Femtosecond to millisecond structural changes in a light-driven sodium pump

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Summary

Light-driven sodium pumps actively transport small cations across cellular membranes¹. They are used by microbes to convert light into membrane potential and have become useful optogenetic tools with applications in neuroscience. While resting state structures of the prototypical sodium pump *Krokinobacter eikastus* rhodopsin 2 (KR2) have been solved^{2,3}, it is unclear how structural alterations over time allow sodium translocation against a concentration gradient. Using the Swiss X-ray Free Electron Laser⁴, we have collected serial crystallographic data at ten pump-probe delays from femtoseconds to milliseconds. High-resolution structural snapshots throughout the KR2 photocycle show how retinal isomerization is completed on the femtosecond timescale and changes the local structure of the binding pocket in the early nanoseconds. Subsequent rearrangements and deprotonation of the retinal Schiff base open an electrostatic gate in microseconds. Structural and spectroscopic data in combination with quantum chemical calculations indicate transient binding of a sodium ion close to the retinal within one millisecond. In the last structural intermediate at 20 ms after activation, we identified a potential second sodium binding site close to the extracellular exit. These results provide direct molecular insight into the dynamics of active cation transport across biological membranes.

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43 **Introduction**

44 The preservation of sodium gradients across cellular membranes is critical to various
45 biological functions. In living cells, the controlled flow of sodium is thus maintained by a
46 series of specialized membrane channels and pumps. For example, glucose uptake in the guts
47 and kidneys of mammals is fueled by a sodium gradient making glucose-sodium-symporters
48 important pharmacological targets in the treatment of diabetes⁵. The opening and closing of
49 voltage-gated sodium channels is responsible for the generation and propagation of neuronal
50 signals. This has enabled the field of optogenetics, where light-sensitive microbial cation
51 channels from the rhodopsin family are used as a key component for the manipulation of
52 physiological responses in neurons or even living animals by light⁶.

53 Rhodopsins are a functionally diverse family of proteins that rely on a retinal chromophore to
54 harvest and sense light energy in microbes⁷ and higher organisms⁸. In 2013, the family was
55 extended by the discovery of light-driven sodium pumps from marine bacteria, where they
56 maintain a low intracellular sodium ion concentration and generate membrane potential¹. In
57 optogenetic applications, the controlled light-induced outward pumping of sodium ions leads
58 to neuronal inhibition under more physiological conditions compared to the use of related
59 proton or chloride pumps². The optogenetic application of the prototypical member of the
60 class, *Krokinobacter eikastus* rhodopsin 2 (KR2), has been demonstrated with nematodes and
61 with cortical rat neural cells². Genetically engineered variants provide further possibilities to
62 optimize KR2 for optogenetic applications^{1-3,9-12}.

63 The pumping cycle of KR2 has been studied by a variety of time-resolved spectroscopic
64 techniques^{1,13-19}. High-resolution structures of the resting state have been determined in
65 various forms by X-ray crystallography^{2,3,20}. However, in these studies the sodium substrate
66 is not bound within the retinal binding pocket, indicating a substantially different pumping
67 mechanism than in related ion pumps. Additional structural information on the reaction
68 intermediates is required to understand how sodium can be actively transported out of the cell
69 against substantial concentration gradients.

70 Time-resolved serial femtosecond crystallography (TR-SFX) allows the determination of
71 structural snapshots of proteins that can be assembled into molecular movies of protein
72 function. A series of classical targets have been characterized using the method including
73 myoglobin²¹, photoactive yellow protein^{22,23} and bacteriorhodopsin (bR)²⁴⁻²⁷. In our present
74 work, we used the Swiss X-ray Free Electron Laser (SwissFEL) to study structural changes in
75 the more recently discovered sodium pump KR2 within a wide temporal window from 800 fs
76 to 20 ms. Ten structural snapshots positioned at temporal delays coinciding with the

77 accumulation of intermediates identified by time-resolved absorption spectroscopy on
78 crystals show how the energy captured by the retinal leads to structural rearrangements.
79 Structural, spectroscopic and quantum chemical data indicate sodium binding between N112
80 and D251 followed by release via a second binding site between E11, N106 and E160 on the
81 extracellular side. Our integrated work thus elucidates the structural changes associated with
82 sodium ions being actively transported across a biological membrane.

83

84 **Results and Discussion**

85 **Photocycle in KR2 crystals**

86 As benchmark for the study of KR2 activation in the crystalline environment, we employed
87 time-resolved absorption spectroscopy in the infrared (IR) and in the ultraviolet/visible
88 (UV/Vis) region. As under the original acidic crystallization conditions KR2 exhibits an
89 accelerated photocycle, we developed a soaking protocol to increase pH in the presence of
90 sodium ions (**Extended Data Fig. 1**). The treatment changes the color of crystals from blue
91 to red with associated changes in the retinal binding pocket. Most importantly in terms of
92 function, KR2 in treated crystals follows a photocycle (**Fig. 1**) identical to that observed by
93 our absorption spectroscopy on purified KR2 in agreement with previous reports^{19,18}.

94 Sodium is expected to bind after deprotonation of the retinal Schiff base (SB) in the M
95 intermediate followed by release in the late O intermediate because the transition between
96 these spectroscopic intermediates is dependent on sodium concentration²⁸. At acidic pH,
97 characteristic O-related bands are absent (**Extended Data Fig. 2**), while at higher pH in the
98 presence of sodium these bands reach maximal amplitude a few milliseconds after activation.
99 A dependency on sodium concentration is evident in the TR-IR data through changes in both
100 amplitude and kinetics of the M-O transition (**Fig. 1, d**). Clearly, KR2 in treated crystals
101 responds to the presence of sodium with a kinetic profile compatible with light-driven sodium
102 pumping. Accordingly, all crystals for the dynamic measurements described below were
103 rebuffed in the presence of sodium before injection across the X-ray laser pulses.

104

105 **Structural changes over time**

106 In three days of beamtime during the first user run of SwissFEL, we collected 158'832 dark
107 (pump laser off) and 496'904 light (pump laser on) indexable diffraction patterns with an
108 anisotropic resolution up to 1.6 Å (**Extended Data Tab. 1, Extended Data Fig. 3, a**). The
109 light data were distributed over ten time delays (Δt = 800 fs, 2 ps, 100 ps, 1 ns, 16 ns, 1 μ s, 30

μs , 150 μs , 1 ms and 20 ms) between the optical pump and the X-ray probe pulses (**Extended Data Fig. 4, a**). These time delays were selected based on our TR-IR data and on previous ultrafast stimulated Raman spectroscopic experiments¹⁶, to cover critical steps in the KR2 photocycle. The serial crystallographic structure of the KR2 resting state closely resembles structures solved by conventional cryo-crystallography (**Extended Data Fig. 5**). Progressing from this starting point, isomorphous difference electron density maps ($F_o(\text{light}) - F_o(\text{dark})$) allow to follow structural changes over time. Extrapolated data were used to refine molecular structures for each individual time delay. The light-activated structures follow a continuous evolution of structural rearrangements that we combined into five different stages (800fs+2ps, 1ns+16ns, 30 μs +150 μs , 1ms and 20ms) based on root-mean-square deviations between the models (**Extended Data Fig. 4, b**). These five structural intermediates provide direct molecular insights into the sequence of structural rearrangements during the KR2 pumping cycle.

Retinal and transmission of light energy

The first stage in the structural evolution of KR2 activation ranges from femto- to picoseconds with structural rearrangements centered at the retinal chromophore, the principal photochemical switch of all rhodopsins. Retinal is covalently bound via a protonated SB linkage to K255 in helix G in the core of KR2. Similar to other microbial rhodopsins, absorption of a photon in KR2 leads to retinal isomerization at the C13=C14 bond (**Extended Data Fig. 3, b**). This photochemical process is faster than in the related proton pump bR, with formation of the earliest photointermediate already after about 200 fs¹⁶, which is consistent with a fully isomerized 13-*cis* retinal in our earliest difference electron density at $\Delta t = 800$ fs and after structural refinement at $\Delta t = 800\text{fs} + 2\text{ps}$ (**Fig. 2, a**). Within the early time delays, we further observed a shift of w406 and the retinal counterion D116, similar, but less pronounced compared to the ultrafast adaptation of bR²⁵. A clear difference to bR is that the isomerized retinal is pointing in the opposite direction in KR2 with the C20 methyl group tilting towards helix C instead of helix G. The direction could already be pre-determined in the resting state as, compared to bR, the retinal polyene KR2 is bend in the opposite direction.

The second distinct stage within the structural evolution takes place in the nanosecond range (**Fig. 2, b**), when early conformational changes of the protein backbone occur. In bR, adaptation of the energetically more favorable planar 13-*cis* conformation pushes the

straightening retinal “upwards” against W182 in helix F to displace it towards the cytoplasmic side²⁴. In KR2, the changes involving the structurally equivalent W215 are absent, instead, the retinal C20 methyl group pushes “sideways” in the membrane plane against V117. Starting at $\Delta t = 1 \mu s$ and rising until the later microsecond delay, the difference density maps indicate a flip of V117 and an established transmission of structural changes into helix C (**Fig. 2, c**). In this way, the light energy stored in the early photoproducts propagates into the seven-transmembrane helical bundle to fuel larger conformational changes at later times.

Sodium translocation and gating

The third and fourth stages of the structural evolution from microseconds to milliseconds correlate with the temporal range relevant for sodium translocation^{19,28}. Starting from $\Delta t = 1 \mu s$, clear electron difference density peaks above 3.5σ show how Y218 in helix F and S254 in helix G approach the position of retinal in the resting state. Changes are further transmitted several turns along helix G towards the intracellular side. Structural refinement resulted into small shifts of helix C in the order of $1 \text{ \AA}$ in the $\Delta t = 30 \mu s + 150 \mu s$ structural intermediate and additional changes along helix D occur in the two millisecond delays. These rearrangements (**Fig. 3**) are of particular interest because they are close to the putative entry and exit routes for sodium^{2,3}.

One half of the translocation pathway connects the retinal binding pocket between helices C, F and G with a water-filled cavity on the intracellular side. It passes N61 and G263 at the entry side and Q123 of the NQD motif, three residues important for ion selectivity^{3,11,13}. Native KR2 pumps lithium and sodium ions, yet not larger cations like potassium¹. In their hydrated forms the size of sodium is larger than that of potassium, but the dehydrated sodium ion is smaller than potassium^{29,31}. This suggests that the selective pumping of sodium ions must involve dehydration, which likely happens at the entry of the conducting pathway³¹.

The narrowest part of the sodium translocation pathway in the light-activated structures of KR2 passes between the retinal and the side chains of D116 and K255, acting as a counterion and covalent link for the retinal, respectively. Neutralization of the retinal SB through proton transfer and transient widening at this position likely acts as an electrostatic gate (**Fig. 4, a, Extended Data Vid. 1**) that allows the selective passage of cations^{2,3,32}. The distance between the retinal SB and D116 contracts by about $0.5 \text{ \AA}$ at $\Delta t = 1 ns + 16 ns$, which favors proton transfer in the transition to the M intermediate³³. At $\Delta t = 30 \mu s + 150 \mu s$ the distance

widens again in agreement with the spectroscopic data indicating deprotonation of the SB and occurrence of the M state in the early microseconds (**Fig. 1**). With 1.4 Å, the opening seems small for a 1.9 Å sodium ion to pass. At this point we cannot exclude that the gate does not fully open in our crystals formed from monomeric KR2, since pentameric KR2 can adopt a more open conformation in the SB region^{3,20} and mutations in the oligomerization interface can affect pumping efficiency^{1,17,20} (for further discussion see **Extended Data Fig. 5**). However, stable structural intermediates that accumulate in time-resolved studies of molecule ensembles do not necessarily reveal all functional steps³⁴ and our integrated structural, spectroscopic and computational analysis is compatible with sodium binding in the later stages (see below). Most probably, approach of a sodium ion and electrostatic weakening of the helix C-SB interaction allows formation of a transient pathway within the L/M equilibrium in the microseconds.

The fifth stage in the late milliseconds is related to changes in the section of the translocation pathway extending from the retinal binding site towards the extracellular side of the protein. Here the bottlenecks run along the side chain of R109 in agreement with mutations at this positions transforming KR2 into a light-gated inward facing potassium channel³⁵. A rotamer change of R109 and Q78 together with a shift of helix D at $\Delta t = 20$ ms indicates an opening which connects the water-filled cavity in vicinity of the retinal with a second water-filled cavity close to E11, N106, E160 and R243 towards the exit site on the extracellular side of the membrane.

Formation of sodium binding sites

In the alternate access model of active membrane transport, the substrate is bound while the protein rearranges to allow release without backflow. Light-driven sodium pumps like KR2 do not bind substrate close to the retinal SB in the resting state¹⁻³. Hence, the question remains where the transported sodium ion is located and how it moves across the membrane with time. Time-resolved IR spectroscopy on a protein film (this work and¹⁹) and crystals provides evidence (through a marker band at 1688 cm⁻¹) of changes in the environment of an asparagine residue peaking in the O intermediate within milliseconds under sodium pumping conditions (**Fig. 1, Extended Data Fig. 2**). Mutagenesis of N112 and D251 close to the retinal binding pocket abolish sodium pumping^{13,35,36} and the two residues have been suggested as potential sodium coordination partners based on molecular dynamic simulations³² and structural comparisons to other rhodopsins³⁷ (**Extended Data Fig. 6**).

Analysis of our TR-SFX data shows an interesting evolution of the electron density in this region (**Fig. 3 c, d**). At $\Delta t = 1$ ms a clear positive difference peak is located about 1 Å away from w406 in the resting state and close to N112 and D251. The electron densities of water and sodium ions have an identical signature in X-ray crystallographic data, yet the environment can provide clues to the nature of the detected atoms³⁸. At $\Delta t = 1$ ms the density peak is shifted away from the amine group of W113 and positively charged R109, both coordinating w406 in the resting state but unable to bind sodium. The new position is with 2.5 Å towards N112 and D251, which is close to the ideal coordination distance for a sodium ion³⁹ concurring with our spectroscopic data. We validated the position of the putative sodium ion using hybrid quantum mechanics/molecular mechanics (QM/MM) calculations. Inclusion of the sodium in the $\Delta t = 1$ ms structure resulted in a large spectral red shift of 55 nm with respect to the electronic absorption band of the resting state, while placing water at this position yields a 11 nm blue shift (**Fig. 4, b, c**). The experimental value obtained from the transient spectroscopic data is a red shift of 66 nm and hence inclusion of the sodium ion is necessary to reproduce the absorption maximum of the O intermediate ($\lambda_{\text{max}} = 592$ nm). The absorption shift is not a direct effect but due to changes in the D116-SB interaction through N112. It is reasonable to suggest that through alteration of this H-bonding network sodium binding favors SB reprotonation, and with this blocks the backflow of ions.

At $\Delta t = 20$ ms, the electron density close to the retinal binding pocket fades below the 3σ level indicating release of the sodium ion. Further along the translocation pathway we observe the formation of a second sodium binding site close to the extracellular side of the membrane. Here, a clear positive difference peak appears between E11, N106 and E160. In the same temporal regime, the shift of R243 moves a positive charge away to facilitate sodium binding (**Fig. 3 e, f**). Again, the coordination distances of 2.4 Å to N106 and 2.5 Å to E11 support our assignment of a sodium ion in this putative binding site. Of particular note is the observation that both sodium binding sites employ displacements of arginine residues to favor sodium binding over water. The corresponding positions of these residues have functional equivalents in bR (**Extended Data Fig. 6**) with R109 close to the retinal binding pocket corresponding to R82 critical for proton transfer in bR. The position of R243 in KR2 is occupied by E194 in bR, which is part of the proton release group. Some key sites in the seven-transmembrane helical bundle seem to be functionally conserved throughout evolution but are approached at different times within their respective pumping cycles.

Conclusions

Our data allow to assemble a molecular movie of structural changes in KR2 and to propose a basic mechanistic model of light-driven sodium transport (**Video 1**). The unidirectional flow of ions is achieved by minimal structural changes that generate ion selectivity and prevent ion back leakage into the cell. Our observation of active ion pumping is consistent with general concepts of ion pumping across a biological membrane by the alternate access model, illustrating them with high-resolution structures of the intermediate steps. It will be an interesting topic for future studies how pH and long-range cooperative effects between protomers influence these structural dynamics. X-ray lasers now provide the means to study how single point mutations allow translocation of larger ions like potassium³ and cesium¹¹ or turn KR2 from an active pump into a passive channel³⁵. Deeper insights into the transport mechanisms found in microbial rhodopsins will demonstrate how nature adapted a common leitmotif to different functions and facilitate the design of variants for neurobiological applications in optogenetics.

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Author contributions

The project was initiated and coordinated by J.S. following input on crystallographic data evaluation from T.W., quantum chemical calculations from I.S., pump-probe experiments at

the ALVRA endstation from C.M. and time-resolved spectroscopy from J.H.. The KR2 expression construct was cloned by R.M.B.. The protein was expressed, prepared and crystallized by P.S. with help from A.F.. A constant supply of sample during the beamtime was secured with the help of A.F., T.G., M.W., D.E., D.K. and R.M.B. using protocols developed by P.S.. Crystal injection was optimized by P.S. and D.J.. The lipidic cubic phase injector was operated and aligned during the beamtime by D.J., F.D., I.M. and D.G.. The endstation including the laser system were aligned and operated by P.S., K.N., G.K., C.C. and C.M who also designed the Alvra prime pump-probe station. C.A. and P.J.M.J. operated the timing diagnostics during the experiment. The SFX data analysis pipeline was built and operated by D.O. and K.N. Data processing during the beamtime was done by T.W., P.N. and S.B.. S.M., V.P. and X.D. recorded progress during data collection and/or in a previous test beamtime. Final structures were refined by P.S. and T.W. and interpreted together with J.S.. Quantum chemical calculations were done by R.K.K. and I.S.. The time-resolved spectroscopic experiments were done by D.E. and P.S. and interpreted together with J.H.. The manuscript was written by J.S. with direct contributions from P.S., T.W., D.E., J.H., R.K.K., I.S. and further suggestions from most of the other authors. All authors read and acknowledged the manuscript.

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Competing interests

The authors declare to have no competing interests.

Data availability

Resting state coordinates and structure factors have been deposited in the PDB database under accession code 6TK7 (acidic pH) and 6TK6 (neutral pH). Together with the neutral pH resting state structure the light activated data for all time points (800 fs, 2 ps, 100 ps, 1 ns, 16 ns, 1 μ s, 30 μ s, 150 μ s, 1ms and 20 ms) was deposited in the mmCIF file. For the refined structures using combined data (800fs+2ps, 1ns+16ns and 30 μ s+150 μ s) and single (1ms and 20ms) light activated data sets, coordinates, light amplitudes, dark amplitudes and extrapolated structure factors have been deposited in the PDB database under accession codes 6TK5 (800fs+2ps), 6TK4 (1ns+16ns), 6TK3 (30 μ s+150 μ s), 6TK2 (1 ms) and 6TK1 (20 ms).

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Fig. 1: Time-resolved absorption measurements on KR2 in solution and crystals. Spectra from (a) purified KR2 and (b) in the crystalline phase prepared in analogy to the TR-SFX experiment. The upper panel depicts experiments in the infrared covering the C=C stretch mode of the retinal up to amide I vibrations originating from the protein backbone. The middle panel presents changes in the UV/Vis region on the same sample. A global fit analysis of the infrared data shown in the bottom panel revealed the presence of intermediate states K_L , L/M, O_1 and O_2 . (c) Model of the photocycle of KR2 derived from time-resolved absorption spectroscopy. (d) Sodium-dependency of the 1516 cm^{-1} marker band for the O intermediate in KR2 crystals under TR-SFX conditions. Time traces were normalized to the ground state bleach signal and fitted by the sum of three connected exponentials.

Fig. 2: Early steps in the activation of a light-driven sodium pump. Difference electron density maps ($F_o(\text{light}) - F_o(\text{dark})$, negative density in gold and positive density in blue shown at $3.5\text{ }\sigma$) and structural refinements (resting state shown in grey and light activated in color) show the early rearrangements at (a) $\Delta t = 800\text{ fs} + 2\text{ ps}$, (b) $\Delta t = 1\text{ ns} + 16\text{ ns}$, (c) $\Delta t = 30\text{ }\mu\text{s} + 150\text{ }\mu\text{s}$. Superposition of structures illustrates how retinal isomerization translates the light energy into structural changes (d) via V117 in helix C in case of KR2 (resting state shown as grey sticks, the structures at $\Delta t = 2\text{ ps}$ shown as blue sticks and $\Delta t = 1\text{ }\mu\text{s}$ shown as green sticks) and (e) via W182 in helix F in case of bR (data and coordinates taken from^{24,25}, resting state shown as grey sticks, the structures at $\Delta t = 10\text{ ps}$ shown as blue sticks and $\Delta t = 0.8\text{ }\mu\text{s}$ shown as green sticks). Arrows indicate structural changes discussed in the main text.

Fig. 3: Structural changes along the sodium translocation pathway. (a) Overview of the KR2 structure with the suggested route of sodium translocation across the membrane identified using the program Caver⁴⁰ (blue and red surfaces for the two halves plotted on the structure at $\Delta t = 1\text{ ms}$ with changes in color indicating functionally critical regions). Selected residues and the retinal chromophore are shown as sticks. (b) Structural refinements and comparison to the resting state show how the protein rearranges from nanoseconds to milliseconds (blue to red gradient and ribbon width indicate rmsd to the resting state). Close-up view into the region of the retinal SB (insets c, d) and the extracellular side of the membrane (insets e, f). The structural rearrangements are compatible with the formation of a transient sodium binding site between N112 and D251 at $\Delta t = 1\text{ ms}$ (c) and a second site further along the translocation pathway between E11, N106 and E160 at $\Delta t = 20\text{ ms}$ after photoactivation (f). Difference electron density maps ($F_o(\text{light}) - F_o(\text{dark})$) shown with negative density in gold and positive density in blue countered at $3.2\text{ }\sigma$) and arrows highlight structural changes discussed in the main text.

Fig. 4: Electrostatic gating mechanism. (a) Circles show the retinal binding pocket at critical steps in the KR2 photocycle. The positive charge of the retinal SB is shown in blue and the negative charge at the D116 counterion is shown in red. Arrows indicate steps in sodium translocation including light-induced retinal isomerization and conformational rearrangements in the retinal binding pocket, entry of a sodium ion after proton transfer from the SB to the counterion, binding of the sodium ion and reprotonation of the retinal SB and ion release. QM/MM optimized geometry with water (b) or sodium ion (c) in the binding site between N112 and D251. The spectral shift is only in line with the spectroscopic data on the redshifted O intermediate (**Compare Fig. 1 and Extended Data Fig. 6, h**) when sodium is included. The shift is not a direct effect but is due to the change in the D116-SB interaction through N112 via the shown hydrogen-bonding network (dashed lines). For a dynamic illustration of the described rearrangements compare **Video 1**.

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Methods

Cloning, protein expression and purification

The KR2 construct with a TEV-cleavable C-terminal 6xHis-tag was cloned into the pStaby1.2 vector (Delphi Genetics, Belgium). Protein expression was performed in C41(DE3) *E. coli* cells. The cells grew in shaking smooth flasks with Luria Broth at 37 °C. Expression was induced by addition of 1 mM β -D-thiogalactopyranoside (IPTG) at an OD600 of ~0.8. Following overnight expression at 37 °C in the presence of 10 μ M all-*trans* retinal, the bacterial cultures were harvested by centrifugation at 5,000 g for 15 minutes. The cell pellets were disrupted with Avestin EmulsiFlex-C3 homogenizer at 15,000 psi in lysis buffer (20 mM Tris pH 8.0, 5% glycerol, 0.5% Triton X-100, 5 μ g/ml DNase I and cOmpleteTM protease inhibitor tablets, Roche), and the membrane fraction was collected with ultracentrifugation at 90,000 g. The membrane pellet was resuspended with IKA T 25 Ultra-Turrax disperser in solubilization buffer that contained 50 mM Tris pH 8.0, 300 mM NaCl, cOmpleteTM protease inhibitors, 1.0% n-Dodecyl β -D-maltoside (DDM, Anatrace), 0.2% cholesteryl hemisuccinate (CHS, Anatrace), and stirred overnight at 4 °C. The overnight suspension was subjected to a second round of ultracentrifugation before the supernatant was applied to Immobilized Metal Affinity Chromatography (IMAC), and further washed with IMAC buffer (50 mM Tris pH 8.0, 150 mM NaCl, 100 mM Imidazole, 0.02% DDM, 0.04% CHS). The bound protein was eluted by addition of 500 mM imidazole in IMAC buffer. TEV protease was added, and the KR2-TEV cleavage solution was sealed in an 8 kDa M_w cutoff dialysis membrane and dialyzed against 50 mM Tris pH 8.0, 150 mM NaCl, 0.02% DDM, 0.04% CHS buffer overnight. The TEV-cleaved solution was re-applied to the IMAC column, and the flow-through was collected and concentrated with a centrifugal filter device (Millipore 100 kDa M_w cutoff). The concentrated protein sample was loaded onto a HiLoad Superdex 75 prep grade 16/600 column (GE Healthcare) equilibrated with SEC buffer (50 mM Tris pH 8.0, 150 mM NaCl, 0.05% DDM, 0.01% CHS). The elution profile was monitored at 280 nm and 530 nm with Shimadzu UV-2401PC spectrophotometer (Kyoto, Japan), and the purest fractions were concentrated to ~100 mg/ml, flash frozen in liquid nitrogen and stored at -80 °C until further crystallization.

499

500 **Crystallization and TR-SFX sample preparation**

501 Crystallization was carried out in lipidic cubic phase (LCP) using conditions similarly as
502 described in ref². The purified protein buffer and monoolein (1-Oleoyl-rac-glycerol, Nu-Chek
503 prep) were thoroughly mixed in a 2:3½ v/v ratio through coupled gas-tight Hamilton
504 syringes. The formed LCP was extruded through Hamilton needles into plastic B-Braun
505 Omnifix®-F syringes loaded with precipitant (200 mM Sodium Acetate pH 4.4, 150 mM
506 MgCl₂, 35% PEG 200). Crystallization occurred overnight in the dark at 20 °C and yielded
507 plate-like blue KR2 crystals with dimensions of 10-30×10-25×1-3 μm³ (**for a size**
508 **distribution see Extended Data Fig. 1, c**).

509 Following formation of crystals, the precipitant solution was washed out by soaking the LCP
510 in excess 150 mM NaCl, 35% PEG 200 solution, two times for 48 hours in total. The washed
511 phase with unbuffered crystals having blue color was further harvested into gastight
512 Hamilton syringes in 60 μl fractions, and doped with 33 μl monoolein and 3.0 μl 50% PEG
513 1500 to form a stable jetting phase. Prior to data collection, the phase with crystals was
514 mixed with LCP prepared from monoolein and 1 M Tris pH 9.0, 150 mM NaCl, 35% PEG
515 200 through a three-way syringe coupler⁴¹. The volumes of the mixed phases were picked
516 such that the water fraction of the mesophase would contain 200 mM Tris, 150 mM NaCl,
517 35% PEG 200 and PEG 1500 and the blue KR2 crystals changed color to red upon mixing
518 (**Extended Data Fig. 1**). While the mixing was done with Tris solution at pH 9.0, the final
519 pH of the preparation was close to pH 8 as confirmed by litmus paper. We attribute this shift
520 by one pH unit and the shift in color transition as compared to KR2 solution to possible
521 residual buffer trapped in LCP and/or buffering capacity of monoolein molecules. Jetting
522 stability of the mixed phase was confirmed before the XFEL experiment with a high-speed
523 camera setup described in⁴¹ (**Video 2**).

524

525 **Experimental setup and XFEL data collection**

526 The TR-SFX data on KR2 crystals were collected in February 2019 in three days at the Alvra
527 experimental station of SwissFEL. X-ray pulses with a photon energy of 12 keV and a pulse
528 energy of 400 μJ at a repetition rate of 50 Hz were used for the experiment. On average 180
529 μJ (9×10¹⁰ X-ray photons) per pulse were deposited onto a 2.6 × 3.6 μm² spot (FWHM),
530 focused by Kirkpatrick-Baez mirrors. The X-ray intensity was adjusted using solid
531 attenuators to maximize diffraction signal without disrupting the sample injector flow or
532 damaging the detector. To reduce X-rays scattering, the air in the sample chamber was

pumped down to 100-200 mbar while being substituted with helium. To reduce the amount of data the Jungfrau 16M detector was run in 4M mode excluding the outer panels.

KR2 crystals were loaded into a high viscosity injector connected to an HPLC pump⁵⁴. The crystals were extruded into the pump-probe interaction point through a 75 μm capillary at a flow rate of 3.35 μl per minute. In the interaction point, the probe XFEL beam intersected with a circularly polarized pump beam originating from an optical parametric amplifier producing laser pulses with 150 fs duration ($1/e^2$), 575 nm wavelength and 3 μJ total energy in a focal spot of $80 \times 80 \mu\text{m}^2$ beam ($1/e^2$), corresponding to a maximal laser fluence of 59 mJ/cm^2 and laser power density of 397 GW/cm^2 . Approximating the dose per KR2 molecule with the Lambert-Beer law for an average $19 \times 16 \times 2 \mu\text{m}^3$ sized crystal, we estimated that 8.3-3.5 photons per retinal are absorbed, depending on the crystal's orientation and the position of the individual chromophore within the optically dense crystal. However, the average photon dose is certainly lower because scattering and reflection on the extruded material further reduces the doses with estimates ranging from 20%²⁷ to 90%⁵⁵. Our previous best estimate was 80% for TR-SFX experiments on bR²⁵, which would reduce the calculated doses to 1.7-0.7 photons per retinal. Another point to consider is that the $\text{Sn} \leftarrow \text{S1}$ excited-state absorption is in the 400–500 nm region¹⁵. As such the excited state is unlikely to absorb a 575 nm photon further minimizing the chance of multi-photon absorption.

To cover the KR2 photocycle time delays between the pump laser and the probing XFEL pulses were chosen at $\Delta t = 800 \text{ fs}, 2 \text{ ps}, 100 \text{ ps}, 1 \text{ ns}, 16 \text{ ns}, 1 \mu\text{s}, 30 \mu\text{s}, 150 \mu\text{s}, 1 \text{ ms}$. An additional time delay at 20 ms was created by shifting the laser focus position and using the 50 Hz XFEL repetition rate to create a delay to the pump laser.

Every fifth pulse of the pump laser was blocked, so that a series of four light-activated and one dark diffraction pattern were collected in sequence. Roughly 50,000 light-activated patterns were collected for each time delay, a high-quality dark dataset was obtained by merging patterns of the fifth pulse, and $\sim 50,000$ patterns with pump laser off. These laser off images were also acquired for comparison, to confirm that the dark data in each cycle was not illuminated. Finally, about 40,000 patterns were obtained with no laser activation for untreated crystals at acidic pH, to compare with crystals soaked as described above. For clarity, the applied data collection scheme is illustrated in **Extended Data Fig. 4**.

Data processing

All data were indexed, integrated and merged using Crystfel^{56,57} version 0.8.0. Data were indexed using the xgandalf⁵⁸ and taketwo⁵⁹ algorithms. Data were integrated using the --rings

option in indexamajig. Patterns were merged using partialator with the following options: --model=unity, --iterations=3. No per-pattern resolution cutoff was applied. Data showed diffraction anisotropy of about 2.2 x 2.2 x 1.6 Å along a*, b* and c*. The general resolution cutoff of 1.6 Å has been chosen after evaluating all uncut datasets using the staraniso server⁴⁸, showing a maximal resolution of 1.6 Å for most datasets (**Extended Data Table 1**). Diffraction intensities of each individual dataset output from partialator were cut at 1.6 Å resolution and subjected to the staraniso server again. The staraniso server determines the resolution cutoff for each dataset based on the signal-to-noise ratio in a given resolution shell and then truncates data along these directions, in case of KR2 this resulted in data truncation very close to the given ellipsoid dimensions. It is currently not possible to generate a merged dataset from serial crystallographic data using staraniso, therefore we generated datasets containing only reflections that were kept by staraniso and generated statistics from them using CrystFEL. Refinements for dark data collected at acidic and neutral pH were carried out to the full resolution range against data obtained after anisotropic truncation. Since data extrapolation lowers the data quality, we lowered the resolution cutoff for structural refinements of the combined data (800fs+2ps, 1ns+16ns and 30µs+150µs) to 2.25 Å resolution and of the smaller datasets at 1 ms and 20 ms to 2.5 Å. The truncated datasets were deposited to the world wide Protein Data Bank (wwwPDB)⁶⁰ together with the structures (**Extended Data Table 2**) refined against original (resting acidic pH and resting neutral pH) and extrapolated data (800fs+2ps, 1ns+16ns, 30µs+150µs, 1 ms and 20 ms).

Calculation of difference density maps

All $F_o(\text{light}) - F_o(\text{dark})$ difference maps were calculated using PHENIX⁶¹ using the multi-scaling option excluding amplitudes smaller than 3 σ and resolutions lower than 10 Å in the anisotropy corrected data and phases of the refined neutral resting state.

Structure determination and refinement of KR2 resting state

The structure of the KR2 neutral resting state was solved using molecular replacement with pdb 3x3c² as search model. The structure of the KR2 acidic resting state was refined directly using the neutral model as a starting point. Structural refinements were done using PHENIX⁶¹ with iterative cycles of manual adjustments made in Coot⁶².

Data extrapolation

Extrapolated data were calculated using the anisotropy corrected data and a linear approximation as follows: $F_{\text{Extra}} = 100/A \times (F_0(\text{light}) - F_0(\text{dark})) + F_0(\text{dark})$, where A is the activation level in percent, F_{Extra} are the extrapolated structure factor amplitudes. $F_0(\text{light})$ was scaled to $F_0(\text{dark})$ prior to calculation of F_{Extra} . The activation level A (the percentage of molecules that did neither stay in nor return to the dark state after the laser pulse) was determined by calculating extrapolated maps with phases of the dark state and light data at different activation levels in steps of 2 % in the calculation of F_{Extra} , until features of the dark state appeared at the retinal. Based on this analysis, an activation level of 14 % was chosen for extrapolated maps.

Refinement of time-resolved states

Negative amplitudes resulting from the extrapolation procedure were removed from the TR-SFX data before model building and refinement. The models were manually adjusted to best fit observed difference map features as well as extrapolated maps. For residues with multiple conformations present in the resting state the prevalent conformation was chosen based on electron density maps, followed by refinement in PHENIX⁶¹. Initial models were refined against extrapolated data from all ten different time delays. Using a pairwise comparison at all time delays (**compare Extended Data Fig. 4, b**) of structures based on their rmsd, in combination with manual inspection of electron density maps and input from the time-resolved spectroscopic data (**compare Fig. 1**) we identified structural transition points and combined delays at 800 fs with 2 ps, 1 ns with 16 ns and 30 μ s with 150 μ s to further improve density maps and refinement statistics and model quality for the first three deposited intermediates (**Extended Data Tab. 2**). In the final refinement of the two delays in the millisecond regime restraints on sodium distance have been used. However, this only marginally affected results, as the sodium atom close to the retinal binding pocket refined to a distance of 2.4 Å to N112 and 2.7 Å to D251 with an average near identical to the 2.5 Å from restrained refinement. A similar result was obtained within the second binding pocket where the sodium atom refined to a distance of 2.4 Å to N106 and 2.5 Å to E11 with constraints and 2.4 Å to both residues without them.

Time-resolved spectroscopy

Crystals for the spectroscopic characterization have been prepared in a similar fashion as to those used in the time-resolved SwissFEL experiments. A few microliters of crystals immersed in LCP (lipidic cubic phase) were sandwiched between two BaF₂ windows and sealed with vacuum paste immediately after extrusion to prevent drying⁶³. Similar to our previous studies^{64,65}, KR2 solubilized in 0.02% DDM, 0.004% CHS with 100 mM Tris pH 8.0, 150 mM NaCl, was dried from a concentrated protein solution on a BaF₂ window. The dried film was rehydrated via the vapor phase generated by a glycerol/water mixture in a 3:2 ratio and sealed with a second window using vacuum grease.

Time-resolved IR experiments were recorded on a home-built spectrometer based on tunable Quantum Cascade Lasers (QCLs) as described in⁴². We traced transient absorption changes in the frequency range of 1510-1690 cm⁻¹ in steps of 2 cm⁻¹ for crystals and 1 cm⁻¹ for protein film across the time range of 5 ns - 200 ms. For experiments on rehydrated KR2 films in KCl (used for comparison in **Extended Data Fig. 2**) the lower time resolution of rapid-scan spectroscopy was sufficient.

The repetition rate was set to 2 Hz and each kinetic was averaged 25 times. After reaching 1690 cm⁻¹ the scanning direction was reversed and the two datasets were merged accounting for possible protein bleaching. The frequency-doubled emission of a Q-switched Nd:YAG (Neodymium Yttrium Aluminum Garnet, Minilite; Amplitude, Pessac, France) laser emitting at 532 nm was used for photoactivation with an energy density set to ~3 mJ/cm².

Absorption changes in the visible were recorded using a commercial flash photolysis setup (LKS70; Applied Photophysics, Leatherhead, UK) essentially as described in⁶⁴. The photoreaction was induced by a short laser pulse emitted by a Nd:YAG laser (Quanta-Ray; Spectra-Physics, Santa Clara, USA), which drives an optical parametric oscillator tuned to 523 nm with an energy density of ~3 mJ/cm². Transients were recorded from 380 to 650 nm in 10 nm steps omitting the wavelength around the exciting laser pulse due to light scattering (i.e. 510-550 nm). Each trace was recorded 10x with a repetition rate of 2 Hz and subsequently averaged.

Time-resolved step-scan and rapid-scan FTIR experiments on KR2 films were conducted using a Vertex 80v spectrometer (Bruker Optics, Ettlingen, Germany). Excitation laser source was the same as for the QCL measurements with a repetition rate of 2 Hz for the step-scan mode. In the case of rapid-scan experiments a slower repetition rate of 0.018 Hz taking account for the slower photocycle of KR2 in the presence of KCl.

We reconstructed the recorded data by applying singular value decomposition (SVD). Kinetic analysis was done by fitting the data to a model consisting of a unidirectional sequence of states. This yields a concentration profile of the involved and spectroscopically observable states over the course of our measurement⁶⁵.

Hybrid quantum mechanics/molecular mechanics (QM/MM) calculations

Crystallographic coordinates from TR-SFX were used as the initial input for the hybrid quantum mechanics/molecular mechanics (QM/MM) calculations⁶⁶. A subsystem of the protein was chosen and treated using a quantum chemical method (QM region), while the remaining part was treated using a classical force-field (MM region), namely AMBER ff14SB⁶⁷. The QM region includes the retinal chromophore, the side chain of K255, and side chains of residues D116, N112, and D251. The link atom was placed at the QM/MM boundary between the C α -C β atoms of the amino acids. To test the effect of sodium binding we included either a sodium ion or a water in the QM region of the 1 ms structure.

The geometry optimization was performed at the B3LYP/cc-pVDZ/AMBER level of theory. In these simulations, the protein backbone position was fixed to the crystallographic structure, whereas the QM region and side chains of amino acids within the 5 Å region of the retinal protonated Schiff base in the MM region was relaxed. Resolution of identity for Coulomb integrals (RI-J) and chain of sphere approximation for the Exchange integrals were applied (RIJCOSX)⁶⁸. Corrections for dispersion effect was included with D3/B-J damping variant⁶⁹. The calculation of vertical excitation energies uses the simplified TD-DFT method⁷⁰ developed by Grimme and co-workers at CAM-B3LYP/cc-pVTZ level of theory. All the QM/MM calculations were performed with quantum chemistry package Orca interfaced with DL_POLY module of ChemShell⁷¹ for the force-field.

696 **Extended Data Items available for this paper.**

697 Extended Data Figures 1-6

698 Extended Data Tables 1-2

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Extended Data Items

Extended Data Fig. 1: TR-SFX sample preparation scheme and characterization. (a)

Two lipidic phases, the first containing KR2 crystals where the acidic buffer had been washed away and the second containing soaking buffer, were mixed through a three-way coupler⁴¹. The sodium concentration was adjusted to 150 mM and tests with litmus paper indicate a pH close to 8 in the final mixture. (b) Left column: Crystals after TR-SFX sample preparation with varying Tris buffers with pH values from 7.0 to 9.0. Right column: KR2 in 200 mM Tris, 150 mM NaCl, 0.02% DDM, 0.004% CHS solution with varied pH of Tris buffer. Note that crystals grown in LCP reach red color after mixing with Tris pH 9.0, whereas the solution reaches red color at pH 8.0. Likely this shift is due to residual buffering capacity from the original crystallization conditions as confirmed by a litmus paper test. (c) Size distribution of crystals determined by microscopic inspection after TR-SFX sample preparation. (d) Microscopic picture of KR2 crystals grown at acidic pH and in absence of NaCl, after washing out the crystallization buffer, the TR-SFX sample prepared via the procedure shown in (a), and LCP with KR2 crystals soaked directly as control. The color change upon increasing pH has been confirmed in five independent experiments. (e) Overview of TR-IR traces and absorption spectra obtained from blue KR2 crystals in the original acidic crystallization condition, red crystals prepared in analogy to the TR-SFX experiment and in hydrated film at pH 8 in presence of sodium chloride. The corresponding lower panels show a kinetic analysis of KR2 intermediates obtained by singular value decomposition (SVD) of the spectroscopic data. Time constants are given in brackets. Under acidic conditions, KR2 in crystals exhibits an accelerated photocycle. In treated crystals on the other hand the critical deprotonation step in the M intermediate is occurring with similar kinetics as in purified KR2. (f, g) Detailed view of the retinal binding pocket in the serial crystallographic room temperature structures of KR2 obtained from (f) blue crystals at acidic pH and (g) red crystals after soaking in neutral pH and NaCl. Critical hydrogen bonds with a distance ≤ 3.2 Å are indicated by black dotted lines. Arrows signify the distance of the SB to w406 and the D116 counterion. In neutral conditions w406 has shifted away from the SB while D116 and N112 are now within hydrogen bonding distance. The resulting change in electrostatic environment is responsible for the color change as reported previously².

Extended Data Fig. 2: Spectroscopic analysis of sodium binding mode. Time-resolved infrared spectra from KR2 microcrystals at (a) pH 8 and at (b) pH 4 recorded with tunable Quantum Cascade Lasers as described in⁴². The maximum concentration of the O intermediate is reached at around 1-20 ms after pulsed excitation at pH 8 and is characterized by specific marker bands that are absent at pH 4, in particular the C=C stretching vibrational band of retinal at 1516 cm^{-1} of the O state. The band at 1688 cm^{-1} has previously been suggested to originate from the C=O stretching mode due to sodium binding to an asparagine residue, presumably N112⁴³. The band at 1554 cm^{-1} is tentatively assigned to the asymmetric stretching vibration of a carboxylate that rises upon binding of a sodium ion in bidentate or pseudo-bridging fashion where one of the carboxylate oxygens is interacting with another partner^{44,45}. For a detailed analysis of the ligation, the corresponding symmetrical mode needs to be assigned as the frequency difference between the COO^- asymmetric and symmetric stretch is dependent on the mode of sodium ligation^{46,47}. (c) O(like)-KR2 (ground state) difference spectra recorded under different conditions. Spectra have been scaled to the ground-state bleaching band measured between 1530 and 1540 cm^{-1} . It is well-established

that KR2 operates exclusively as a sodium pump at neutral pH and in the presence of sodium ions. KR2 acts as proton pump in the presence of KCl but has no (known) function at pH 4^{1,28}. It is evident that the band at 1688 cm⁻¹ is most pronounced if sodium is pumped, which supports the assignment to the C=O stretching vibration of N112 upon binding of sodium. (d) Time traces of the ethylenic stretch of the O state vibrating at 1518 cm⁻¹ and the two candidate vibrational bands at 1420 cm⁻¹ and 1392 cm⁻¹ of the symmetric carboxylate stretching vibration. The band at 1420 cm⁻¹ exhibits similar kinetic behaviour as the one at 1518 cm⁻¹. Hence, the former vibrational band is tentatively assigned as the symmetrical mode that relates to the asymmetrical vibration at 1554 cm⁻¹ of deprotonated D251 upon ligation of a sodium ion. The difference in frequency of the symmetric and asymmetric modes is with 135 cm⁻¹ at the edge of binding in bidentate to pseudobridging mode of model compounds^{2,4,5}. This indicates that the two oxygen of D251 are not equidistant to the sodium ion. Such asymmetric ligation is expected in the heterogenous environment of the protein interior as documented by our X-ray structures in the millisecond regime.

Extended Data Fig. 3: Comparison of data truncation schemes and changes in retinal over time. (a) The top panels show F_o-F_c simulated annealing omit maps of the resting state at 4 σ. No truncation (left) shows the map using all data up to 1.6 Å resolution along a*, b* and c*. Spherical truncation (middle) shows the map using all data up to 2.2 Å resolution along a*, b* and c*. Anisotropic truncation (right) shows the map using data up to 2.3 Å, 2.2 Å and 1.6 Å resolution along a*, b* and c*, respectively, as truncated by the staraniso server⁴⁸. The lower panels show the F_o(light)-F_o(dark) difference density maps of the 1 ms time delay at the region around retinal and V117 at 3 σ. The structure is shown as sticks (resting state salmon and 1 ms refined structure in cyan). No truncation (left) shows the map using all data up to 1.6 Å resolution. Spherical truncation (middle) shows the map using all data up to 2.2 Å resolution. Anisotropic truncation (right) shows the map using data up to 1.6 Å resolution in c* as truncated by the staraniso server. Overall the truncated data processed by result in better electron density maps (both for 2F_o-F_c maps and F_o(light)-F_o(dark) difference maps) with finer features being resolved. This effect is likely because noise along the missing directions is removed when compared to no truncation, while retaining the high-resolution data along c* when compared to spherical truncation. (b) The panels show the evolution of electron density around the retinal chromophore over time. Retinal and K255 of the refined structures are shown as sticks and the electron density is displayed around them (blue mesh, 2σ). The dark panel on the top shows the original 2F_o-F_c electron density map and the panels below show extrapolated 2F_{extra}-F_c maps. The extrapolated maps allow to follow retinal isomerization in detail. In the dark state, the middle section of the retinal polyene chain is slightly bent downwards. In the picoseconds range, the isomerization is completed and the polyene appears to be straightened. In our ultrafast data, we did not observe retinal with a pronounced twist in the C13=C14 bond as in bR, with retinal in KR2 reaching a near planar 13-*cis* conformation much earlier along the activation pathway. In the time delays from nanoseconds to milliseconds, the electron density reveals a bend in the retinal molecule resulting from two planes which are twisted against each other. While the exact dihedral angles cannot be refined realistically based on the extrapolated data, the bend seems to originate from the C9=C10-C11=C12 dihedral angle as suggested for the L, M and O intermediates based on time-resolved FTIR¹⁸ and resonance Raman spectroscopy³³. After 20 milliseconds, a definite conclusion concerning the retinal isomer is difficult. The extrapolated maps suggest that a fraction of the retinal molecules may have already re-isomerized to the all-*trans* conformation, while it is still bend sideward.

Extended Data Fig. 4: Experimental setup and evolution of structural changes over time. (a) KR2 crystals were extruded in a stream of LCP from a high viscosity injector. At the interaction point, the crystals were pumped with 575 nm femtosecond laser pulses before probing for structural changes with near parallel 12 keV XFEL pulses arriving from SwissFEL with a specific time delay Δt . The diffraction patterns are collected with a Jungfrau 16 M detector in a series of four light-activated patterns and one dark pattern, for which the visible pump laser had been blocked. To reduce background through diffuse scattering of XFEL radiation in air, the experimental chamber was pumped down to 100-200 mbar while the residual air is replaced by helium. (b) The evolution of structural changes in KR2 over time can be followed in a matrix of root-mean-square deviations (rmsd) between all protein and retinal atoms (total of 2703) in individual KR2 structural snapshots. The numerical rmsd values have been determined using the program pymol and are colored in a blue-white-red gradient. The black boxes highlight time delays where we combined data based on manual inspection of electron density maps and the evolution of photo intermediates in KR2 crystals determined by TR-IR spectroscopy. The approach was inspired by a recently published tool for visualizing protein motions in time-resolved crystallography⁴⁹ but relies on refined atom positions instead of electron density changes. (c) Difference electron density maps ($F_o(\text{light}) - F_o(\text{dark})$), negative density in gold and positive density in blue, contoured at 4σ and shown together with the KR2 resting state) obtained at the indicated time delays. The first panel is included as a control and shows difference electron density obtained from 50k patterns collected with laser off and 100k images from dark patterns obtained by the 4 light / 1 dark cycle used during the TR-SFX data collection. For orientation important residues discussed in the main text are shown as sticks.

Extended Data Fig. 5: Comparison of monomeric and pentameric KR2 structures.

Overall the structures at neutral pH reported in this work (left), Kato *et al.*², (middle) and Kovalev *et al.*²⁰ (right) are very similar (rmsd of C α atoms of 0.30 and 0.72 Å) with the position of residues and hydrogen bonding pattern (lower insets, black dotted lines, defined as distance ≥ 3.2 Å) in the binding pocket well preserved. Depending on conditions, the pocket in the pentameric structure can adopt the shown extended conformation. Here N112 is rotated out into the interaction interface between two KR2 protomers (colored red) and the space is filled with water molecules. Since the pentameric resting state can adopt a more open conformation in the Schiff base region³, we cannot exclude that the electrostatic gate does not fully open in our crystals formed from monomeric KR2 (see main text). However, a yet unpublished steady-state structure of pentameric KR2 under continuous illumination (6XYT, Gordeliy group, IBS Grenoble) shows N112 rotated back into the binding pocket in a conformation very similar to our time-resolved millisecond states. As the retinal is modelled in the *trans* configuration the structure may represent a later intermediate compared to what we observe. The opening along the retinal (calculated by the program Caver⁴⁰) in our 30 μ s+150 μ s structure is 1.4 Å, which is very close to the 1.9 Å needed for a sodium ion to pass. Stable structural intermediates that accumulate in time-resolved studies of molecule ensembles do not necessarily reveal all functional states³⁴ and it seems reasonable that a gate should only form when sodium is in close proximity to the SB. This would allow fast transfer of sodium to the extracellular side upon deprotonation. The gate would then collapse immediately upon sodium binding on the extracellular side, coinciding with reprotonation of the SB. Such a mechanism would efficiently prevent sodium back leakage. Differences in the position of N112 between the monomer and pentameric resting states could furthermore explain the long-range effects of mutations in the oligomerization interface on the photocycle¹⁷ and sodium pumping efficiency^{1,17,20}.

Extended Data Fig. 6: Comparison of ion binding sites in selected members of the rhodopsin family and QM/MM simulations. (a) Sodium binding site in *Krokinobacter eikastus* rhodopsin 2 (KR2, this work), (b) chloride binding site in the related bacterial pump *Nonlabens marinus* rhodopsin 3 (NM-R3, pdb code 5G28⁵⁰), (c) protonated Schiff base in bacteriorhodopsin (bR, pdb code 6RQP²⁶), (d) chloride binding site in halorhodopsin (HR, pdb code 1E12⁵¹). The retinal chromophore together with selected interactions and amino acid side chains are shown as dotted lines or sticks. Besides overall similarity in ion binding between bacteria and archaea, the suggested binding site in KR2 is in line with quantum chemical calculation of absorbance changes upon sodium binding. The QM/MM optimized geometry of (e) the resting state and the 1 ms structure with (f) water or (g) sodium bound to N112, D251. (h) Comparison of the absorption shifts of the states measured in UV-Vis difference spectroscopy with the calculated QM/MM excitation wavelengths. Absolute values are given in brackets. Extent and location of structural changes in (i) KR2 at 20 ms and (j) bR at 15 ms after activation (blue to red gradient and ribbon width indicate rmsd to the resting state, bR coordinates taken from²⁶). The light-driven pumping against a concentration gradient is achieved with smaller conformational changes in the order of 1-2 Å in sodium pumping KR2 compared to the more elusive protons pumped by bR^{24,26}. The translocation path for sodium and protons (indicated by arrows), however, is similar for both proteins and includes three critical sites (for reviews on bR structural dynamics and activation mechanism, see ref⁵² and ref⁵³, respectively). Close to the water-filled cavity on the cytoplasmic side of KR2, Q123 of the NDQ motif is the most likely location where the sodium ion is losing its water coordination shell. The position is analogous to D96 of the DTD motif in bR, which is the primary donor for reprotonation at the end of the pumping cycle. The second critical site is formed by D116, N112 and D251 which correspond to D85, T89 and D212 in bR and coordinate sodium and proton transport in the SB region. The role of R109 in switching from water to sodium binding in KR2 is analogous in position and function to R82, which is regulating the transfer of protons towards the release group in bR. The proton release group in bR is formed by E194 and E204 with the position overlapping well with the second sodium binding site between E11, N106 and E160 at the extracellular side of KR2. Beside these similarities, the sequence of events is clearly different between the two outward ion pumps. Whereas light-driven pumping in bR starts with (1) a prebound proton followed by (2) release and (3) reloading. In KR2 the process is shifted in sequence with (1) entry, (2) binding and (3) release with a corresponding adaptation of the photocycle intermediates. The similar ion binding modes between bacteria and archaea together with how retinal isomerization is used to drive them, provides an exciting example for the evolutionary economy of nature. The adaptation of this common leitmotif is particularly interesting in the case of KR2 where substrate binding has been shifted from the stable resting state into a transient intermediate.

Extended Data Table 1: X-ray statistics.

Extended Data Table 2: Refinement statistics. *Data are anisotropic, see extended statistics Table 1 and Materials and Methods.

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a.

QM/MM optimized structures of the 1 ms intermediate

absorbance shift vs resting state:
-11 nm (blue)

absorbance shift vs resting state:
+55 nm (red)

This work
R.T. / neutral / mono

Kato et al., 2015
cryo / neutral / mono

Kovalev et al., 2019
cryo / neutral / pent

Bacterial sodium pump
(KR2, 1 ms)

Bacterial chloride pump
(NM-R3 resting)

Archaeal proton pump
(bR resting)

Archaeal chloride pump
(hR, resting)

Optimized KR2 resting state

Optimized KR2 1 ms state + water

Optimized KR2 1 ms state + sodium

Retinal peak absorption shift

	Dark-acidic	Dark-neutral	800 fs	2 ps	100 ps	1 ns	16 ns	1 μs	30 μs	150 μs	1 ms	20 ms	800fs + 2ps	1ns + 16ns	30μs + 150μs
Data collection															
Space group	I222														
Cell dimensions															
a, b, c (Å)	41.5, 84.5, 235.6														
α, β, γ (°)	90, 90, 90														
Indexed patterns	47'518	158'832	47'801	47'595	43'162	45'045	50'165	55'041	50'754	48'383	60'629	48'329	95396	95210	99137
Indexing rate (%)	12.51	11.30	10.68	15.28	9.65	14.11	14.28	11.87	9.00	9.61	12.23	9.16	-	-	-
Overall statistics excluding anisotropic shell 12.4 Å – 2.3 Å (Overall statistics 12.4 Å – 1.60 Å including anisotropic shell)															
Resolution along a*; b*; c* (Å)	2.0; 2.3; 1.6	2.2; 2.3; 1.6	2.1; 2.3; 1.7	2.1; 2.3; 1.7	2.1; 2.20; 1.6	2.1; 2.20; 1.6	2.1; 2.3; 1.7	2.1; 2.3; 1.7	2.1; 2.3; 1.7	2.1; 2.3; 1.7	2.1; 2.2; 1.7	2.1; 2.20; 1.6	2.0; 2.2; 1.6	2.0; 2.2; 1.6	2.0; 2.2; 1.6
No. reflections	18'807 (28'904)	18'873 (31'741)	18'840 (26'799)	18'840 (27'107)	18'872 (29'755)	18'847 (28'057)	18'827 (26'141)	18'828 (26'237)	18'793 (25'531)	18'812 (26'123)	18'847 (27'051)	18'863 (29'034)	18'874 (29'870)	18'872 (30'047)	18'861 (28'402)
Completeness (%)	99.7 (52.3)	100 (57.5)	99.8 (48.5)	99.8 (49.1)	100 (53.9)	99.9 (50.8)	99.8 (47.3)	99.8 (47.5)	99.6 (46.2)	99.7 (47.3)	99.9 (49.0)	99.9 (52.6)	100 (54.1)	100 (54.4)	99.9 (51.4)
Multiplicity	608 (505)	1'640 (1'328)	522 (444)	497 (419)	475 (395)	507 (431)	547 (469)	563 (481)	505 (439)	479 (413)	594 (505)	531 (445)	1018 (834)	1053 (869)	983 (827)
R _{split} (%)	6.1 (6.8)	4.3 (4.9)	8.0 (8.6)	8.3 (9.0)	8.4 (9.1)	7.8 (8.4)	7.3 (7.8)	7.4 (8.0)	7.1 (7.7)	7.8 (8.4)	6.9 (7.5)	7.6 (8.2)	5.7 (6.3)	5.3 (5.9)	5.3 (5.9)
CC _{1/2}	1.00 (1.00)	1.00 (1.00)	0.99 (0.99)	0.99 (0.99)	0.99 (0.99)	0.99 (0.99)	0.99 (0.99)	0.99 (0.99)	0.99 (0.99)	0.99 (0.99)	0.99 (0.99)	0.99 (0.99)	1.00 (1.00)	1.00 (1.00)	1.00 (1.00)
<I/σ(I)>	11.4 (8.3)	16.5 (11.1)	9.2 (7.1)	9.1 (7.0)	9.2 (6.7)	9.2 (7.0)	9.6 (7.5)	9.7 (7.6)	9.4 (7.5)	9.3 (7.3)	10.4 (7.9)	9.8 (7.2)	12.8 (9.1)	13.1 (9.2)	13.1 (9.6)
High resolution statistics of the anisotropic shell (2.3 Å - 1.60 Å)															
No. reflections	10'097	12'867	7'959	8'267	10'883	9'210	7'314	7'409	6'738	7'311	8'204	10'171	10'996	11'175	9'541
Completeness (%)	27.7	35.4	21.9	22.7	29.9	25.3	20.1	20.4	18.5	20.1	22.6	28.0	30.2	30.7	26.2
Multiplicity	331	872	261	241	256	274	269	272	256	244	302	285	519	559	518
R _{split} (%)	38.3	30.0	43.8	44.3	38.2	41.0	45.2	44.0	45.8	43.8	42.1	38.2	37.0	35.4	37.1
CC _{1/2}	0.90	0.95	0.86	0.84	0.89	0.89	0.84	0.86	0.85	0.86	0.87	0.89	0.91	0.92	0.91
<I/σ(I)>	2.5	3.1	2.2	2.2	2.5	2.4	2.2	2.2	2.2	2.2	2.3	2.5	2.6	2.7	2.6

	Dark-acidic	Dark-neutral	800fs + 2ps	1ns + 16ns	30μs + 150μs	1ms	20ms
Data collection							
Space group	I222						
Cell dimensions							
a, b, c (Å)	41.5, 84.5, 235.6						
α, β, γ (°)	90, 90, 90						
Resolution (Å)	12.36 – 1.6* (2.3 – 1.6)	12.36 – 1.6* (2.3 – 1.6)	12.36 – 2.25 (2.33 – 2.25)	12.35 – 2.25 (2.33 – 2.25)	12.35 – 2.25 (2.33 – 2.25)	12.36 – 2.5 (2.58 – 2.5)	12.36 – 2.5 (2.58 – 2.5)
<i>R</i> _{split}	6.8 (38.3)	4.9 (30.0)	5.8 (24.29)	5.4 (24.1)	5.4 (27.3)	6.6 (17.3)	7.4 (17.1)
< <i>I</i> /σ(<i>I</i>)>	8.3 (2.5)	11.1 (3.1)	12.2 (3.5)	12.5 (3.7)	12.5 (3.3)	12.2 (5.1)	11.3 (5.6)
<i>CC</i> _{1/2}	1.00 (1.00)	1.00 (1.00)	0.99 (0.95)	0.99 (0.96)	0.99 (0.96)	0.99 (0.97)	0.99 (0.96)
Completeness (%)	52.3 (27.7)*	57.5 (35.4)*	99.9 (98.6)	99.9 (98.6)	99.7 (97.5)	99.9 (100)	99.9 (100)
Multiplicity	505 (331)	1328 (872)	994 (643)	1028 (660)	960 (616)	647 (443)	579 (400)
Refinement							
Resolution (Å)	12.36 – 1.6 (1.64 – 1.6)	12.43 – 1.6 (1.64 – 1.6)	12.36 – 2.25 (2.35 – 2.25)	12.35 – 2.25 (2.35 – 2.25)	12.35 – 2.25 (2.35 – 2.25)	12.36 – 2.5 (2.66 – 2.5)	12.36 – 2.5 (2.66 – 2.5)
No. reflections	28'895 (123)	31'728 (203)	18'021 (1874)	18'073 (1906)	17'917 (1920)	13'446 (2007)	13'268 (1871)
<i>R</i> _{work} / <i>R</i> _{free}	17.67 / 21.3	18.8 / 21.7	27.2 / 32.8	26.5 / 33.2	26.7 / 33.5	25.8 / 32.4	27.8 / 35.3
No. atoms							
Protein	2139	2105	2103	2111	2099	2099	2099
Ligands	242	242	242	242	242	243	243
Water	99	60	53	50	49	49	47
<i>B</i> -factors							
Protein	31.8	36.1	42.1	40.1	33.7	41.7	52.1
Ligands	61.5	62.1	65.6	65.5	53.2	63.6	69.9
Water	46.9	46.1	41.6	43.6	36.3	43.6	50.1
R.m.s. deviations							
Bond lengths (Å)	0.011	0.006	0.008	0.007	0.007	0.007	0.008
Bond angles (°)	1.063	0.735	0.938	0.928	0.928	0.865	0.921
PDB Code	6TK7	6TK6	6TK5	6TK4	6TK3	6TK2	6TK1

* Data are anisotropic, see extended statistics Table 1 and Materials and Methods