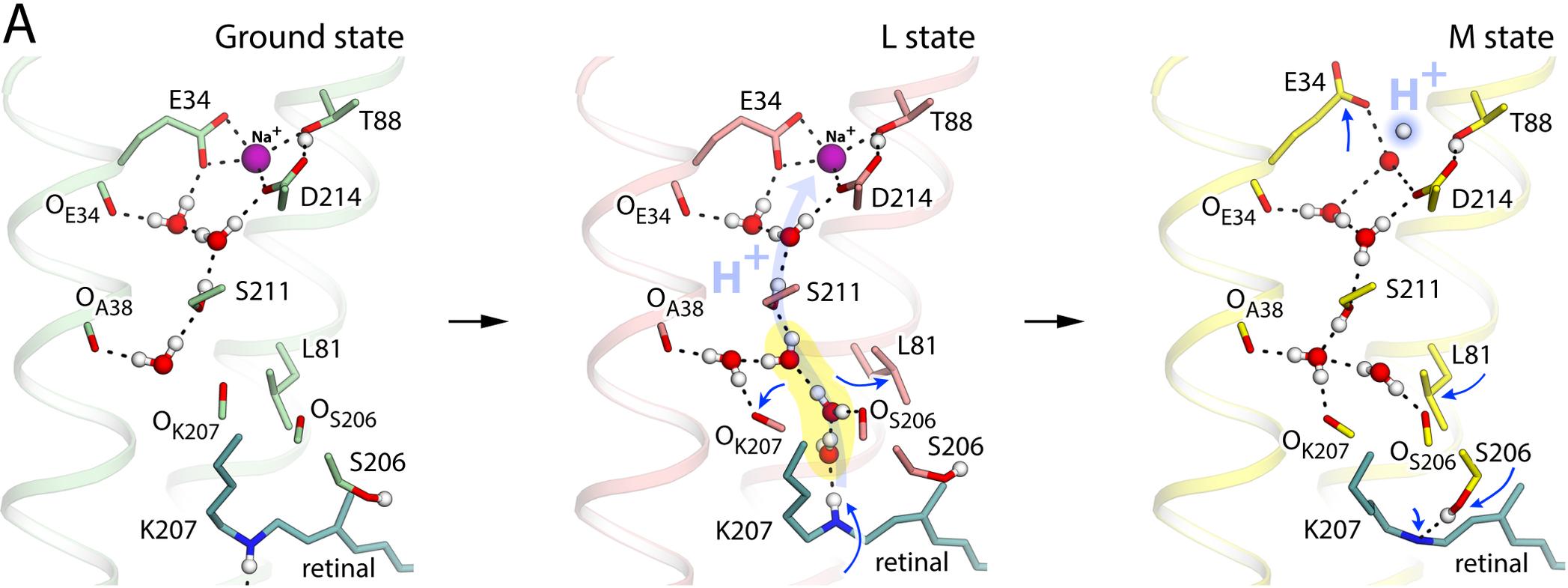


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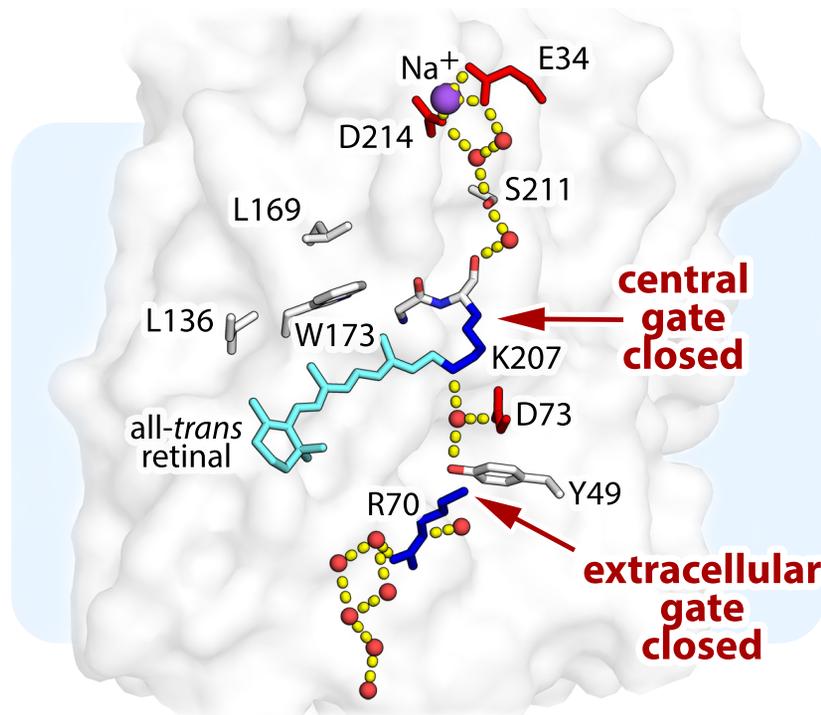


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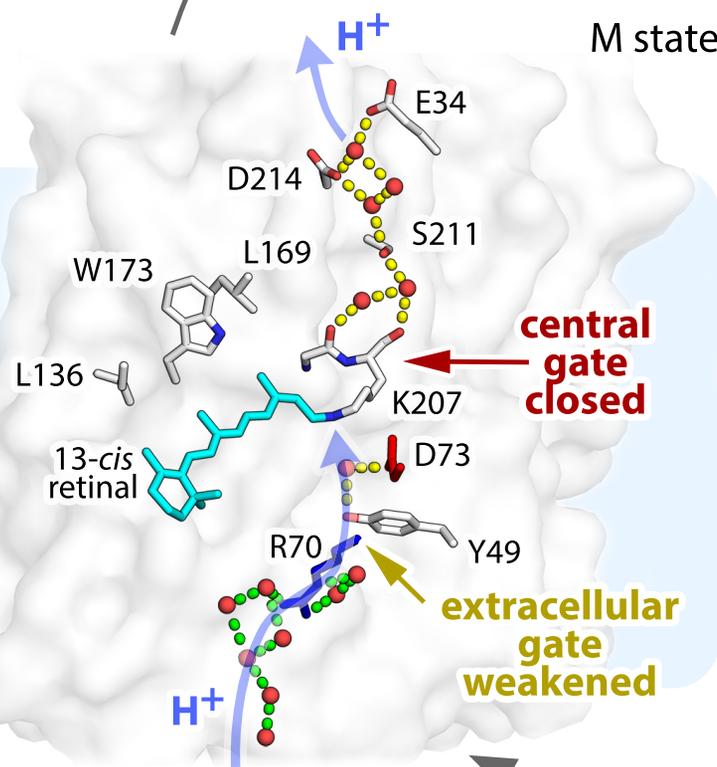


Ground state

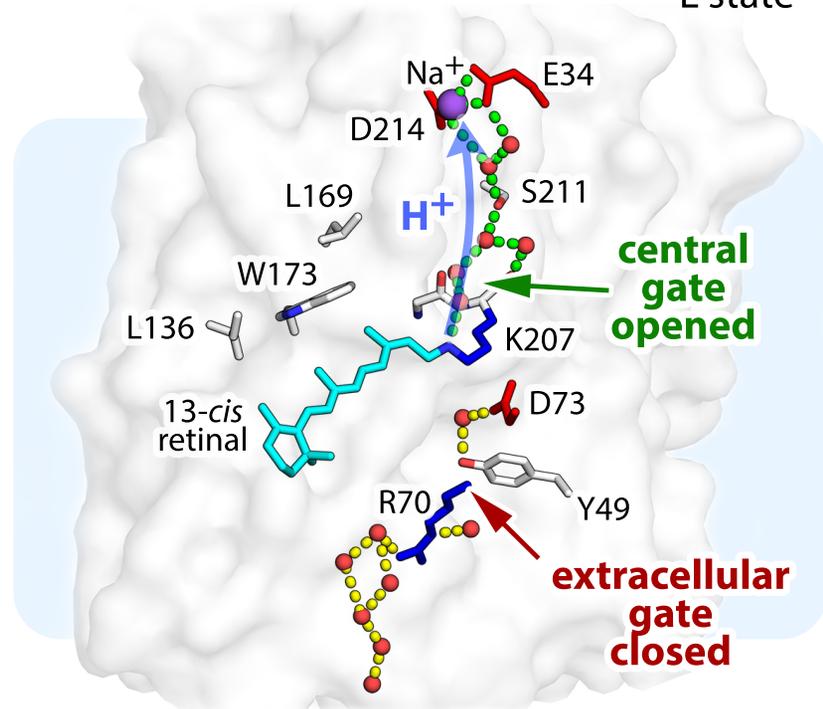


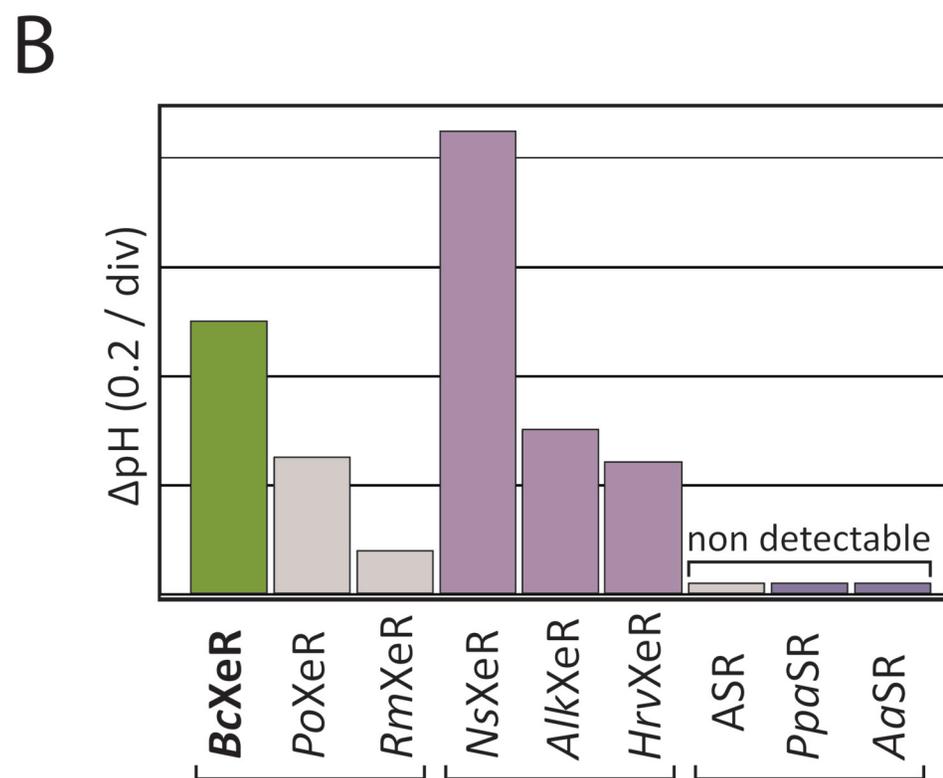
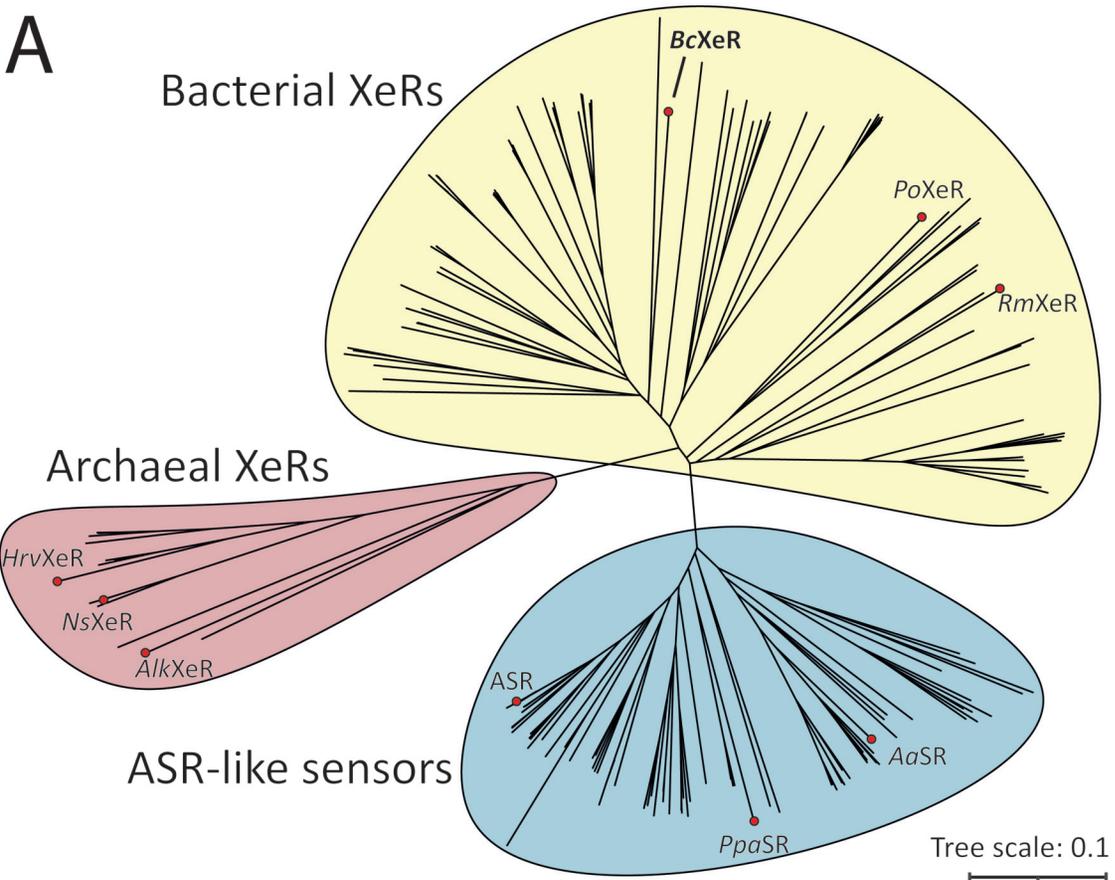
$h\nu$

M state



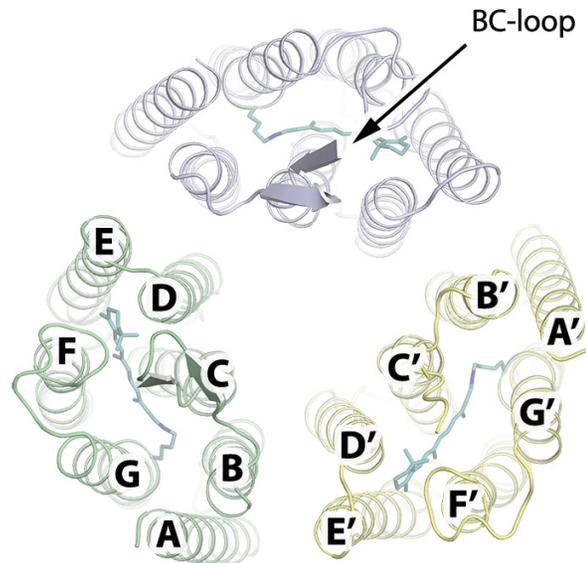
L state



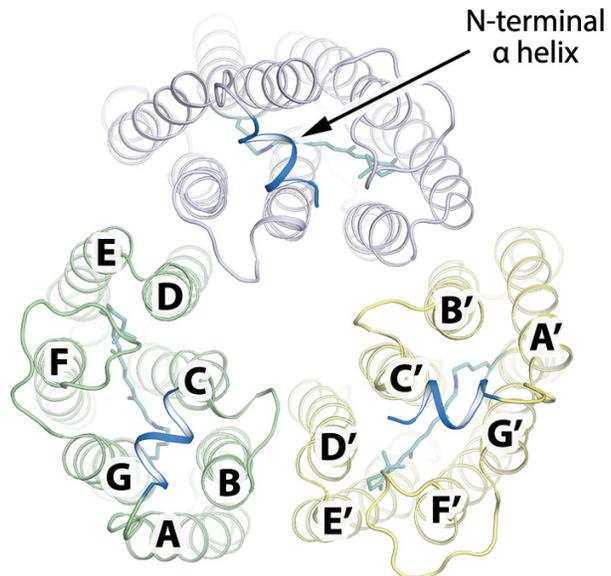


View from the extracellular side

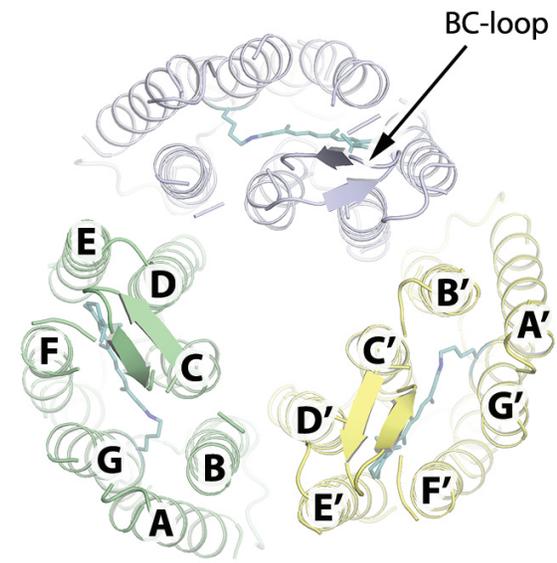
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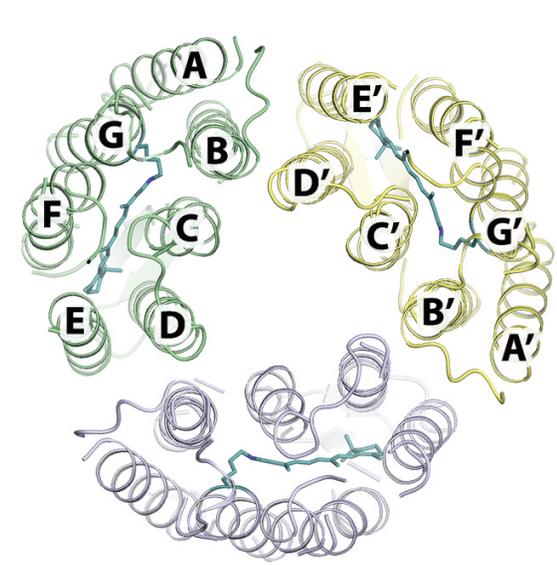
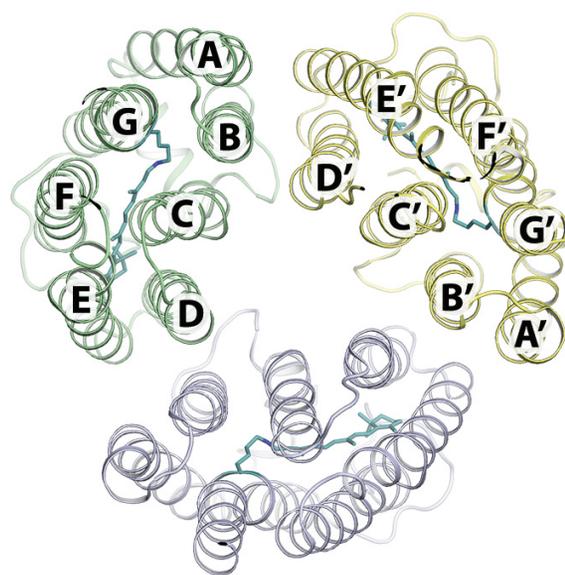
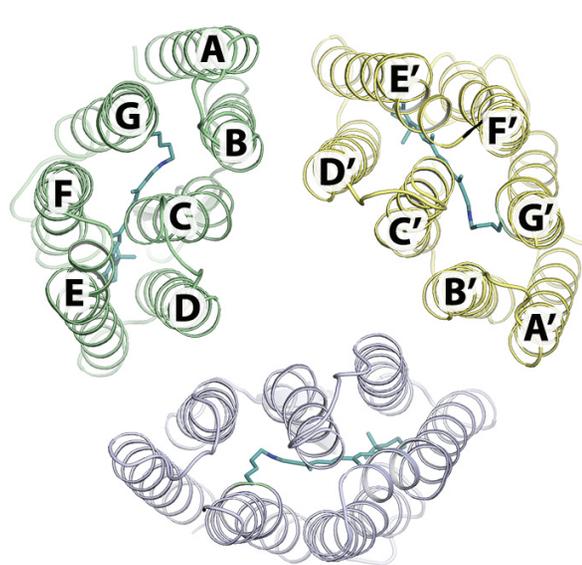
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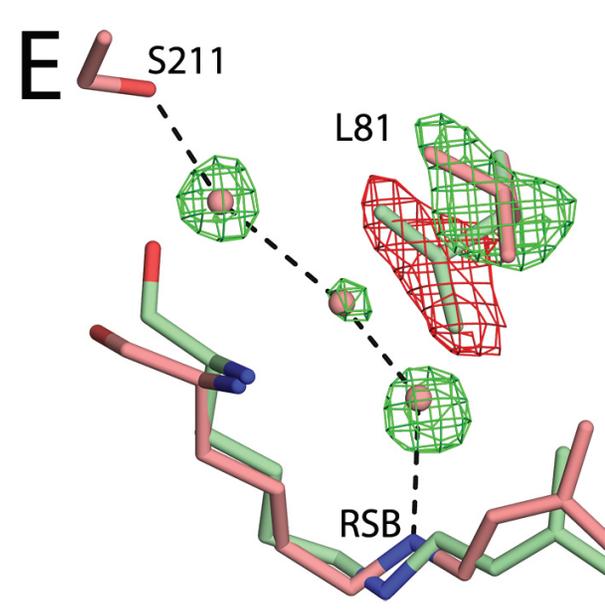
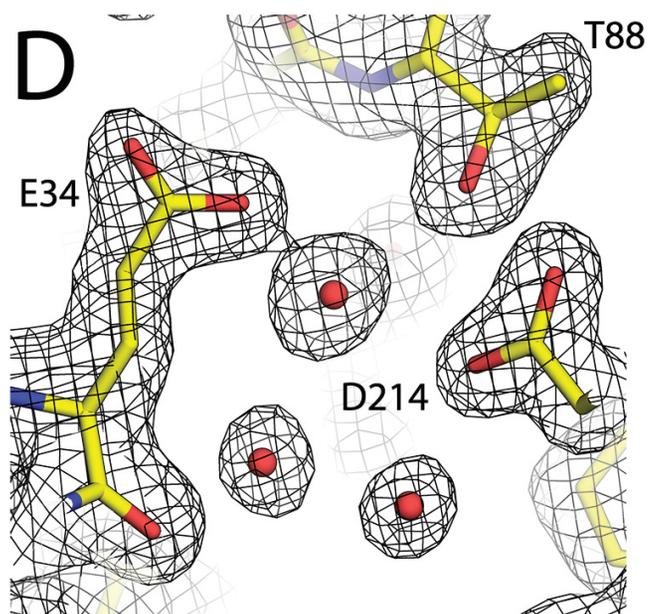
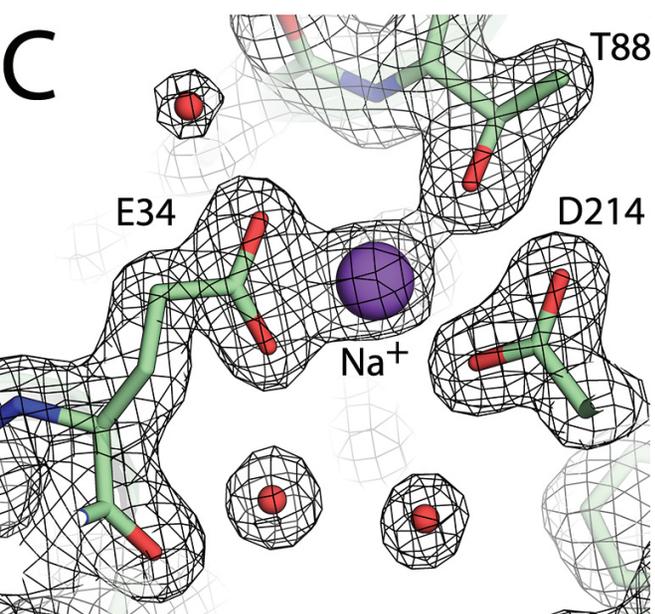
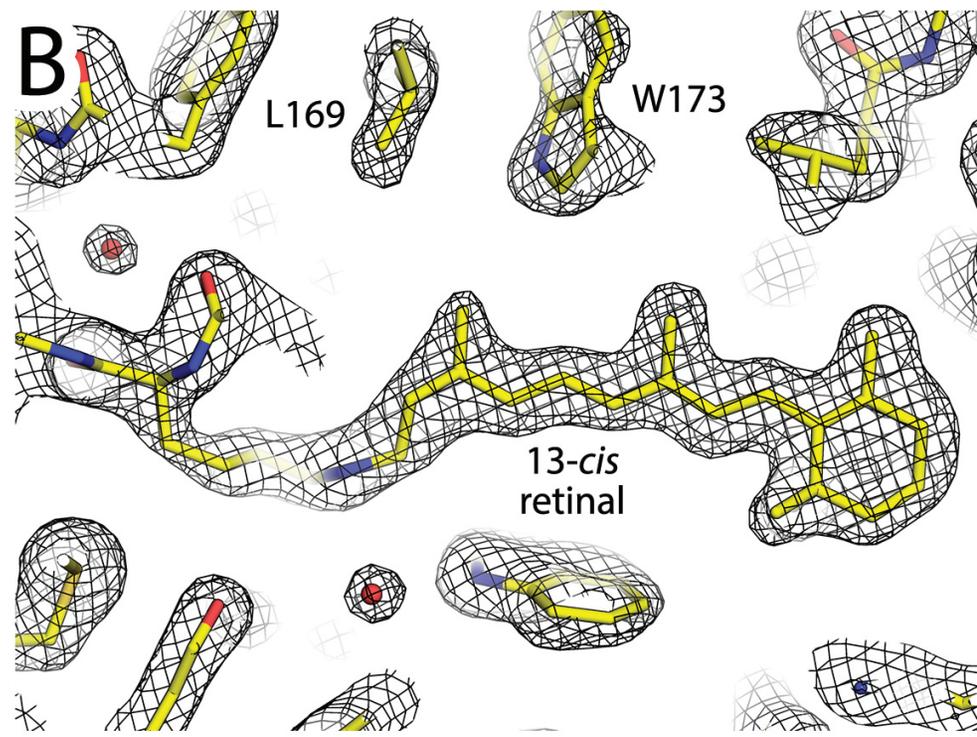
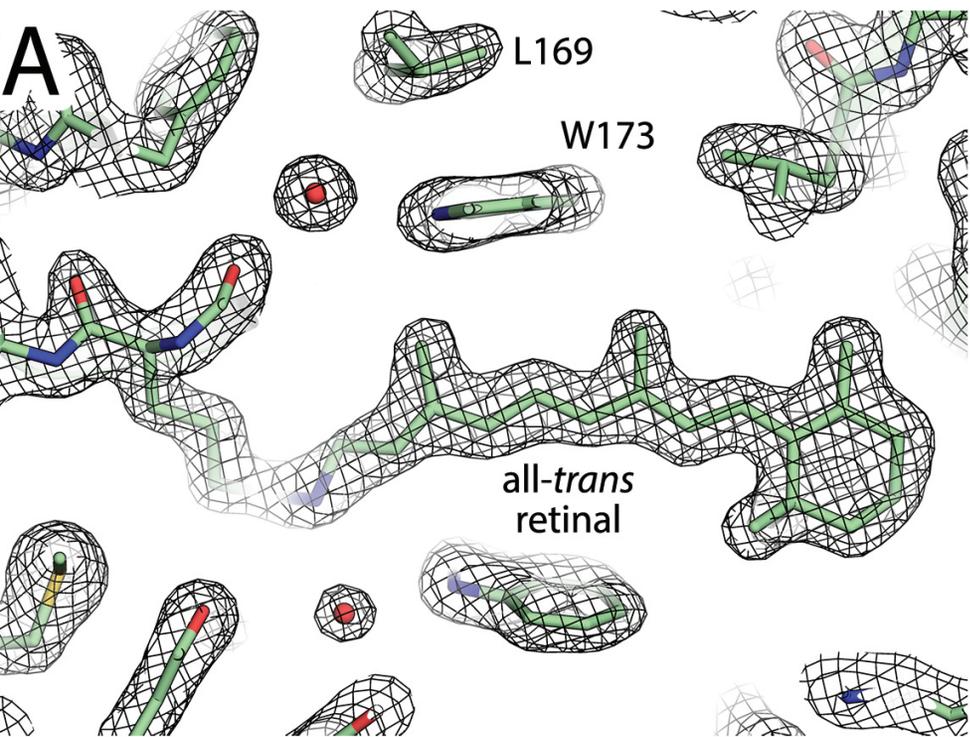


HsBR

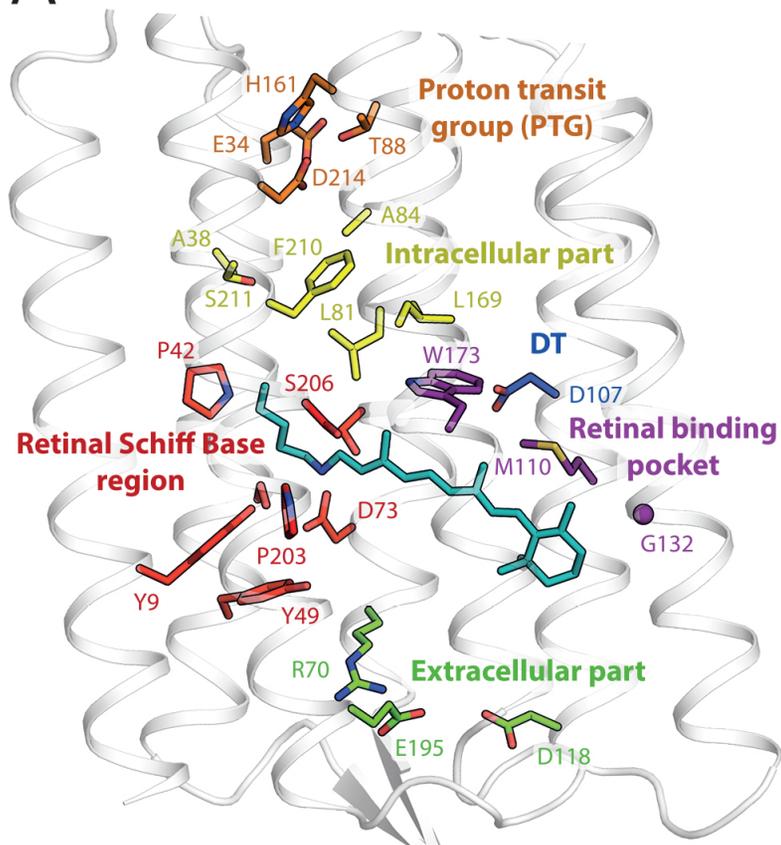


View from the cytoplasmic side





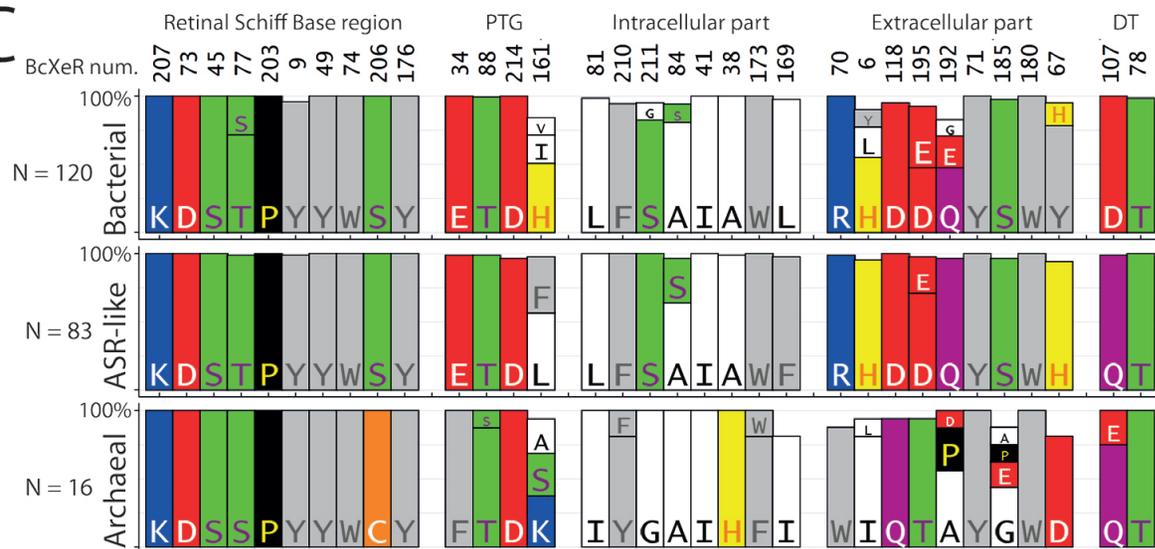
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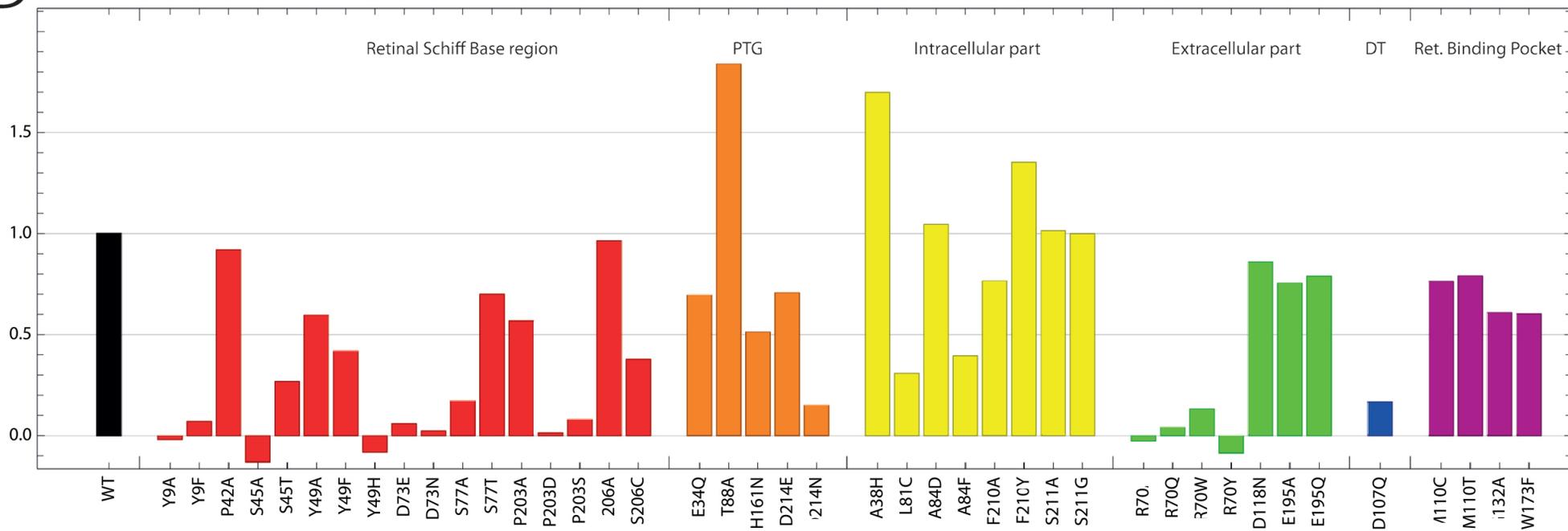
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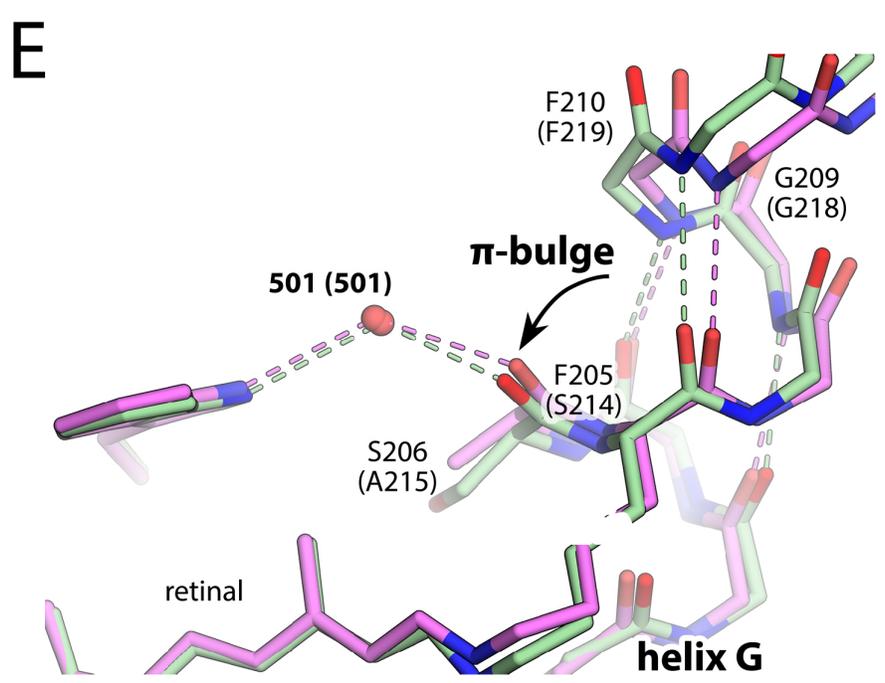
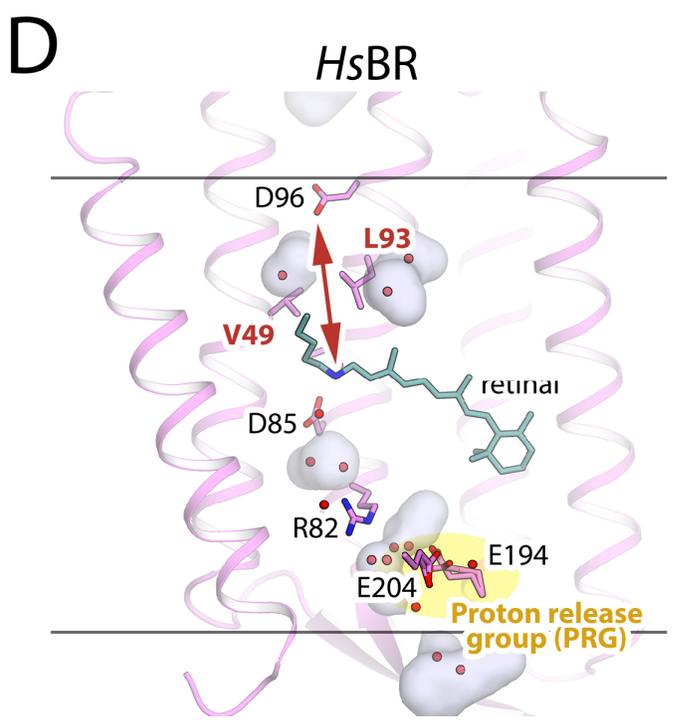
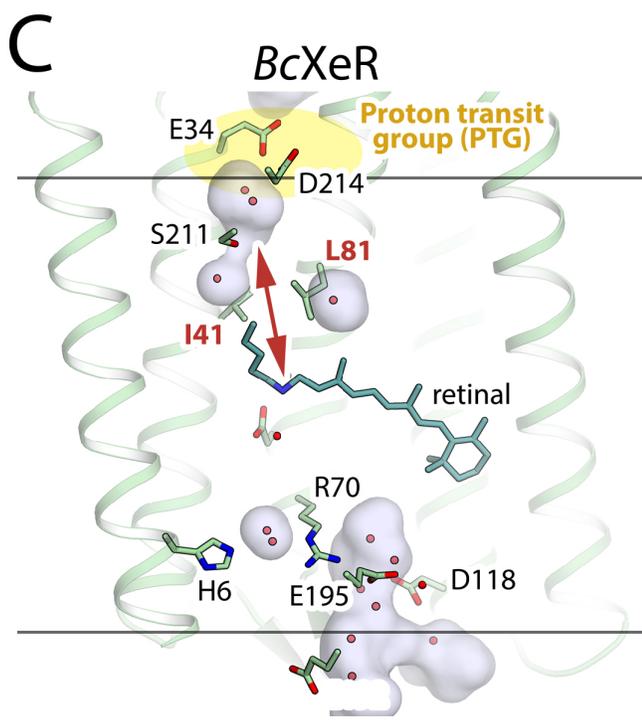
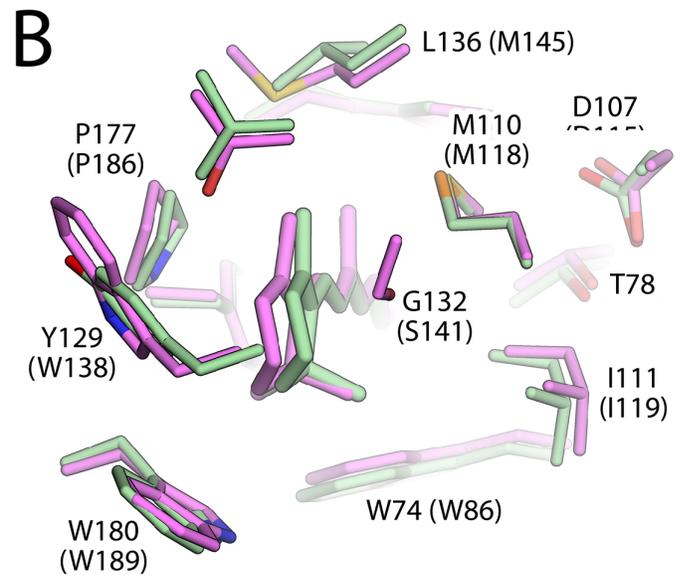
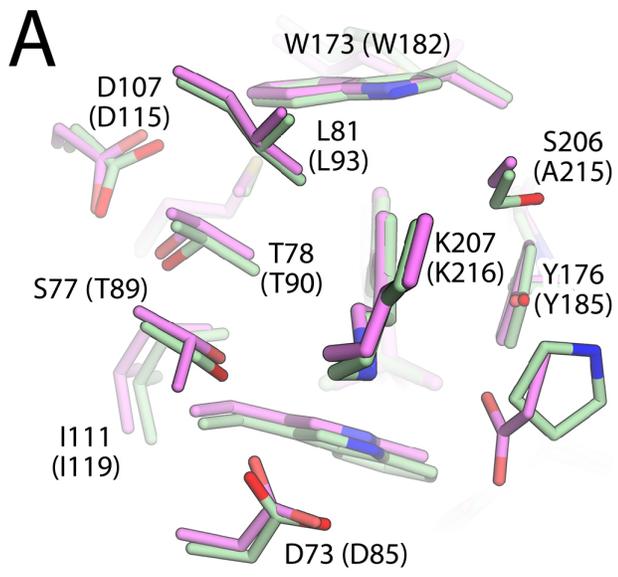
BcXeR num.	Retinal Schiff Base region							PTG			Intracellular part					Extracellular part					DT												
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BcXeR	K	D	S	S	P	Y	Y	W	S	Y	E	T	D	H	L	F	S	A	I	A	W	L	R	H	D	E	E	Y	S	W	Y	D	T
PoXeR	K	D	S	T	P	Y	Y	W	S	Y	E	T	D	T	L	F	S	L	I	A	W	L	R	L	D	S	P	Y	S	W	Y	D	T
RmXeR	K	D	S	T	P	Y	Y	W	S	Y	E	T	D	I	L	F	S	A	I	A	W	L	R	Y	D	D	Q	Y	S	W	Y	D	T
ASR	K	D	S	T	P	Y	Y	W	S	Y	E	T	D	L	L	F	S	S	I	A	W	F	R	H	D	D	Q	Y	S	W	H	Q	T
PpaSR	K	D	S	S	P	Y	Y	W	S	Y	E	T	D	L	L	F	S	A	I	A	W	V	R	Y	D	D	P	Y	S	W	Y	Q	T
AaSR	K	D	S	T	P	Y	Y	W	S	Y	E	T	D	F	L	F	S	A	I	A	W	F	R	H	D	D	Q	Y	S	W	H	Q	T
AlkXeR	K	D	S	S	P	Y	Y	W	C	Y	F	S	D	E	I	F	G	A	I	H	W	V	F	I	H	V	D	Y	-	W	E	Q	T
NsXeR	K	D	S	S	P	Y	Y	W	C	Y	F	T	D	A	I	Y	G	A	I	H	F	I	W	I	Q	T	P	Y	G	W	D	E	T
HrvXeR	K	D	S	T	P	Y	Y	W	C	Y	F	T	D	K	I	Y	G	A	I	H	F	I	W	I	Q	T	A	Y	E	W	D	Q	T

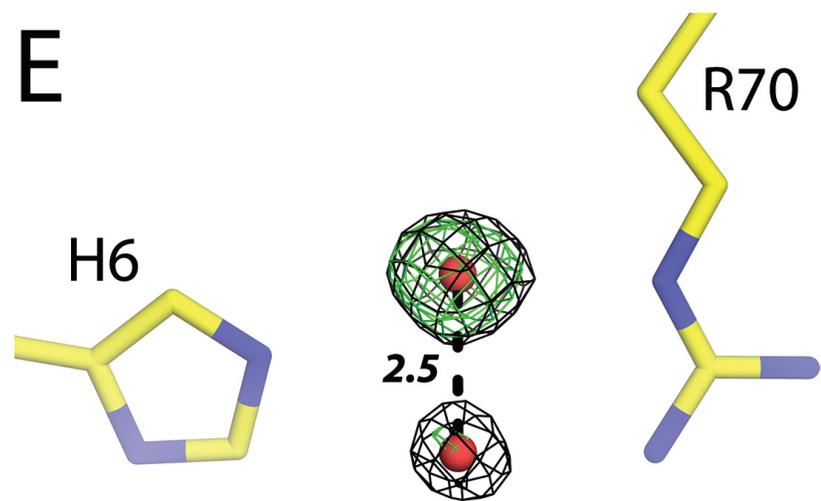
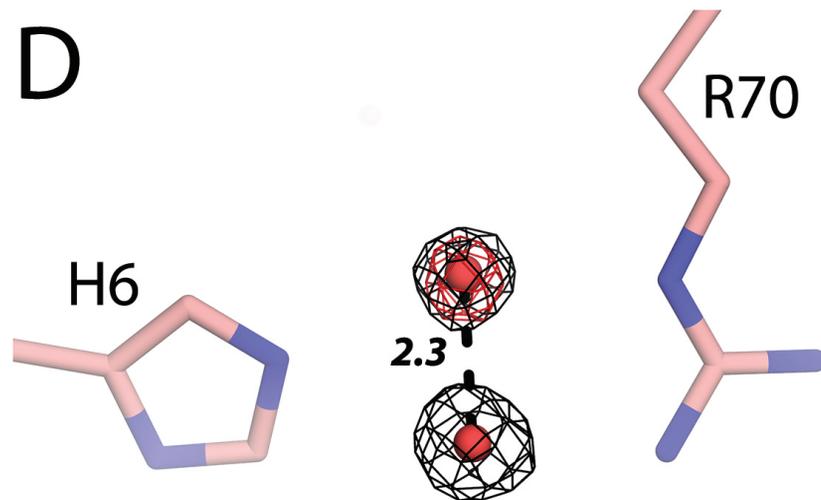
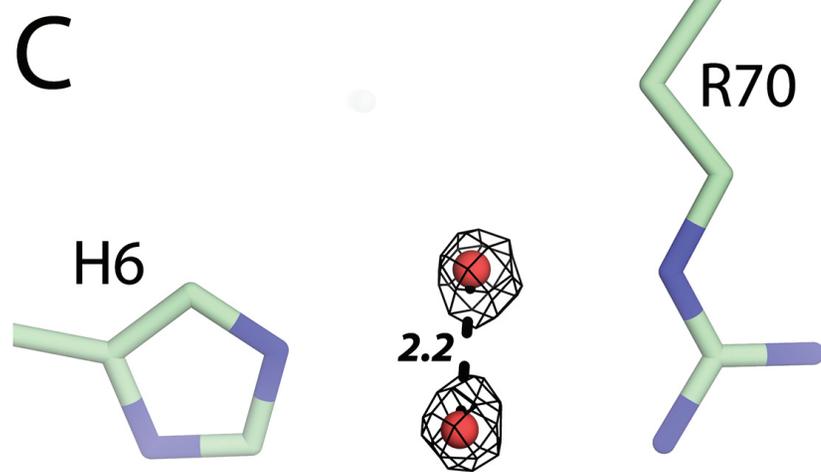
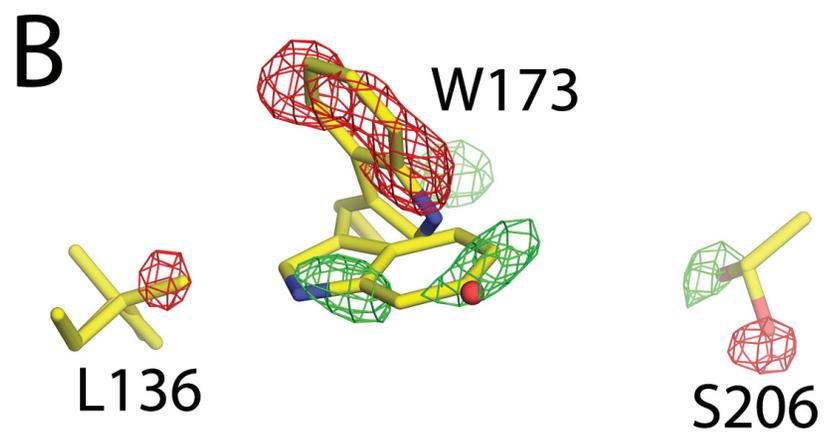
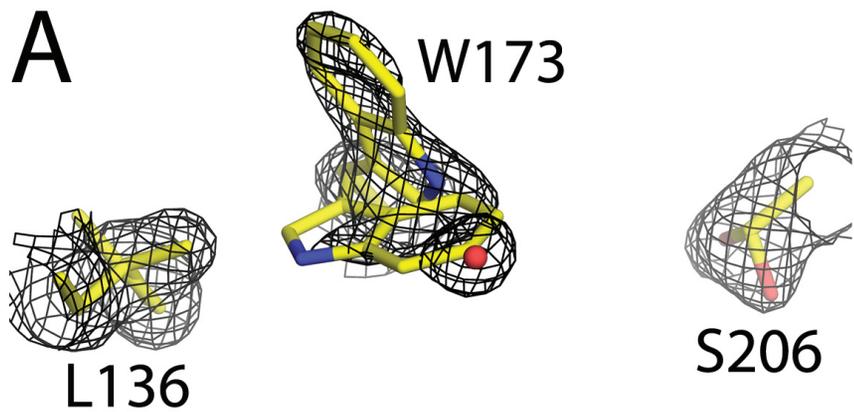
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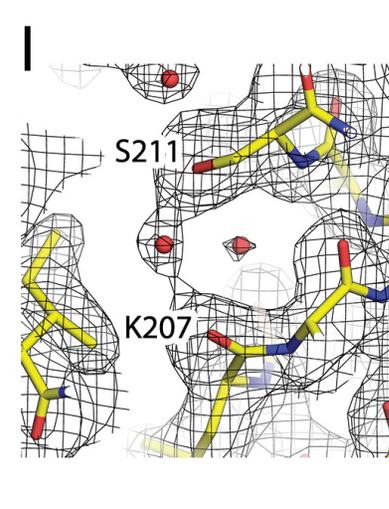
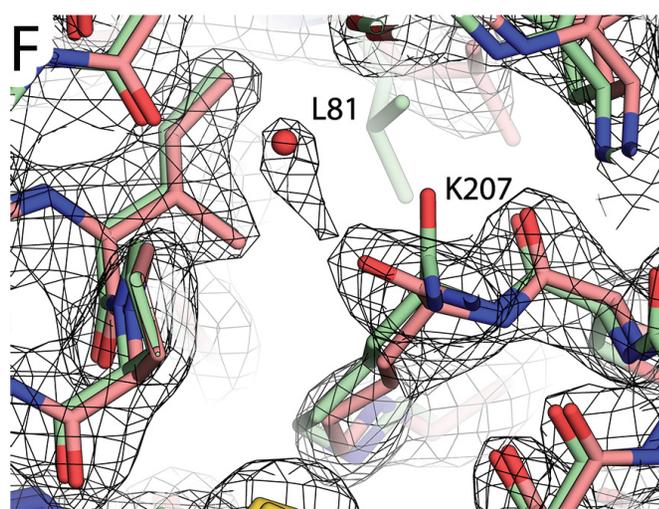
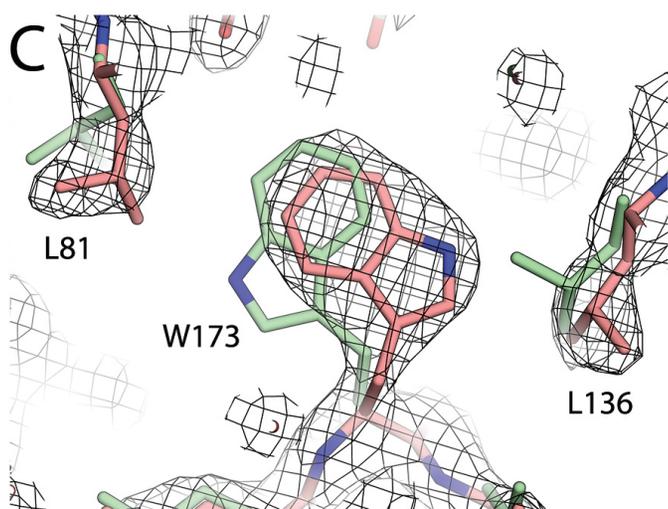
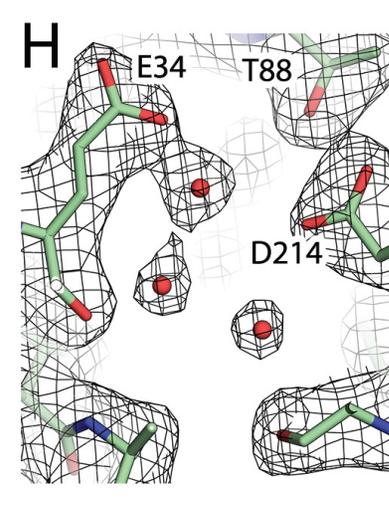
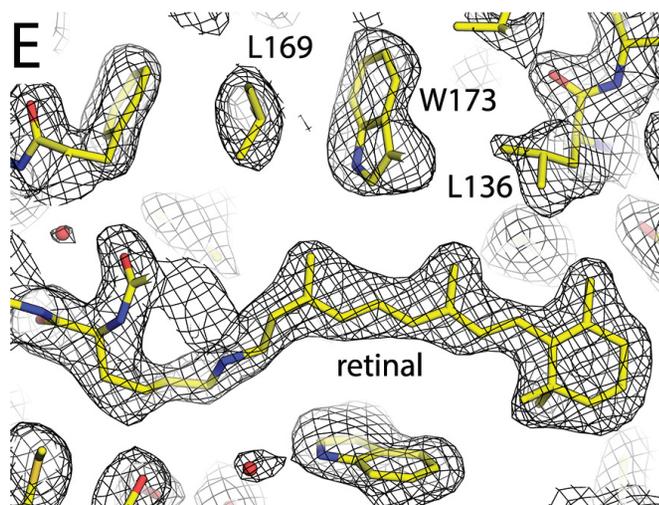
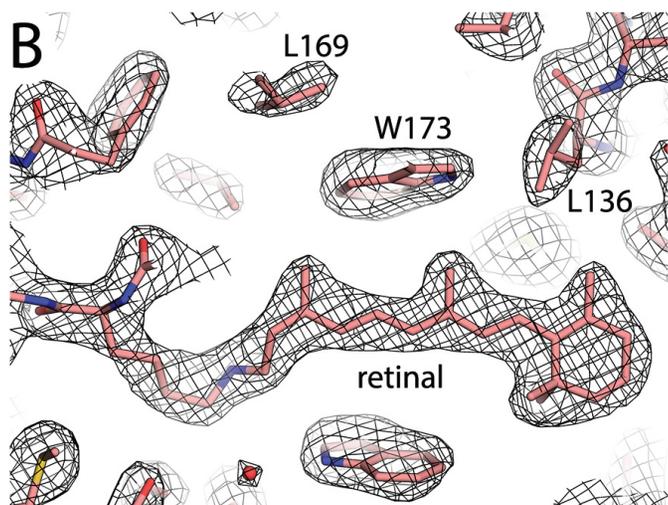
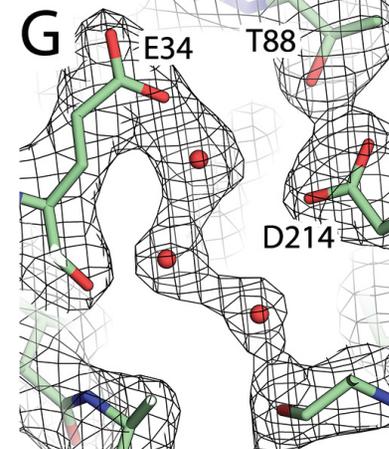
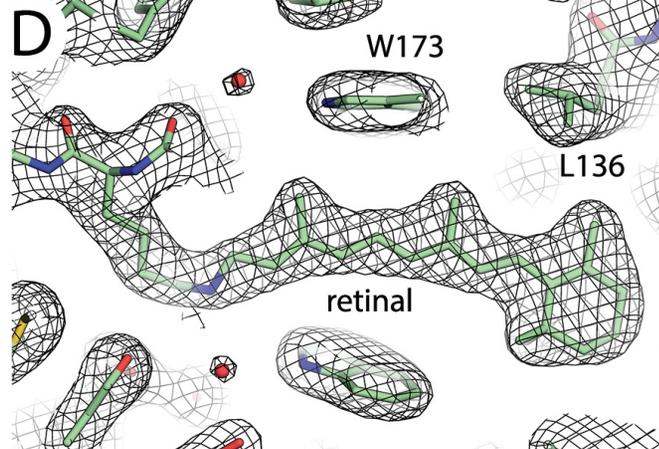
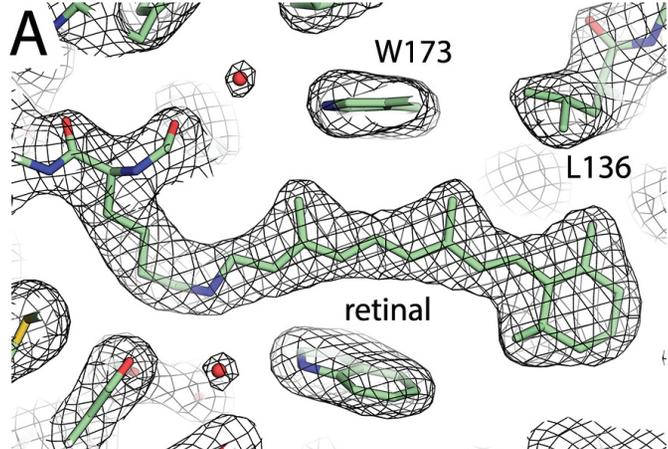


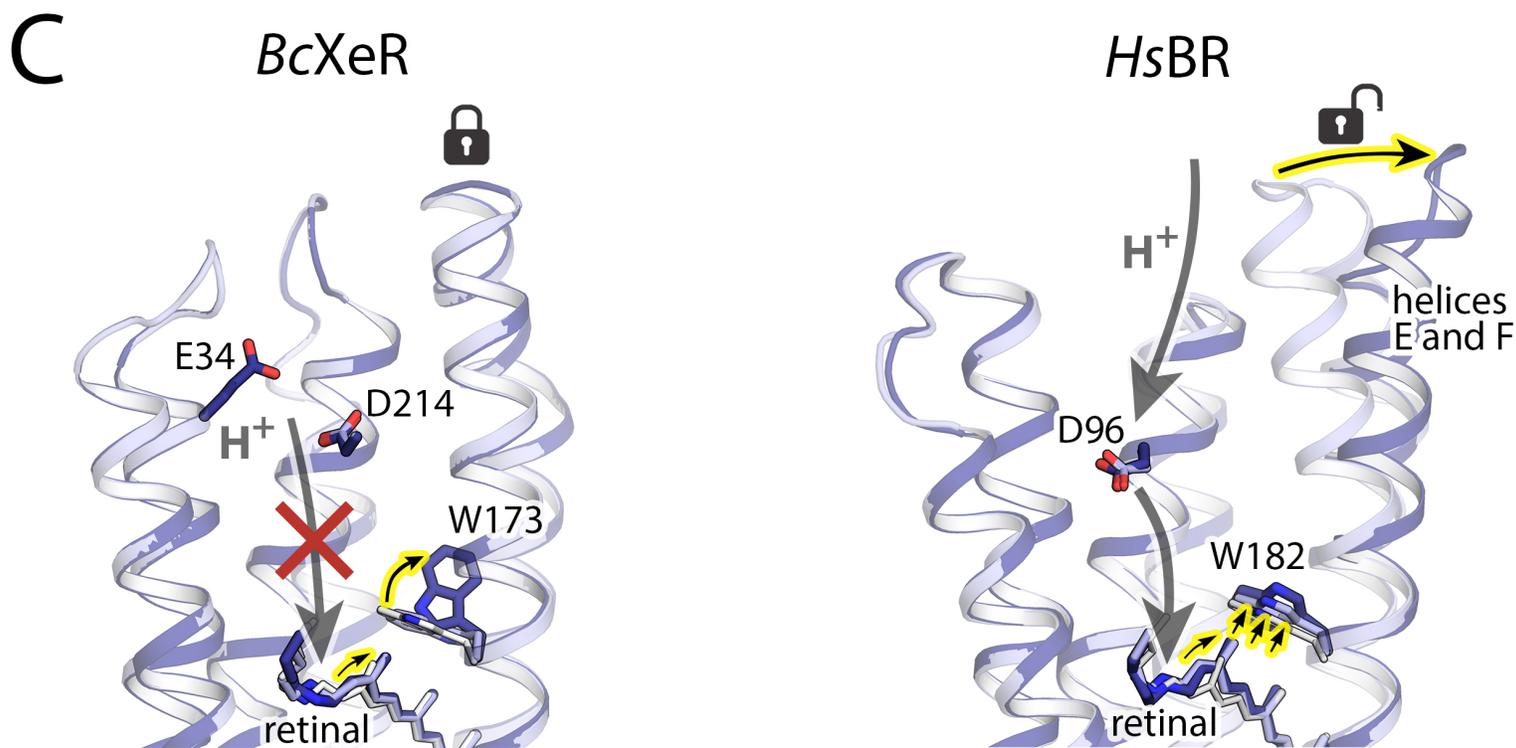
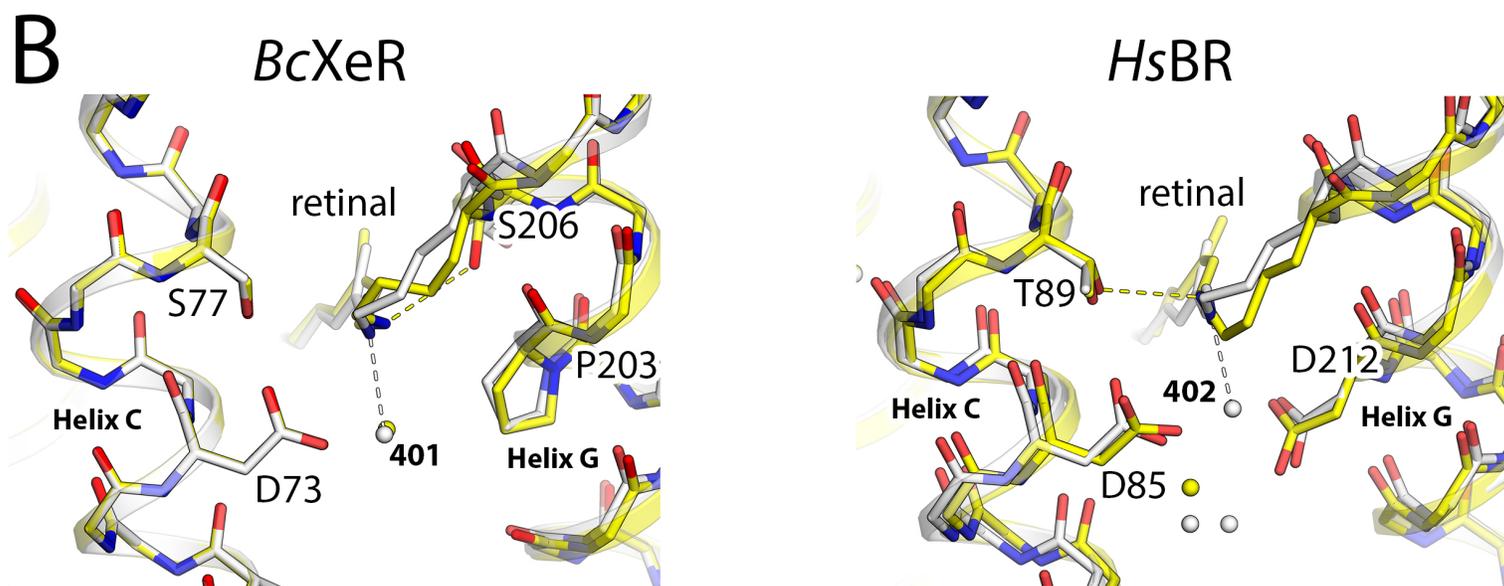
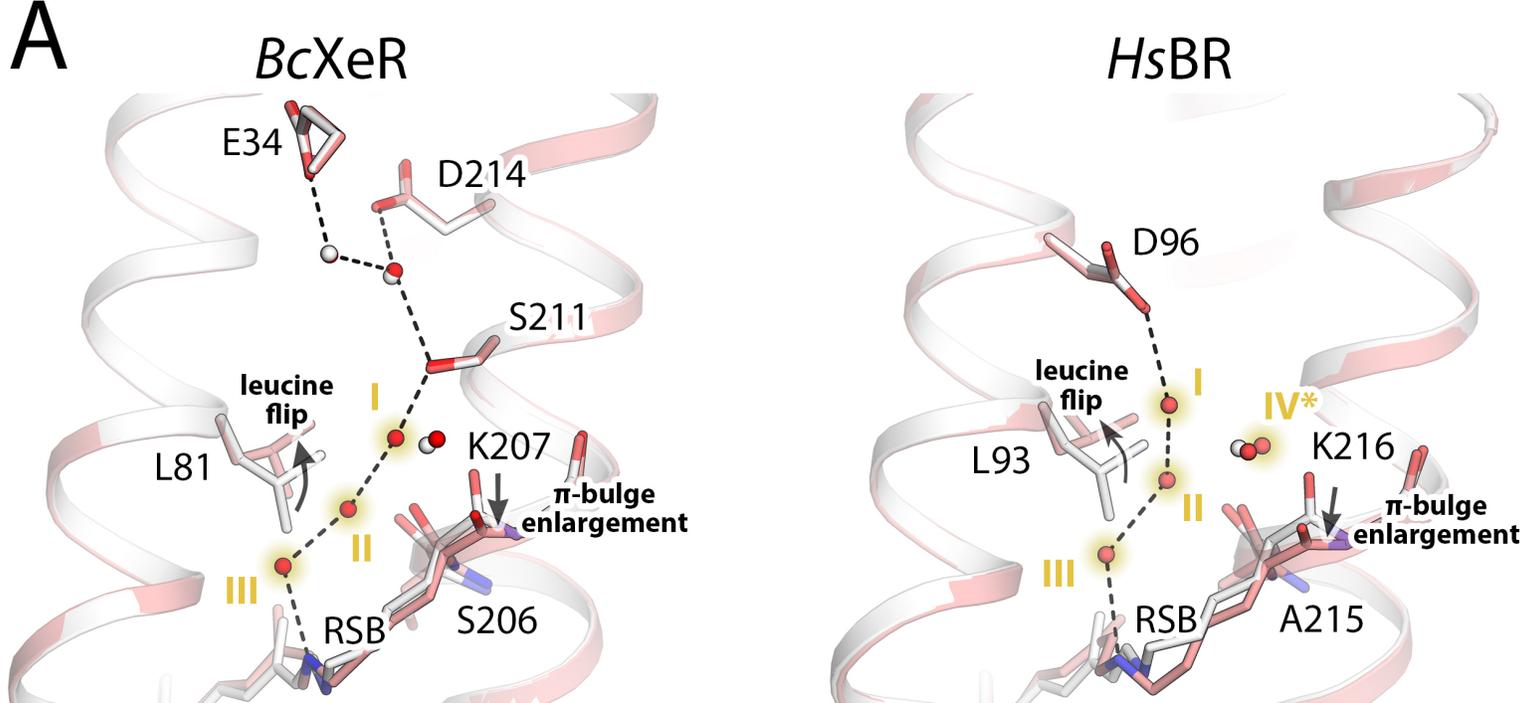
D









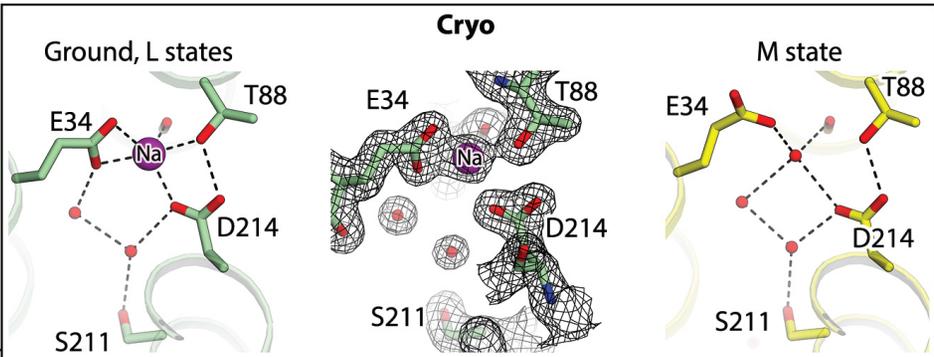


pH

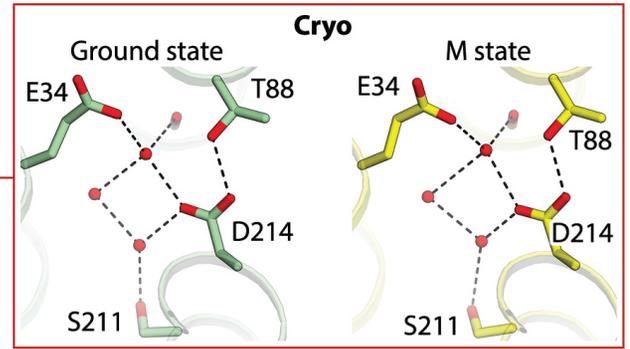
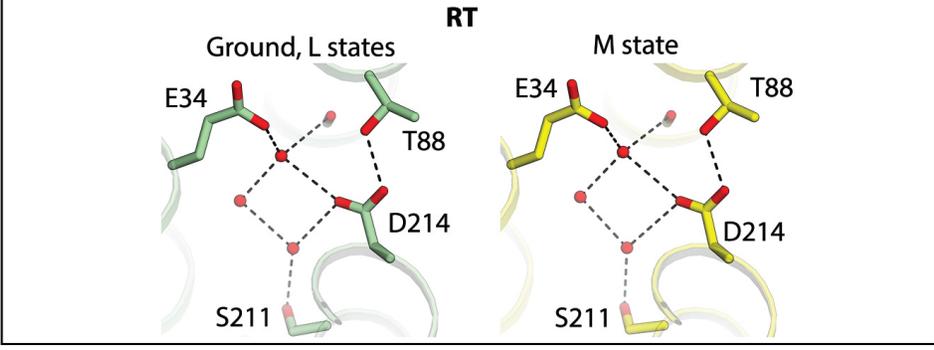
Sodium

No sodium

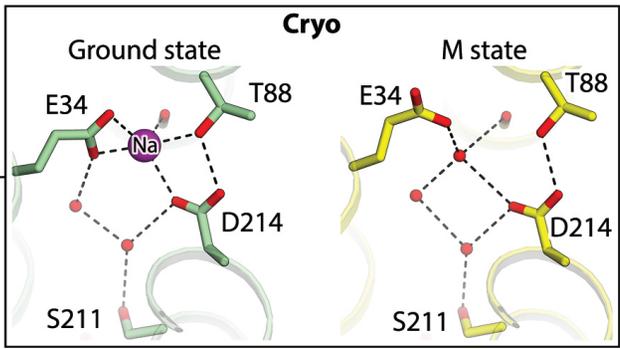
8.2



7.6



7.0



5.2

