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Conformational alterations in unidirectional ion transport of a light-driven chloride pump revealed using X-ray free electron lasers

Toshiaki Hosaka, Takashi Nomura, Minoru Kubo, Takenori Nakane, Luo Fangjia, Shun-ichi Sekine, Takuhiro Ito, Kazutaka Murayama, Kentaro Ihara, Haruhiko Ehara, Kazuhiro Kashiwagi, Kazushige Katsura, Ryogo Akasaka, Tamao Hisano, Tomoyuki Tanaka, Rie Tanaka, Toshi Arima, Ayumi Yamashita, Michihiro Sugahara, Hisashi Naito, Yoshinori Matsuzawa, Susumu Yoshizawa, Kensuke Tono, Shigeki Owada, Osamu Nureki, Tomomi Kimura-Someya, So Iwata, Eriko Nango, and Mikako Shirouzu

*RIKEN Center for Biosystems Dynamics Research, Yokohama, Kanagawa 230-0045, Japan; †RIKEN SPring-8 Center, Sayo-cho, Sayo-gun, Hyogo 670-5148, Japan; ‡Department of Life Science, Graduate School of Science, University of Hyogo, Hyogo 678-1297, Japan; §Department of Biological Sciences, Graduate School of Science, The University of Tokyo, Tokyo 113-0034, Japan; ¶Department of Cell Biology, Graduate School of Medicine, Kyoto University, Sakyo-ku, Kyoto 606-8501, Japan; ¶Division of Biomedical Measurements and Diagnostics, Graduate School of Biomedical Engineering, Tohoku University, Sendai 980-8575, Japan; ‡Atmosphere and Ocean Research Institute, The University of Tokyo, Chiba 277-8564, Japan; ††Japan Synchrotron Research Institute, Sayo-cho, Sayo-gun, Hyogo 679-5188, Japan; and ‡‡Institute of Multidisciplinary Research for Advanced Materials, Tohoku University, Aoba-ku, Sendai 980-8577, Japan

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Light-driven chloride-pumping rhodopsins actively transport anions, including various halide ions, across cell membranes. Recent studies using time-resolved serial femtosecond crystallography (TR-SFX) have uncovered the structural changes and ion transfer mechanisms in light-driven cation-pumping rhodopsins. However, the mechanism by which the conformational changes pump an anion to achieve unidirectional ion transport, from the extracellular side to the cytoplasmic side, in anion-pumping rhodopsins remains enigmatic. We have collected TR-SFX data of *Nonlabens marinus* rhodopsin-3 (NM-R3), derived from a marine flavobacterium, at 10-μs and 1-ms time points after photoexcitation. Our structural analysis reveals the conformational alterations during ion transfer and after ion release. Movements of the retinal chromophore initially displace a conserved tryptophan to the cytoplasmic side of NM-R3, accompanied by a slight shift of the halide ion bound to the retinal. After ion release, the inward movements of helix C and helix G and the lateral displacements of the retinal block access to the extracellular side of NM-R3. Anomalous signal data have also been obtained from NM-R3 crystals containing iodide ions. The anomalous density maps provide insight into the halide binding site for ion transfer in NM-R3.

**Microbial ion-pumping rhodopsins are integral membrane proteins that actively transport ions across membranes upon light stimulation (1). Bacteriorhodopsin (bR) and halorhodopsin (HR) are well-known microbial ion-pumping rhodopsins found in halophilic archaea (2, 3). bR is a light-driven outward proton pump and HR is a light-driven inward anion pump, specific for chloride ion. Microbial ion-pumping rhodopsins possess common structural features consisting of seven α-helices with an all-trans retinal covalently bound to a lysine residue as the chromophore, despite the transport of different ions (4). The retinal undergoes photoisomerization from the all-trans to 13-cis configuration, which initiates the photocycle accompanied by several intermediates to export ions (4, 5). Its light-controllable function is suitable for optogenetic applications for manipulating cells, such as neurons, by changing the ion concentration inside or outside the membrane (6, 7). In fact, microbial rhodopsins, including channelrhodopsins and HRs, are employed as optogenetic tools (8–10).

*Nonlabens marinus* rhodopsin-3 (NM-R3) is a light-driven chloride pump recently discovered in a marine flavobacterium (11). It is a distinct chloride pump from HRs and shows low amino acid sequence homology with HRs (11). To date, HR-type chloride pumps have been found in haloarchaea, marine bacteria, and cyanobacteria, including *Halobacterium salinarum*, *Natronomonas pharaonis*, and *Mastigocladus laminosus* repens, with sequence identities of 20%, 21%, and 20% to NM-R3, respectively (3, 12–15). Interestingly, NM-R3 has higher sequence identity (36%) to *Krokinobacter rhodinus* 2 (KR2), a sodium pump found in *Krokinobacter eikastus* (16). NM-R3 possesses a unique NTO

**Significance**

Light-driven chloride pumps have been identified in various species, including archaea and marine flavobacteria. The function of ion transportation controllable by light is utilized for optogenetics tools in neuroscience. Chloride pumps differ among species, in terms of amino acid homology and structural similarity. Our time-resolved crystallographic studies using X-ray free electron lasers reveal the molecular mechanism of halide ion transfer in a light-driven chloride pump from a marine flavobacterium. Our data indicate a common mechanism in chloride pumping rhodopsins, as compared to previous low-temperature trapping studies of chloride pumps. These findings are significant not only for further improvements of optogenetic tools but also for a general understanding of the ion pumping mechanisms of microbial rhodopsins.
motif (Asn98, Thr102, Gln109) in the third helix (helix C), which corresponds to key residues (DDT motif, Asp85, Thr89, Asp96) for proton transport in bR (11, 17, 18) (SI Appendix, Table S1). Asp85 acts as the primary proton acceptor of bR from the protonated Schiff-base (PSB), with assistance from Thr89 and Asp96, which is the proton donor (5, 17, 18). HRs from halarchaea have a highly conserved TSA (Thr, Ser, Ala) motif, while the Ala residue is replaced by Asp in HR from cyanobacteria (19). In the X-ray crystal structure of NM-R3 (SI Appendix, Fig. S1A), a chloride ion located between the PSB and Asn98 (SI Appendix, Fig. S1B) is stabilized by the positive charge of the PSB (20). The position of this chloride ion is similar to those in the H. salinarum HR and N. pharaonis HR (NpHR) structures except for Thr and Ser, which correspond to Asn98 and Thr102 in NM-R3, respectively (20–22). Several amino acid residues around the retinal, including Arg95, Trp99, Trp201, and Asp231, are highly conserved among ion-pumping rhodopsins. Previous spectroscopic studies suggested that NM-R3 displays a similar sequence of intermediates, with K−, I−, N−, and O-like species, as in other HRs (23) (Fig. 1I). Recently, intermediate structures of NM-R3 obtained by low-temperature trapping X-ray crystallography and serial femtosecond crystallography (SFX) have been reported (24, 25). However, the detailed ion-pump mechanism still remains unclear, due to the lack of dynamic structures of anion transport at atomic resolution.

Time-resolved serial femtosecond crystallography (TR-SFX) is a powerful tool for visualizing reactions and motions in proteins at the atomic level (26–28). In SFX, myriad of microcrystals are continuously injected by a sample injector into an irradiation point of X-ray free electron lasers (XFELs) at room temperature, thus providing diffraction patterns before the onset of radiation damage by the intense X-ray pulse. Combined with a visible-light pump laser for reaction initiation, TR-SFX has been applied to light-driven ion pumps to observe the structural dynamics during the ion transfer. While TR-SFX has revealed femtosecond/millisecond structural dynamics in light-driven cation pumps, including bR and KR2 (29–31), TR-SFX studies of anion pumps have been limited to early-stage structures adopted at picoseconds after light illumination (32).

Results

Xfel Structures of Bromide-Bound or Iodide-Bound NM-R3 in the Resting State. In 2016, we reported the crystal structure of NM-R3, determined at 1.58-A resolution from data collected using synchrotron radiation (SR) (20). The X-ray structure of NM-R3 from crystals grown in a lipidic cubic phase (LCP) indicated that one Cl− is bound to the PSB of the all-trans retinal (SI Appendix, Fig. S1). That same year, Kim et al. also determined the X-ray structure of NM-R3 at 1.56-A resolution using SR, which showed two Cl− ions at one position and the other by the loop between helix A and helix B on the cytoplasmic side (33). To date, several structures of NM-R3, including the structures from NM-R3 crystals obtained at lower pH and the bromide-bound NM-R3, have been reported (33). Recently, the Xfel structure of chloride-bound NM-R3 was also solved at the Linac Coherent Light Source (25).

We have determined the structures of the bromide-bound and iodide-bound forms of NM-R3 crystals grown in LCP at 2.1-A resolution, using XFEL pulses at the SPring-8 Angstrom Compact Free Electron Laser (SACLAA) (SI Appendix, Fig. S24). The dissociation constants (Kd) for Cl−, Br−, and I− in NM-R3 were previously estimated as 24 mM, 10 mM, and 2.5 mM, respectively, by Tsukamoto et al. (23). At first, NM-R3 crystals embedded in LCP were soaked in a solution of 1 M sodium iodide to prepare the iodide-bound form and then injected from a high-velocity sample injector into the XFEL irradiation area (34). However, the interaction of the sample with XFEL emitted light and the sample stream flow was very unstable and formed droplets, presumably due to the phase transition of LCP. Since the highly concentrated halide ions caused liquefaction of sample streams, the concentration used for soaking needed to be decreased to the extent that the phase transition and illumination of the samples subsided. Consequently, NM-R3 crystals soaked in buffer containing 200 mM sodium iodide or 1 M sodium bromide were successfully injected, without phase transition and illumination.

The overall Xfel structures of the bromide-bound NM-R3 (NM-R3-Br) and iodide-bound NM-R3 (NM-R3-I) are similar to the SR structure of the chloride-bound NM-R3 (NM-R3-Cl) (PDB ID: 5B2N), with Co rmsds of 0.41(Br) and 0.32(I) Å (20) (SI Appendix, Fig. S3). Although the number of water molecules was decreased from 96 to 53, most of the internal water molecules were consistent with those in the SR structure. Three halide ions were detected in both the NM-R3-Br and NM-R3-I Xfel structures (25) (SI Appendix, Fig. S2). The first halide ion is bound to the PSB, as in the NM-R3-Cl SR structures (SI Appendix, Fig. S4 A–C). The second halide ion is in the loop between helix A and helix B in both the NM-R3-Br and -I structures, as in the NM-R3-Cl SR structure reported by Kim et al. (SI Appendix, Fig. S2B) (33). The third halide ion is on the cytoplasmic side of helix E (SI Appendix, Fig. S2C).

Fig. 1. TR-visible absorption spectroscopy for microcrystals. (A) Photocycle model of NM-R3 in the 1 M NaCl buffer solution (23). (B) TR difference spectra ΔA upon the 532-nm excitation. The difference was calculated by subtracting the spectrum of NM-R3. (C) Global fitting analysis with two exponentials. The A1 and A2 amplitude spectra correspond to the differences of [ΔA0 − ΔA0 (μs)] and [ΔA0 (μs) − ΔA0], respectively. Here, ΔA0 represents the difference spectrum of the O intermediate minus NM-R3. (D) The isomeric forms of the retinal chromophore in bacterial-type rhodopsins.
In contrast, the distances between the anion and the key residues around the retinal differ in the NM-R3-Br and -I XFEL structures, as compared with the SR structures (NM-R3-Cl and NM-R3-Br). \( \text{Br}^- \) and \( \Gamma^- \) are bound to the PSB with 3.18- and 3.37-Å distances, respectively, depending on their ionic radii (SI Appendix, Fig. S4 A and B). Interestingly, the distances between \( \text{Br}^- \) (or \( \Gamma^- \)) and two residues (Thr102, Asn98) are relatively shorter or almost the same as those of the SR structures (SI Appendix, Fig. S4 C and D). Particularly, the interaction with Asn98 is tightened, with distances of 3.10 (Br\(^-\)) and 3.30 (\( \Gamma^- \)) Å, as compared to that of NM-R3-Cl (4.04 Å) (SI Appendix, Fig. S4 A–C). The NM-R3-Cl XFEL structure (5ZTL) reported previously also indicates this tendency (SI Appendix, Fig. SAE). As a significant difference, only one water molecule is bound to the halide ion in our XFEL structures, as in the other NM-R3 SR structures, whereas an additional water molecule bound to Asp231 was observed in 5ZTL (25).

**Time-Resolved Spectroscopy Analysis of NM-R3 Microcrystals.** Previously, Tsukamoto et al. reported that the photocycle of NM-R3-Cl in a solution including n-dodecyl-\( \beta \)-D-maltopyranoside (DDM) consists of five kinetically defined states: L, N(1), O1, O2, and O3. The intermediate O1 present in the 13-cis/15-syn configuration of the 13-cis/15-syn intermediate was adopted by the N-like intermediate term (N\( ^* \)) (18). In the photocycle model, C1 is released during the transition from L(N1) to O1 (23). Subsequently, a conformational change may occur to prevent the reverse ion transfer and to prime the next uptake of C1 from the extracellular side during the O1–O2 transition.

In this study, we investigated the photocycle kinetics of NM-R3-Br in the crystalline phase by TR-visible absorption spectroscopy. The TR difference spectra after 532-nm excitation are shown in Fig. 1B. At 10 μs, a broad positive difference peak appears at a relatively long wavelength around 610 nm, suggesting the possibility of the partial existence of an O intermediate together with earlier intermediates (K or L/N). The positive peak then increases with a slight blue shift with a time constant of 150 μs and vanishes with a τ of 44 ms, as confirmed by a global fitting analysis with two exponentials (Fig. 1C). This spectroscopic result demonstrates the accumulation of the O intermediate and its following movement to the initial state of NM-R3. It should be noted that our spectral analysis detects only one O intermediate, while two O intermediates, O1 and O2, have been proposed (23). Although it is difficult to identify which type of O intermediate could be accumulated in the microcrystals, we tentatively assigned the observed species to O1, according to its characteristic long wavelength (>600 nm) of the positive difference peak. This assignment will be discussed further in the next section and in Discussion.

**Structural Changes in NM-R3-Br Crystals after Photoexcitation at \( \Delta t = 1 \text{ ms} \).** TR-SFX data were collected from NM-R3-Br and NM-R3-I microcrystals, using a TR-SFX setup at SACLAY (35). The microcrystal stream was continuously ejected from the nozzle of the high-viscosity sample injector and then irradiated with an XFEL pulse at 1 ms after photoactivation by a 540-nm, nanosecond laser pulse.

TR-SFX data of NM-R3 crystals with a bromide ion were recorded to 2.1-Å resolution. In the \( f_{\text{obs}}^{\text{light}} - f_{\text{obs}}^{\text{dark}} \) difference electron density map, most of the positive and negative features are visible around the retinal and on helices C, F, and G (SI Appendix, Fig. S5). The paired negative and positive electron density peaks appearing around the C12–C14 and C13 methyl groups of the retinal reveal that the retinal moves toward helix C (Fig. 2A–C). The negative and positive difference features indicate that the side chain and backbone of Lys235, which is covalently attached to the retinal, are slightly displaced toward the cytoplasmic side. The time-resolved difference absorption spectra from NM-R3 crystals demonstrate that the O intermediate (O1 or O2) is dominant at \( \Delta t = 1 \text{ ms} \) (a delay time of 1 ms after photoexcitation). The reisomerization of the retinal from the 13-cis to all-trans configuration was previously observed in previous TR-SFX studies (29). On the other hand, the weaker positive electron density features reveal that the C13 methyl group of the retinal moves toward the cytoplasmic side by 1.70 Å, with a corresponding 1.05 Å displacement of Trp201 indole ring in helix F (Fig. 2B). The side chain of Trp201 interacts with Wat501 in the resting state, but the displacement of Wat501 is observed at \( \Delta t = 1 \text{ ms} \) as a negative difference electron density peak (Fig. 2D). Similar movements of Trp182 in bR and Trp215 in KR2, which are genetically and structurally conserved in most bacterial-type rhodopsins, were observed in previous TR-SFX studies (29, 31). The negative and weakly positive difference electron density peaks along Met197 show that the Met197 side chain slightly moves toward the cytoplasmic side upon the sequential displacement of Trp201 and the retinal (Fig. 2A and B).

The structural model for \( \Delta t = 1 \text{ ms} \) indicates that the PSB is oriented toward the cytoplasmic side and interacts with Thr102. As a result, the distance between Thr102 and the PSB becomes closer, from 4.21 Å to 3.45 Å. The strongly negative difference electron density features reveal around the bromide ion bound to the PSB position also reveal that the L(N1) to O1 transition (Fig. 2B). Consequently, the negative and positive electron densities associated with Asn98 of helix C indicate that Asn98 moves toward the empty space created when the bromide ion bound to the PSB is dislodged (Fig. 2B). On the other hand, the weaker paired negative and positive electron density features around the cytoplasmic side of helix C shows that residues Ile103-Leu108 move slightly outward, away from the retinal (Fig. 3B). This outward movement of the cytoplasmic side of helix C could create an ion translocation pathway in the cytoplasmic interhelical space.

The paired negative and positive difference electron densities along helix G demonstrate that this helix is slightly shifted toward helix C (Fig. 3D). A positive difference electron density feature appears between Asn98 of helix C and Asp231 of helix G. We modeled the feature as a movement of the side chain of Asp231 toward Asn98 (Fig. 2B and E). The distance between Asn98 and Asp231 becomes closer (from 10.27 Å to 4.74 Å, Asn98 OD1-Asp231 OD1) with the movements of helices C
Fig. 2. Structural change of NM-R3-Br near retinal at Δt = 1 ms. (A) View of the \(|F_{\text{obs}}|^2 - |F_{\text{calc}}|^2|\) difference Fourier electron density map near the retinal in NM-R3-Br for Δt = 1 ms. The blue electron density map indicates positive electron density, and orange denotes negative electron density (contoured at ±3.3σ). The resting state NM-R3 model (yellow sticks and white helices) was used for phases when calculating this map. (B and C) Crystallographic structural models derived from partial occupancy refinement are superimposed upon the resting state NM-R3 structure (yellow) for Δt = 1 ms (pink). View of the \(|F_{\text{obs}}|^2 - |F_{\text{calc}}|^2|\) difference Fourier electron density map at the water molecules Wat501 (D) and Wat401 and Wat402 (E) for Δt = 1 ms. The blue electron density map indicates positive electron density, and orange denotes negative electron density (contoured at ±3.0σ). Crystallographic model for the time point Δt = 1 ms (magenta) superimposed on the resting state model (yellow, partially transparent). A bromide ion and water molecules are depicted by blue and red spheres, respectively. The movements of the retinal and water molecules are depicted by a dashed arrow. Numbers indicate the distance (in angstroms) between two atoms.

and G, which block the pathway inside the protein, like a gate closing. The gate composed of the two hydrophilic amino acids may play a role in preventing backflow or overflow of anions by forming a steric or electrostatic barrier.

A negative difference electron density peak visible on a water molecule, Wat402 between Asn98 and Asp231, indicates a displacement of Wat402 (Fig. 2E). In the resting state, Wat402 forms hydrogen-bonding interactions with the bromide ion, Asp231, Trp99, and Arg95. Correlated displacements are observed on Trp99 and Arg95. The paired negative and positive difference electron density features reveal that Trp99 moves toward the retinal. The negative difference electron density and two \(|F_{\text{obs}}|^2 - |F_{\text{calc}}|^2|\) electron density features along Arg95 show that the side chain of Arg95 is disordered by the loss of hydrogen-bonding interactions. The similar disordering of Arg95 was seen in low-temperature studies of NM-R3 in SR and TR-SFX experiments with KR2 (corresponding to Arg109 in KR2) at 1 ms following photoactivation (24, 31). The equivalent arginine residue is presumably involved in the ion transfer in bR and NpHR (38, 39). The disordering of Arg95 may facilitate anion uptake from the extracellular side. The sequential movements on the extracellular side trigger displacements of water molecules, detected as positive or negative difference electron density peaks, indicating the disordering of Wat401 in addition to Wat402 and the ordering of new water molecules, Wat491 and Wat492 (Fig. 2E). Low-temperature trapping crystallographic studies of NM-R3 have shown that negative difference electron density peaks appear on Wat401, Wat402, and Wat442, but no positive electron density peak arises around the water molecules except for Wat402 (24), which is inconsistent with our observations.

Implications for Structural Changes and Ion Transport Pathway in NM-R3-I after Photoexcitation at Δt = 10 μs and 1 ms. Bromine and iodine display anomalous dispersions in specific X-ray wavelength ranges. The K absorption edge of bromine (at 13.5 keV) was beyond the easily accessible wavelength at the XFEL facility. Moreover, such shorter wavelengths are prone to yield low-resolution data due to the lower quantum efficiency of the detector, including the multiport charge-coupled device detector used at SACLA (38). On the other hand, iodine has a sufficiently anomalous \(f''\) value of 8.6 e\(^{-}\) at 7 keV, which is often used at SACLA (\(f''\) value is taken from http://skuld.bmsec.washington.edu/scatter/). We attempted to measure an anomalous signal from NM-R3-I crystals to identify an anion transfer pathway and obtained TR-SFX data at \(Δt = 10 \mu s \) and 1 ms after photoactivation. In both datasets, negative and positive difference electron density features around the retinal are visible, but become weaker as compared with the difference electron density map of the TR-SFX data of NM-R3-Br recorded at \(Δt = 1 ms\). Furthermore, in the difference electron density map of NM-R3-I at \(Δt = 1 ms\), very weak negative electron density peaks are observed on Wat401 (3.3σ) and Wat402 (3.3σ) and positive electron peaks corresponding to Wat491 or...
Wat492 are not detected, which implies the lower accumulation of a photointermediate in NM-R3-I. The binding affinity of I$^-$ (2.5 mM) estimated from previous spectroscopic studies is higher than that of Cl$^-$ (24 mM) (23). Therefore, it may cause lower ion pumping efficiency in NM-R3.

As remarkable difference electron density features, the paired negative and positive electron densities along the retinal show that the retinal moves toward helix C at $\Delta t = 10 \mu$s ($SI$ Appendix, Fig. S7A). Strong paired negative and positive electron density peaks appearing on the iodide ion interacted with the PSB, indicating a displacement of the iodide ion from the retinal toward Thr102 on the cytoplasmic side at $\Delta t = 10 \mu$s ($SI$ Appendix, Fig. S7A). The paired negative and positive electron density features around Asn98 suggest that the side chain of Asn98 in helix C also moves toward the iodide binding site ($SI$ Appendix, Fig. S7B), which results in the distortion of helix C (Arg95-Leu108). As with the difference electron density changes observed in the difference map of NM-R3-Br at $\Delta t = 1$ ms, the paired negative and positive electron density features along Trp201 show that Trp201 of helix F also shifts toward the cytoplasmic side ($SI$ Appendix, Fig. S7). No difference electron density peaks are detected around these locations, and thus they are not a major position for I$^-$ at the time points but may instead indicate part of an anion transport pathway.

In low-temperature trapping studies of NM-R3-I, a small anomalous peak (4.1$\sigma$) is situated between Ile190-Val193 of helix F and Ile245 of helix G ($SI$ Appendix, Fig. S8B). These peaks are located at the anion release exit on the intracellular side. However, no difference electron density peaks are detected around these locations, and thus they are not a major position for I$^-$ at the time points but may instead indicate part of an anion transport pathway.

Conformational alterations in unidirectional ion transport of a light-driven chloride pump revealed using X-ray free electron lasers

Fig. 3. Conformational changes of helices C and G. (A) View of the $|F_{\text{obs}}^\text{light}| - |F_{\text{obs}}^\text{dark}|$ difference Fourier electron density map along helix C and the resting state structural model (yellow). The blue electron density map indicates positive electron density, and orange denotes negative electron density (contoured at $\pm 3.0 \sigma$) for $\Delta t = 1$ ms. (B) Structural model of helix C on the intracellular side (Asn98-Leu106) for the time point $\Delta t = 1$ ms (pink) superimposed upon the resting state model (yellow). (C) Structural model of helix C on the extracellular side (Tyr94-Asn98) for the time point $\Delta t = 1$ ms (pink) superimposed upon the resting state model (yellow). (D) Structural model of helix G near the retinal for the time point $\Delta t = 1$ ms (pink) superimposed upon the resting state model (yellow). The helices C and G move toward the inside of the protein in association with the movements of Asn98 and Lys235, respectively.

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A and B adopt an apo form, due to the lack of Cl− bound to the retinal (SI Appendix, Fig. S9B). The side chain of Asn98 in helix C is located at the anion binding site instead of Cl−, in association with the displacement of Thr102 (SI Appendix, Fig. S9B). This induces the movement of helix C toward helix G, as in the O2-intermediate structure obtained by TR-SFX. However, the conformations of the other helices are almost the same as those in the resting state structure, except for helix C. A previous structural analysis of the anion-depleted form of NpHR indicated that the side chain of Thr126 (equivalent to Asn98) moves toward the halide binding side, accompanied by the inward movement of helix C (39). Asn98 is likely to serve as a stabilizer in the absence of a halide ion. On the other hand, the retinal of chain C binds Cl− and is very similar to the structure in the C2 crystal. An overlay of the chain C structure of the P222121 crystal with the C2 crystal structure reveals a rmsd of 0.49 Å for the backbone Ca atoms.

**Discussion**

Low-temperature trapping studies of NpHR have provided multiple intermediate structures, including the L1, L2, N, and O states (37, 40). The L1 structure indicated that Cl− remains bound to the PSB, as in the resting state structure (39). The L2 structure, the anion slightly moved to a position between Ser130 and the PSB, but no other movements were observed (37). Similarly, our NM-R3-I structure at Δt = 10 ms indicates that I− moves toward Thr102, a conserved residue in the NTQ motif that is equivalent to Ser130 of the TSA motif in NpHR. In contrast, the Asn98 side chain moves toward the anion binding site in the resting state, in association with the large movement of helix C. Previous spectroscopic studies of NM-R3 indicated the presence of the intermediate states K, L(N), O1, O2, and NM-R3/C0 (23) (Fig. 1A). The NM-R3-I structure at Δt = 1 ms might be the same as the (N) state, since the K state appears at an earlier step after light illumination. Interestingly, TR-SFX studies of NM-R3 that measured early-stage dynamics (1 to 100 ps) showed that Cl− is already translocated to Thr126 upon retinal isomerization at Δt = 100 ps and the Co of Asn98 slightly moved within 1 Å (32), in contrast to the movements in the NM-R3-I structure at Δt = 10 μs. Meanwhile, Trp99 and Trp201, located around the retinal, were displaced from 1 ps to 100 ps as observed in the NM-R3-I data at Δt = 10 μs (32). Therefore, photo-isomerization of the retinal may trigger the displacements of Cl− toward Thr102 and two tryptophan residues, followed by the movement of Asn98 in helix C to fill in the space left after the Cl− moved.

We assigned the NM-R3 at Δt = 1 ms to the O1 intermediate, based on the 13-cis/15-syn configuration of the retinal and the elimination of the halide ion bound to the PSB (Fig. 2B and SI Appendix, Fig. S6). This halide ion is released to the extracellular side at Δt = 1 ms, since no noticeable peak is visible in the difference electron density map and the anomalous density map. Interestingly, the NpHR N-like [X(N)] structure determined from low-temperature trapping studies also indicated the 13-cis/15-syn configuration of the retinal and the elimination of the halide ion (37). In the NpHR X(N) structure, large movements of helix C originating by the displacement of Thr126 were observed, resembling the movement of helix C including Asn98 (equivalent to Thr126) in NM-R3 at Δt = 1 ms. Likewise, the side chain of Thr126 in NpHR moved toward the space created by the elimination of the halide ion. Although NM-R3 shares low sequence identity to NpHR with the different key motif, they have obvious similarities in terms of the structural changes that take place during halide ion transfer. In addition, light/dark adaptation does not occur in both NM-R3 and NpHR, unlike the light/dark adaptation cycle of bR, which is attributed to retinal isomerization from the all-trans to 13-cis configuration (23, 41). These two chloride pumps might have a common mechanism for halide ion transfer, although NM-R3 evolved independently from NpHR. On the other hand, helices C and F underwent large conformational changes in the NpHR X(N) structure, thus altering the ion accessibility from the cytoplasmic side to the extracellular side (22). However, in the NM-R3 structure, the mobile extraendrical side (F and S) is smaller than those of helix C. The restricted movements of helix F might be caused by the tight crystal packing of NM-R3 in the LCP, since NpHR was crystallized by vapor diffusion.

Our study revealed that the Asp231 side chain moves toward the halide ion site around the PSB in NM-R3 at Δt = 1 ms, accompanied by the displacement of Asn98 (Figs. 2E and 3D). These movements have not been observed in previous NM-R3 studies. To our knowledge, this is a unique observation of the movement of Asp231 in a chloride pump rhodopsin. Previous studies of NpHR mutants demonstrated that the mutation of Asp252 (equivalent to Asp231 in NM-R3) has no detectable light-driven chloride ion transportation activity (42). Therefore, Asp231 may be one of the important residues for anion transportation. The movement of Asp231 toward the halide binding site dislodges Wat402, thus disordering Arg95 and Wat401 (Fig. 2E). Changes in the hydrogen bonding networks facilitate anion transportation. Furthermore, the side chain carboxylate group of Asp231, which is negatively charged in the apo state, might serve as a key driving force for the halide ion transportation around the PSB. Asn98 and Asp231 approach each other within 5 Å by the movements of helices C and F, which block water molecules and ions from accessing the extracellular side, like closing a gate, and prevent the backflow of halide ions (Fig. 4). The gate closure by Asn98 and Asp231 is a distinctive change, supporting the alternative access model proposed by Jardetzky (43). The principle of the model is that the protein is initially accessible on one side of the membrane and switches conformations to a low-affinity binding site that is accessible to the other side. In fact, the structural model of the NM-R3-Br reveals the outward movements of helix C at Δt = 1 ms, which allow access to the cytoplasmic side.

Interestingly, the displacement of the retinal toward helix C is observed at Δt = 1 ms, thus also suggesting a disconnection between the cytoplasmic and extracellular sides (Fig. 2C). The structural changes of the retinal and helix C in NM-R3 may assist the anion pump and/or disrupt the pathway for the reverse anion transfer. This retinal movement resembles that seen in the TR-SFX study of channelrhodopsin (ChR) at Δt = 1 ms, by Oda et al. (44). However, helix C of ChR was pushed outward by the retinal conformational change, while helix C of NM-R3 slightly moved within 0.5 Å. The authors proposed that the sequential movements of ChR induced water influx from the cytoplasmic side (44). The different structural changes between ChR and NM-R3 may reflect the specific functions of channels and pumps. Alternatively, the structural variations may be due to the differences between the translocated ions, H+ and Na+ in ChR, and the relatively large radius halide ions in NM-R3. Recently, the crystal structure of the light-gated anion channel, GaCr1, with a bromide ion was reported (45). The bromide ion was located among Pro58, Trp246, and Trp250 (equivalent to Thr51, Ile243, and Ile247 in NM-R3, respectively) in the tunnel on the cytoplasmic side. As compared with the apo-type GaCr1, Arg94 (equivalent to Arg95 in NM-R3) around the retinal moiety of the GaCr1-Br complex flipped to form a salt bridge with Asp234 (equivalent to Asp231 in NM-R3), which is very similar to the behavior of NM-R3-Br at Δt = 1 ms, while no halide ion was bound to the PSB in GaCr1-Br. It is quite intriguing that the anion channel and the anion pump adopt similar conformations for ion transport, even though they are functionally different since pumps actively transport ions, unlike channels.
NM-R3 has high amino acid sequence and structural feature similarities with KR2, the light-driven Na\(^+\)-pumping rhodopsin. In particular, the key amino acid residues involved in ion transfer around the retinal are identical, except for Thr102 (Asp116 in KR2), whereas they pump oppositely charged ions. However, the two rhodopsins are different in terms of their photocycles and the initial locations of the ions in the resting state. KR2 forms the intermediate states K, K\(_i\), L/M, O\(_1\), and O\(_2\) during the photocycle, while the M state does not appear in the NM-R3 photocycle (31). A sodium ion is not bound within the retinal binding pocket in the KR2 resting state structures (46, 47). TR-SFX studies of KR2 showed that the sodium ion is located around the deprotonated SB, at a distance of 2.5 Å from Asn112 (equivalent to Asn98 in NM-R3) and Asp251 (equivalent to Asp231 in NM-R3), in the O\(_i\) state at 1 ms after light illumination (SI Appendix, Figs. S10B and S11B) (31). Asn112 is 3.1 Å apart from the PSB in the resting state of KR2, which resembles the positional relationship of Asn98 and the PSB in the anion-depleted structure of the O\(_i\) structure model of NM-R3 (SI Appendix, Fig. S10). This structural similarity might be a common feature of the ion pumping rhodopsins in the absence of an ion around the retinal. A superimposition of the resting state and the O\(_i\) intermediate of KR2 indicated that the Cα atom positions of Asn112 and Asp251 did not change very much (SI Appendix, Fig. S11B). Therefore, the conformational changes in NM-R3 from the resting state to the O\(_i\) state differ from those of KR2 (SI Appendix, Fig. S11).

In both TR-SFX experiments of KR2 and NM-R3, the O\(_i\) states werecontingency captured at 1 ms after light illumination (31). Although these overall structures are very similar, the retinal in KR2 was not in the 13-cis/15-syn configuration seen in NM-R3, but in the 13-cis/15-anti configuration. In addition, the retinal was tilted toward helix C in KR2, which differs from the retinal movement in NM-R3 (SI Appendix, Fig. S11). In the structural changes from the resting state to the O\(_i\) state in KR2, the extracellular side portions of helix C moved inward within 0.5 Å, and the cytoplasmic side moieties of helix C indicated outward movements within 0.8 Å. The inward flex of helix C resembles that of NM-R3, whereas larger inward movements of helix C were observed in NM-R3. Similar movements of helix C were also seen in the M state structure of bR obtained by TR-SFX at 1.725 ms after photoexcitation. As for a time-resolved structure of bR in the latter steps of the photocycle, only one N-intermediate structure was determined using time-resolved serial millisecond crystallography (TR-SMX) at the synchronrons, and a similar inward flex of helix C was also observed as detected in the M state TR-SFX structure of bR (48). Inward movements of helix G toward the helix C occurred in the M and N structures of bR, as in the O\(_1\) structure of NM-R3. Conversely, the cytoplasmic sides of helices E and F moved largely outward within 9 Å in the TR-SMX structure of the N state, which is distinctive in bR. The comparison of three pumps transporting different ions, such as halide ions, sodium ions, and protons, revealed the common structural dynamics including the movements of helices C and G, as well as the unique structural changes.

**Conclusion**
Our TR-SFX data clearly visualized dramatic conformational changes in NM-R3 after halide ion release (Fig. 4). The 13-cis/15-syn configuration of the retinal is displaced upward toward helix C, breaking the connectivity to the extracellular side. The upward displacement of the retinal induces the sequential movements of Trp99 and Trp201 toward the cytoplasmic side, to change the ion channel around the retinal. Asn98 is substantially shifted to stabilize the retinal binding pocket in the absence of the halide ion, accompanied by the large movements of helix C. Meanwhile, Asp231 of helix G approaches the Asn98 side chain, which also blocks the accessibility from the extracellular side. These structural changes in NM-R3 are similar to those of NphR observed in low-temperature trapping studies, but differ from those of KR2 or bR obtained from TR-SFX data. Therefore, the considerable structural changes may be specific to the chloride pump rhodopsins, which transports an anion with a relatively large ionic radius. In addition, the difference electron density map obtained from NM-R3 crystals with iodide ions suggests that the halide ion slightly moves toward Thr102 and the displacement of Asn98 to the halide binding site has already begun at Δt = 10 μs. Our observations of anomalous signals from iodide ions provide clues toward solving the pathway of halide ion transfer in NM-R3.

**Materials and Methods**
Detailed materials and methods are provided in SI Appendix.
NM-R3 for crystallization was synthesized by using an *Escherichia coli* cell-free protein synthesis system, and the protein was crystallized by the in meso method (20). The microcrystals were soaked in crystallization buffer with 1,000 mM NaBr.
The photocycle reaction of NM-R3 crystals with 1,500 mM NaBr crystallization buffer was induced by a 532-nm pump pulse, and the spectral changes during the reaction were measured with a spectrometer. The pump and probe light source and the spectrometer were synchronized by pulse generators, and the pump-probe delay time was adjusted with a timing jitter of ±20 ns.

Data Availability. The coordinates and structure factors for resting state, 1 ns after photostimulation state, and anion-depleted form of NM-R3 have been deposited in the Protein Data Bank (PDB ID code 7VGT, 7VGV, and 7YGC).

TR-SFX experiments were performed at BL3 of SACLA (49). NM-R3 crystals were loaded into the injector (34). A 540-nm wavelength pump laser was introduced through optical fibers. Data collection was monitored by Chesterah and data processing was performed using CryoTEFL (50–52). The structure was solved by molecular replacement with Protein Data Bank (PDB) entry 5SB2 (20), and difference Fourier maps were calculated by the CCP4 and PHENIX suites (53, 54).

Raw diffraction images for this study are deposited to the Coherent X-Ray Imaging Data Bank (CIXDB ID 192). All other study data are included in the article and/or SI Appendix.

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