Non-cryogenic structure of a chloride pump provides crucial clues to temperature-dependent channel transport efficiency

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Running title: Non-cryogenic structure of ClR derived from XFEL.

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Keywords: serial femtosecond crystallography, light-driven chloride pump, non-cryogenic condition, transport efficiency, X-ray free electron laser, anion pump, rhodopsin, temperature dependence, time-resolved XFEL

ABSTRACT

Non-cryogenic protein structures determined at ambient temperature may disclose significant information about protein activity. Chloride-pumping rhodopsin (ClR) exhibits a trend to hyperactivity induced by a change in the photoreaction rate due to a gradual decrease in temperature. Here, to track the structural changes that explain the differences in ClR activity resulting from these temperature changes, we used serial femtosecond X-ray crystallography (SFX) with an X-ray free electron laser (XFEL) to determine the non-cryogenic structure of ClR at a resolution of 1.85 Å, and compared this structure with a cryogenic ClR structure obtained with synchrotron X-ray crystallography. The XFEL-derived ClR structure revealed that the all-trans retinal (ATR) region and positions of two coordinated chloride ions slightly differed from those of the synchrotron-derived structure. Moreover, the XFEL structure enabled identification of one additional water molecule forming a hydrogen bond network with a chloride ion. Analysis of the channel cavity and a difference distance matrix plot (DDMP) clearly revealed additional structural differences. B-factor information obtained from the non-cryogenic structure supported a motility change on the residual main- and sidechains as well as of chloride and water molecules due to temperature effects. Our results indicate that non-cryogenic structures and time-resolved XFEL experiments could contribute to a better understanding of the chloride-pumping mechanism of ClR and other ion pumps.

Introduction

The crystallization and diffraction technologies for membrane proteins have become more advanced, resulting in the accumulation of
membrane protein structure information (1-7). In particular, crystallization of membrane proteins in the lipidic cubic phase (LCP) is a critical method for solving the structures of membrane proteins such as G-protein coupled receptors (GPCRs) (8-9). However, it remains difficult to form appropriately sized, well-ordered crystals suitable for obtaining a complete diffraction dataset at synchrotron facilities. Recently, with the development of LCP injectors (10), membrane protein structure determination has become possible from microcrystals at room temperature using serial femtosecond crystallography (SFX) with X-ray free-electron lasers (XFELs) developed by Chapman and colleagues (11-14). Structural determination of membrane proteins using XFELs showed that XFEL-derived structures are comparable to synchrotron-derived structures (15-19). However, understanding of the structural differences between structures determined using SFX at XFEL facilities at room temperature and those determined using the conventional macromolecular crystallography method (MX) at synchrotrons in a cryogenic state remains limited, along with the implications for molecular functions.

Rhodopsin is an excellent target that could serve as a good model system for the technical development of structural methods such as time-resolved SFX for examining light-activated membrane proteins grown in an LCP environment (20-26). In microbial light-driven ion-pumping rhodopsin, the chromophore all-trans retinal (ATR) of rhodopsin binds covalently to a lysine residue through a Schiff base linkage. Absorption of light induces a conformational change in the retinal from all-trans to 13-cis, leading to overall structural changes in the protein, thereby activating ion transport and a photocycle that traverses several key intermediate states coming back to the dark state (21, 22, 27-29). The motif of the protonated Schiff base (PSB) changes according to the unique residue of the proton acceptor and proton donor in rhodopsin, which induces a difference in the ion transport pathway and pumping mechanism. Several studies have reported proton and chloride-pumping rhodopsins (CIRs) with various PSB motifs, including bacteriorhodopsin (bR) with DTD motifs, thermophilic bacteria rhodopsin (TR) with DTE motifs, and halorhodopsin (HR) with TSA motifs (23, 30-33).

In recent years, XFEL-derived structures for proton-pumping rhodopsins have been reported and compared with those determined at synchrotrons, and the proton-transporting mechanism was clearly identified from the nanosecond to millisecond time scale with retinal photo-isomerization using time-resolved SFX experiments (21, 22, 34). However, the structural information of CIRs close to the native condition (room temperature) and the correct ion-transporting mechanism using time-resolved SFX methods remain unknown. Proton- and chloride-pumping rhodopsin have different directions of ion uptake and exhibit completely different photocycle mechanisms. In particular, most CIRs lack the photochemical intermediate ‘M’ state in the photocycle (33, 35). Therefore, it is very important to clarify the three-dimensional structure and accurate ion transport pathway of CIR at room temperature using XFEL.

Recently, a CIR with a novel PSB (NTQ) motif exhibiting unique ion-transporting activities and pathways have been reported in the marine bacterium Nonlabens marina S1-08T (26, 36-38). Growth of this strain is modulated by environmental temperature, with a growth temperature range of 10–30°C; the optimal temperature for growth is 20–25°C, and no growth occurs below 5°C or above 37°C (39). In early studies investigating the photoelectric signal of bacteriorhodopsin, it had been suggested that the rate constant could be changed according to the pH or temperature change resulting in the modulation of rhodopsin's functions (40). In the present study, we also found that the channel functions of CIR are modulated by temperature in a similar manner. Therefore, it would be of interest to know the correlation between temperature and function of CIR, and in this respect, CIR could serve as a model protein to obtain structural dependency on temperature.

Here, we report the first room temperature crystal structure of CIR containing the NTQ motif in the chloride ion transfer pathway using XFEL.
The structure of CIR at room temperature, determined at a resolution of 1.85 Å, reveals tremendous structural details. We analyzed structural differences by comparing two structures at both room temperature and the cryogenic temperature. Although the overall structure determined by XFEL was comparable to that determined by synchrotron X-ray crystallography, the chloride ion binding sites (one at the active center of the pump and the other near the loop on the cytoplasmic side) were found to be in distinct positions showing different hydrogen bond networks. Interestingly, one additional water molecule forming a hydrogen bond network with a chloride ion (Cl\(^{-}\)) near PSB was clearly observed in the XFEL-derived structure, but not in the structure determined at the cryogenic temperature.

Our findings will be essential in gaining a better understanding of the structural differences and mechanical properties attributed to temperature during data collection by both XFEL and synchrotron analyses, providing hints into the temperature dependence of hydrogen bonding in CIR and its correlation with chloride pumping function. The room temperature structure of CIR thus sets the foundation for further time-resolved SFX studies on chloride ion transportation pathways and their underlying molecular mechanism.

**Results**

**Hyperactivity and anion binding affinity of CIR for anion transport at low temperatures**

The gram-negative, orange-pigmented, and rod-shaped bacteria *Nonlabens marina* S1-08(T) were isolated from seawater of the western north Pacific Ocean (30°11’ N, 145°05’ E; depth 0 m). It is well known that *Nonlabens marina* S1-08(T) ceases cell growth at temperatures below 10°C unlike other marine bacteria (39), and the optimal growth temperature is between 20°C and 25°C (Supplemental Table 1). Thus, to determine how temperature affects the anion transport activity of the CIR derived from *Nonlabens marina* S1-08(T), we measured light-induced pH changes for different monovalent salts, sodium chloride and sodium bromide, at five different temperatures, -20°C, -10°C, 4°C, 10°C and 25°C. As the temperature gradually decreased, the chloride ion transport rate increased and was almost two-fold faster at -20°C than at 25°C in the absence of a protonophore, and the same trend was not observed for bromide ions (Figs. 1A and 1B). Likewise, in the presence of the protonophore carbonyl cyanide 3-chlorophenylhydrazone (CCCP), the pH change was much more drastic during chloride and bromide ion transportation at lower temperatures. The enhanced activity observed in the presence of CCCP completely disappeared following the addition of tetraphenylphosphonium ion (TPP\(^{+}\)), a hydrophobic cationic reagent that disrupts membrane potential. This implied that the physiological function of CIR in pumping activity can be sensitively modulated by temperature changes. In this regard, abnormal functions, such as CIR hyperactivity involved in phototransduction biochemical reactions, could also play a role in cell death or suspended growth of *Nonlabens marina* S1-08(T) by altering the electrochemical balance of the bacterial cell.

The binding affinity of CIR for different anions was measured by ultraviolet (UV)-visible absorption spectrometry to determine whether the hyperactivity was caused by a change in the binding affinity with the anion owing to the temperature difference. The maximum wavelength with and without 1 M sodium chloride was 533 nm and 555 nm, at both room temperature (25°C) and 4°C, respectively (Figs. 2A and 2B). Likewise, the maximum wavelength with and without 1 M sodium bromide was 537 nm and 555 nm at room temperature (25°C) and 533 nm and 555 nm at 4°C, respectively (Supplemental Figs. 1A and 1B). A similar blue shift was observed with bromide and chloride ions in the maximum absorption peak upon titration at both 25°C and 4°C. Anion titration allowed for the determination of dissociation constants (\(K_d\)) from the absorbance changes at 581 nm (Figs. 2C and 2D and Supplemental Figs. 1C and 1D). The apparent \(K_d\) values were estimated using the Hill equation and are summarized in Supplemental Table 2. Surprisingly, the binding affinity of chloride and bromide ions were approximately twice as strong at 4°C than at room
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These binding affinity results were consistent with the anion transport activity results (Supplemental Table 2).

**XFEL- and synchrotron-based structures demonstrate subtle differences in two chloride positions and hydrogen bonding networks**

The ClR structures were determined by XFEL and synchrotron analysis to investigate structural changes that can explain functional differences of ClR at different temperatures. Crystal structures were collected at a resolution of 1.85 Å and 1.75 Å at room temperature (294 K) by XFEL and at cryogenic temperature (93 K) by the synchrotron, respectively. Overall, the two structures showed good agreement with no significant changes in the overall topology. The root mean square deviation (RMSD) of the 253 Cα atoms of a single molecule in the asymmetric unit was calculated to be 0.128 Å with similar overall residual B-factors (Å²) except for some structural points (Supplemental Fig. 2). Specifically, helices A and B and part of helix G showed lower B-factor values, especially, Lys235, which formed a covalent bond with retinal and exhibited the lowest B-factor value in the ClR structure at room temperature. Moreover, we also identified several important structural differences through a detailed comparison of these two structures.

First, we investigated the near-retinal region, which is the main trigger for ion-transporting activity. Interior residues surrounding the retinal chromophore were revealed with high-quality electron density maps, especially with a clear retinal β-ionone ring (Figs. 3A and 3B). The covalent bonding angle between the retinal C15 and Lys235 residue in the two structures differed by about 3° (Figs. 3C and 3D). This difference could be related to the dynamic properties that cause structural changes in response to temperature changes. Moreover, the chloride ion (Cl⁻-I), located in the active core of the XFEL-derived structure, moved toward the PSB region, resulting in a shorter distance between Cl⁻-I and the PSB by approximately 0.3 Å in comparison to the synchrotron-derived structure (Figs. 3C and 3D). Ion pumps such as ClR stabilize chloride and water molecules around the PSB region by interacting with proton acceptors or donor residues for ion transport, and the distance between them significantly differed between the two structures (Supplemental Figs. 3A, 3B, 3D and 3E). Disruption of hydrogen bonds is essentially a temperature-dependent process (41). The rearrangement of water molecules and chloride ions leads to a change in the hydrogen network. Important information gained from the XFEL structure was the discovery of new hydrogen bonding networks with an additional water molecule (W614), having B-factor of 26.4 Å² and near Cl⁻-I (Figs. 3A and 3B). ClR was previously thought to have unique hydrogen bond networks with two water molecules distinct from other chloride pumps such as HR, which have three well-resolved water molecules mediating the hydrogen bond network between Cl⁻-I-PSB and Asp239-Arg108 (in HR from *H. salinarum*) (42). However, the ClR structure derived from XFEL confirms that it also has three well-resolved water molecules, W507, W508, and W614, and forms similar hydrogen bonding networks between Cl⁻-I-PSB and Asp231-Arg95, like other chloride pumps (Supplemental Fig. 3A). The B- factors of W507 and Cl⁻-I were approximately 16.3 Å² and 20.8 Å² in the XFEL-derived ClR structure, compared to the corresponding values of 24.4 Å² and 29.2 Å² in the synchrotron-derived structure, indicating more stable dynamics in this region.

As a result, the XFEL-derived ClR structure determined at room temperature showed much stronger water-mediated hydrogen bonds and considerably more stable dynamic properties near the PSB and Cl⁻-I. In particular, chloride ions exhibited increased interactions with W507 and the PSB in the XFEL-derived structure. The hydrogen bond between Arg95 and the water molecule W508 was similar, whereas the hydrogen bond between Asp231 and the water molecule W507 was very different owing to the presence of the additional water molecule W614 (Supplemental Figs. 3A, 3B, 3D and 3E).

Second, structural rearrangement was
detected around the chloride ion (Cl$^-$-II) in the cytoplasmic region (Fig. 3D). The location of Cl$^-$-II changed by approximately 0.5 Å, resulting in a longer distance between Cl$^-$-II and Lys46 amide nitrogen in the XFEL-derived structure by approximately 0.2 Å in comparison to that in the synchrotron-derived structure. The B-factors of Cl$^-$-II in the two structures were quite comparable: both are about 40 Å$^2$.

Additionally, one water molecule in the cytoplasmic region, W515, showed different positions and B-factor values between the two structures. W515 forms a hydrogen bond between Lys46 and Ile112 by 3.0 and 2.7 Å in the XFEL-derived structure, though it shows a much weaker hydrogen bond between W515 and Lys46 in the synchrotron-derived structure (Supplemental Figs. 3C and 3F). In the XFEL-derived structure, the position of W515 shifted by 1.4 Å toward the backbone carbonyl oxygen of Lys46 and the B-factor was increased from 27.8 Å$^2$ to 47.1 Å$^2$ (Figs. 3E and 3F). The information of hydrogen bonding distance and B-factors from the structure determined at room temperature using the XFEL method provides a structural explanation for the abnormal activities of ClR at low temperature.

**Channel cavities of ClR**

For more in-depth structural comparisons, the volume sizes and side chain orientations of the participating residues were investigated for four core cavities buried in the seven transmembrane domains of ClRs. The volume of individual cavities showed quantitative dependence on the number of atoms (or residues) and water lining the cavity. The cavity volumes (Å$^3$) in the two ClR structures from the XFEL and the synchrotron are marked in Figure 4A and 4B. Overall, cavity 1 and 3 in the XFEL-derived ClR structure were slightly larger (87.79 and 2.76 Å$^3$ vs. 86.05 and 2.63 Å$^3$), whereas cavities 2 and 4 were smaller than that in the synchrotron-derived structure (11.02 and 0.17 Å$^3$ vs. 13.90 and 0.29 Å$^3$).

In addition, although the structures were generally similar, the hydrogen bonds between the residues forming the cavity and the surrounding water differed. In cavity 1, Cl$^-$-I and five water molecules form a hydrogen bond network, and the side chain orientation of participating residues were very similar in the two structures, with some notable variations in the distances between certain residues, as explained in the previous section (Fig. 4C and Supplemental Fig. 3). In cavity 2, different side-chain packing for Ile64, Lys65, Arg95, Asn98, Trp99, Thr102, Asp231, and Lys235 was observed in the hydrogen bond network, with Cl$^-$-I and water molecules depending on the presence of an additional water molecule, W614 (Fig. 4D).

Interestingly, the water molecule W509 did not share the same position, and its B-factor value differed between the two resolved structures (34.2 vs. 41.4 Å$^2$). In cavity 3, Asn3 and Asn92 showed very different side chain orientations, and the position of one nearby water molecule, W501, was not observed in the XFEL-derived structure (Fig. 4E and Supplemental Figs. 4A and 4B). In cavity 4, Met58, Lys106, Gln109, and Lys235 showed slight differences with respect to side chain orientation (Fig. 4F). Thus, the high-resolution ClR structures measured by both XFEL and synchrotron allow accurate comparison of the cavity and side chain orientations.

**Temperature-dependent conformational change of ClR**

Since the overall conformational changes were quite subtle, we constructed a difference distance matrix plot (DDMP) to detect regional structural rearrangements that might be caused by the temperature differences (Fig. 5). DDMP analysis demonstrates dramatic internal distance differences in several regions based on the distances among Cα atoms. According to the DDMP results, structural perturbation resulting from the cryo-cooling conditions occurred during data collection. Specifically, major structural perturbations occurred in the B-C loop, the C-D loop, a part of helix C in the extracellular side, a part of helices E and G in the intracellular side, and the C-terminal helix (Figs. 5A and 5B). The poly-chains Val79-Leu85 and Gln86-Thr89, located in the B-C loop, moved in opposite directions, and this may have affected the π-stacking interaction.
that formed the 3-omega motif of CIR (Fig. 5C). The extracellular section of helix C moved toward the interior of the protein, with the same orientation as poly-chain Val79-Leu85 in the B-C loop (Fig. 5C; upper panel).

The intracellular portion of Helix E is another region that showed the widest conformational change (Fig. 5A and 5B).

Interestingly, C-terminal helix movement was clearly observed in the direction opposite to helix G movement (Fig. 5C; lower panel). These motions were reported to be important for maintaining the function and stability of CIR in previous studies (42) and are similar to the related motion in the function of bR (34). As a result, the structural orientation of the C-D loop, a part of helix E, and C-terminal helix moved toward the exterior of a protein, whereas helix G moved in the opposite direction (Fig. 5C; lower panel).

The detailed structural information of the XFEL-derived CIR provided the basis for revealing the ion channel mechanism of the light-induced photocycle of CIR at room temperature.

**Discussion**

A number of functionally important membrane protein structures have been solved using crystals grown with the LCP method. However, several problems remain unsolved in order for membrane protein crystallization to obtain crystals suitable for synchrotron X-ray diffractions. Recently, SFX crystallography using XFELs has been developed and has emerged as a powerful method for the structural determination of macromolecules, especially membrane proteins such as GPCRs. The experimental environment for XFEL is different from that used for synchrotron-based crystallography which requires cryogenic cooling protection, and this difference could influence the interaction of ligands or ions that are related to membrane protein function. The structure and dynamics for some membrane proteins depend on temperature and pressure.

Studies have indicated that XFEL-derived structures do not suffer from perturbations because of cryogenic cooling or radiation damage from synchrotrons (34, 43). Here, we directly compared structures of CIR at cryogenic and room temperatures and revealed subtle structural differences that could explain the hyperactivity of CIR at low temperatures. The DDMP analysis depicted regional conformation changes for temperature-sensitive residues. The high-resolution data (1.85 Å and 1.75 Å of the structures from the XFEL and synchrotron, respectively) allowed us to accurately compare hydrogen bonding networks mediated by chloride ions and water molecules through direct structure comparison. The overall unit cell volume of microcrystals in the XFEL experiment was larger (by about 3.2%) than that of the crystals used in the synchrotron crystallography experiment, indicating that the cryogenic cooling slightly dehydrates the crystals and increases the crystal packing. In a previous bR study, the electron density map around the C2–C4 atoms of the retinal β-ionone ring was found to be partially depleted in the XFEL structure, indicating the possibility of multiple conformers at ambient temperature (34). In the present study, the CIR structure derived from XFEL showed a very clear electron density map of the retinal β-ionone ring, indicating that the structure was thermodynamically stable at physiological temperature. These findings suggest that bR and CIR may have different dynamic motions of the retinal, leading to a cis-trans transition even at the same temperature conditions (Supplemental Figs. 4C and 4D).

Here, we also identified the hydrogen bond networks mediated by the additional water molecule, W614, near the Cl−-I and PSB regions. In general, the energy associated with hydrogen bonds is 6–30 kJ/mol, and even a slight change in the angle and distance between the relevant atoms causes a drastic change in the energy barrier (44). For this reason, hydrogen bonding is a key element in maintaining the binding strength and ion specificity in a transporter protein, which in turn affects the ion transport efficiency. These differences were also observed at the Cl−-II position in the cytoplasmic region. The weak hydrogen bonding network near Cl−-I in CIR would eventually lead to rapid chloride ion
diffusion into the cytoplasm direction at a reduced energy cost. In this respect, the non-cryogenic structure determined at ambient temperature is helpful to better understand protein functions at the optimum temperature of their activity.

Considering the structural changes observed in the cavity and DDMP analyses, temperature-dependent structural movement of ClR can be inferred. At ambient temperatures, the ClR structure has a relatively wider intracellular portion and narrower extracellular portion compared to its structure at low temperatures.

In this study, we concluded that the ClR structure derived from the XFEL method provided a structural foundation in understanding its chloride ion-pumping activity at physiological conditions.

**Experimental procedures**

**Protein expression and purification**

The ClR gene (GI: 594833795) from *Nocardioides marinus* in the pET21b vector was transformed into *Escherichia coli* BL21-CodonPlus (DE3; Agilent Technologies, Santa Clara, CA, USA), and the cells were grown in high-salt Luria-Bertani medium at 37°C. When the optical density at 600 nm (OD_{600}) was over 1.0, 50 μM of all-trans retinal (ATR) (Sigma Aldrich, St. Louis, MO, USA) and 0.5 mM isopropyl-β-D-thiogalactoside (IPTG) were added to induce ClR expression for 6–8 h at 30°C. Harvested cells were lysed by sonication in lysis buffer containing 50 mM Tris-HCl (pH 7.0) and 150 mM NaCl. The membrane fraction was isolated by ultracentrifugation (Beckman, Brea, CA, USA) at 370,000 × g for 40 min at 4°C, resuspended in solubilization buffer containing 50 mM Tris-HCl (pH 7.0), 150 mM NaCl, 1% n-dodecyl-β-D-maltoside (DDM), and 0.2% cholesteryl hemisuccinate (CHS), and incubated for 2 h at 4°C for solubilization. The solubilized protein was purified by TALON affinity chromatography and the eluate was applied to a Superdex-200 size-exclusion column (GE Healthcare, Little Chalfont, UK) equilibrated with buffer containing 20 mM HEPES (pH 7.5), 150 mM NaCl, 0.05% DDM, and 0.01% CHS (Supplemental Fig. 5A).

**Microcrystal preparation for XFEL and synchrotron analysis**

ClR microcrystals appeared in 30% polyethylene glycol dimethyl ether 500, 0.1 M sodium chloride, and 0.1 M MES (pH 6.0) buffer at a final concentration of 50 mg/mL for conventional synchrotron experiments (42). For the LCP-XFEL approach, a high density of microcrystals is required for efficient data collection. We followed optimized protocols (45) to the obtain samples for a complete dataset collection. The purified protein was mixed with monoolein (1-oleoyl-rac-glycerol) at a 1:1.5 molar ratio (w/w) using a syringe lipid mixer (Hamilton, Reno, NV, USA). After formation of a clear lipidic cubic phase, ~5 μL of the protein-laden LCP sample was injected using a 100-μL syringe filled with 55–60 μL of precipitant solution. After sealing the needle stopper, the syringe was wrapped in a moist tissue to maintain the humidity and placed in a 20°C incubator for 1 week (Supplemental Fig. 5B). Five additional syringes were prepared to produce a large volume of the LCP crystal sample. Microcrystals of ClR were grown in the syringes, and red-colored crystals were identified using a microscope. For the synchrotron experiment, ClR microcrystals were grown on LCP plates (Supplemental Fig. 5C).

**Experimental setup at Linac Coherent Light Source, and data collection from XFEL and synchrotron**

The experiment was performed at the Coherent X-ray Imaging (CXI) end station (46) at the Linac Coherent Light Source (LCLS) of the SLAC National Accelerator Laboratory. Prior to LCP-XFEL data collection, ClR crystals grown in syringes were incubated at 25°C for 1 h. Approximately 40 μL of the LCP sample was transferred into a new syringe after removing the precipitant solution, and ~10 μL of 9.9 MAG was applied to the LCP sample and homogenized. The sample was transferred into an LCP loading needle through an LCP loading needle. Data were collected at 120 Hz using a photon energy of 9.5 keV (λ = 1.3 Å) and a pulse duration of ~60 fs. Because of evaporative cooling, the sample
temperature at the X-ray interaction region (approximately 100 μm downstream of the nozzle exit) was estimated to be slightly below room temperature. The samples were injected using an LCP injector with a 75-μm diameter nozzle at an average flow rate of 2 µL/min. A Cornell-SLAC Pixel Array Detector (CSPAD) was used to collect the diffraction data. The panels of the CSPAD were tiled to form a two-dimensional detector that was placed at 77.5 mm from the interaction plane, allowing for diffraction data collection up to a 1.33 Å resolution at the given wavelength. About 420,465 diffraction patterns were collected in ~1 h from 120 μL of the sample. The Cheetah program (47) was used to preprocess and filter the raw data to select diffraction patterns (actual “hits” of crystals from a liquid background). A pattern was classified as a "hit" if it contained at least 20 peaks with a signal-to-noise ratio larger than 6.0. A total of 22,094 patterns were identified as hits for an overall hit rate of 5.26%. The CrystFEL software suite (48) was used to index the diffraction patterns and merge the intensity to form the three-dimensional diffraction volume. After refinement of the experimental conditions, including detector geometry, 10,805 patterns were indexed using the indexamajig module of the CrystFEL suite, and the unit cell parameters were determined for data merging. Finally, all reflections, including those predicted based on the observed peaks of indexed patterns, were merged into a dataset with a Monte Carlo approach implemented in CrystFEL. For conventional synchrotron data, we collected diffraction data from ClR crystals on the BL17A beamline at the Photon Factory (Tsukuba, Japan) using a 0.03 × 0.01 mm micro-beam. The diffraction data were processed with XDS software (49). We determined the cryogenic structure at Photon factory as described in a previous report (42). All parameters for data collection, model refinement, and statistical analysis of both the XFEL and synchrotron data are summarized in Table 1.

Structure determination and refinement

Crystals grew in space-group \( \text{C}2 \), with \( a = 103.36 \) Å, \( b = 50.09 \) Å, \( c = 69.40 \) Å, \( \alpha = 90.00^\circ \), \( \beta = 109.65^\circ \), and \( \gamma = 90.00^\circ \) for the XFEL-derived structure, and \( a = 102.76 \) Å, \( b = 49.40 \) Å, \( c = 69.33 \) Å, \( \alpha = 90.00^\circ \), \( \beta = 109.85^\circ \), and \( \gamma = 90.00^\circ \) for the synchrotron-derived structure, with one molecule in the asymmetric unit. Initial models of the near-isomorphous crystal data were obtained by molecular replacement using PHASER (50) with a previously solved structure as a search model (PDB 5G28). The solutions of ClR were successfully obtained with a final translation function Z-score of 19.0. The models were refined with the simulated annealing protocol using bulk-solvent correction with data between a resolution of 50.0 Å and 3.0 Å. Electron density was interpreted and traced using COOT (51), and the model was refined with PHENIX (52) and autoBUSTER (53). After several rounds of alternate refinement by autoBUSTER in the absence and presence of ATR, the structure of ClR containing ATR was further refined, resulting in \( R_{work}/R_{free} = 0.24/0.28 \) over a resolution range of 18.52–1.85 Å. Likewise, the synchrotron-derived structure was refined, resulting in \( R_{work}/R_{free} = 0.19/0.22 \) over a resolution range of 23.63–1.75 Å. Solvent molecules were placed at positions where spherical electron density peaks were found above 1.3 \( \sigma \) in the \(|2F_o-F_c| \) map and above 3.0 \( \sigma \) in the \(|F_o-F_c| \) map, and where stereochemically reasonable hydrogen bonds could form. Validation of the final model was carried out using PROCHECK (54). During structure refinement, we tried to reduce the resolution gap and the artifact by applying the same sigma cut off value between the XFEL- and synchrotron-derived structure, especially for selection of chloride ions and water molecules.

Data availability

Coordinates and structure factors have been deposited in the Protein Data Bank under accession codes 5ZTL (XFEL-derived ClR structure) and 5ZTK (synchrotron-derived ClR structure). Remaining data are available from the corresponding authors on reasonable request.

Difference distance matrix plot (DDMP) analysis

The pairwise distance map was computed between all possible amino acid residue pairs in a
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three-dimensional structure and represented with a two-dimensional matrix. We used the difference distance matrix plot (DDMP) (Center for Structural Biology, Yale University, New Haven, CT, USA) program to analyze the protein contact map. In the difference distance map, the changes in distance between Ca-Ca atoms were reflected by the brightness of the colors, and color saturation levels were set to represent Ca shifts of 0.5 Å closer (red) and 0.5 Å away (blue).

Cavity calculation

The PDB coordinates of CIR structures were submitted to the CASTp Web server (http://sts.bioe.uic.edu/castp/calculation.html) for putative substrate-binding pocket and retinal cavity calculation, and identification as a probe radius of 1.4 Å. Each pocket was assigned a unique identification number, roughly corresponding in order of decreasing volume. After selection of the specific pocket, graphical representations of CIR were made using PyMol.

Measurement of pumping activities of CIR

Escherichia coli BL21-CodonPlus (DE3) cells, expressing CIR, were incubated at 37°C in YT media supplemented with 100 mg/mL ampicillin. When the OD600nm was greater than 1.0, the expression of CIR was induced by adding 1 mM IPTG and 50 μM ATR. After additional cultivation for 4 h at 37°C, the cells were collected by centrifugation at 4000 ×g for 5 min, washed three times with 100 mM NaCl or NaBr including 50% glycerol, and suspended in the desired solvents for measurement by adjusting the OD600nm value to 8.0. Cell suspensions were placed in the dark for more than 2 h, under control of desired temperature from -20°C to 25°C, and then, proton ion flux changes in the buffer were measured using a pH electrode F-72G (Horiba, Kyoto, Japan) at five different temperatures, -20°C, -10°C, 4°C, 10°C and 25°C, respectively. The light source used was a 200 mW 520 nm Xeon lamp (Elpisbio, Seoul, South Korea).

UV–visible spectroscopy

CIR was purified and buffer exchange was conducted using 10 mM MOPS (pH 6.5) and 0.05% DDM on a Superdex-200 size-exclusion column (GE Healthcare). The absorption spectra were scanned for the protein samples using a V-650 spectrophotometer (JASCO, Oklahoma City, OK, USA) at two different temperatures, 4°C and 25°C. Anion titration experiments were performed by adding chloride and bromide ions and standard deviations were calculated from triplicate experiments. Anion binding affinities were calculated from absorption changes at a wavelength of 581 nm using the Hill equation. The binding affinities are listed in Supplemental Table 2.

Acknowledgements – We are grateful to the staff scientists at the Coherent X-ray Imaging (CXI) station of the Linac Coherent Light Source of the SLAC National Accelerator Laboratory, BL17A beamline of the Photon Factory. We also appreciate to the staff scientists at the Pohang Accelerator Laboratory X-ray Free Electron Laser (PAL-XFEL) for their technical support on the initial screening. This research was supported by a Tianhe-2JK computing time award at the Beijing Computational Research Center (CSRC). This work was supported by NRF-2017R1A2B2008483 to WL, NRF-2017M3A9F6029755 to HC, and the Basic Science Research Program (NRF-2016R1A6A3A04010213 to JHY) through the National Research Foundation of Korea (NRF) funded by the Ministry of Education. Jae-Hyun Park has been supported by Brain Korea Plus(BK+) program; H. Liu acknowledges support from NSFC (No. 11575021, U1530401, and U1430237); Portions of this research were carried out at the Linac Coherent Light Source (LCLS) at the SLAC National Accelerator Laboratory. This LCLS beam time was part of the Protein Crystal Screening (PCS) program. LCLS is an Office of Science User Facility operated for the US Department of Energy Office of Science by Stanford University. Use of the Linac Coherent Light Source (LCLS), SLAC National Accelerator Laboratory, is supported by the U.S. Department of Energy, Office of Science, Office of Basic
Energy Sciences under Contract No. DE-AC02-76SF00515.; Parts of the sample delivery system used at LCLS for this research were funded by the NIH grant P41GM103393, formerly P41RR001209.

**Conflict of interest** – The authors declare that they have no conflicts of interest with regard to the contents of this article.

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**Abbreviations:** ClR – chloride-pumping rhodopsin; SFX – serial femtosecond X-ray crystallography; XFEL – X-ray free electron laser; ATR – all-trans retinal; DDMP – difference distance matrix plot; LCP – lipidic cubic phase; GPCRs – G-protein coupled receptors; PSB – protonated Schiff base; bR – bacteriorhodopsin; TR – thermophilic bacteria; HR – halorhodopsin; CCCP – carbonyl cyanide 3-chlorophenylhydrazone; TPP+ – tetraphenylphosphonium ion; IPTG – isopropyl-β-D-thiogalactoside; DD M – n-dodecyl-β-D-maltoside; CHS – cholesteryl hemisuccinate; RMSD – root mean square deviation.
Table 1. Data collection and refinement statistics

|                              | XFEL-derived CIR                  | Synchrotron-derived CIR                  |
|------------------------------|-----------------------------------|-----------------------------------------|
| **Data collection**          |                                   |                                         |
| Space group                  | C2                                | C2                                      |
| Cell dimensions              |                                   |                                         |
| a, b, c (Å)                  | 103.36 50.09 69.40               | 102.76 49.40 69.33                     |
| α, β, γ (°)                  | 90.00 109.65 90.00               | 90.00 109.85 90.00                     |
| Number of collected images   | 420,465                           | N.A                                     |
| Number of hits               | 22,094                            | N.A                                     |
| Number of indexed patterns   | 10,805                            | N.A                                     |
| Number of merged patterns    | 10,801                            | N.A                                     |
| Indexing rate (%)            | 48.9                              | N.A                                     |
| Number of total reflections  | 1,644,020                         | 32,667                                  |
| Number of unique reflections | 28,973                            | N.A                                     |
| Resolution (Å)               | 18.52–1.85 (1.92–1.85)*           | 23.63–1.75 (1.81–1.75)*                 |
| Rmerge (%)                   | N.A                               | 8.6 (26.9)                             |
| Rsplit, (%)                  | 16.6 (72.7)                       | N.A                                     |
| Rpim (%)                     | N.A                               | N.A                                     |
| I/σ(I)                       | 4.04 (1.61)                       | 24.47 (5.0)                            |
| Completeness (%)             | 100 (100)                         | 98.4 (97.1)                            |
| Multiplicity                 | 56.7 (37.3)                       | 3.1 (2.5)                              |
| CC1/2 (%)                    | 96.7 (38.6)                       | N.A                                     |
| **Refinement**               |                                   |                                         |
| Resolution (Å)               | 18.52–1.85                        | 23.63–1.75                             |
| No. reflections/test set     | 28,681/1,420                      | 32,667/1,655                          |
| Rwork/Rfree                  | 0.24/0.28                         | 0.19/0.22                              |
| No. atoms                    |                                   |                                         |
| Protein                      | 2,045                             | 2,072                                  |
| Retinal                      | 20                                | 20                                     |
| Water                        | 120                               | 72                                     |
| Chloride                     | 2                                 | 2                                      |
| Lipid                        | 98                                | 99                                     |
| B-factor (Å²)                |                                   |                                         |
| Wilson B/Overall B           | 22.53/23.59                       | 20.92/23.12                           |
| Root mean square deviations  |                                   |                                         |
| Bond lengths                 | 0.01                              | 0.01                                   |
| Bond angles                  | 0.97                              | 0.93                                   |
| Ramachandran plot (%)        |                                   |                                         |
| Favoured                     | 98                                | 99                                     |
| Allowed                      | 2                                 | 1                                      |
| Disallowed                   | 0                                 | 0                                      |

*Values in parentheses are for highest-resolution shell.
FIGURE LEGENDS

Figure 1. The anion-pumping activity of CIR depending on different temperatures. Light-induced pH changes in E. coli cell suspensions expressing CIR in solution containing (A) 100 mM NaCl and (B) 100 mM NaBr observed in the absence (grey solid lines) or presence (black solid lines) of the protonophore CCCP (30 µM), or in the presence of 30 µM CCCP and 50 mM TPP⁺ (black broken lines) at different temperatures from -20 to 25°C. CIR-expressing E. coli cells were light-induced by a green-light laser-on (540 nm) for 5 min and were dark-adapted before and after green-light laser-on for 5 min each.

Figure 2. The UV-visible absorption spectroscopy and binding affinity of CIR at 25°C and 4°C. The UV-visible absorption spectra of CIR following the addition of NaCl up to 1 M at (A) 4°C and (B) 25°C. The maximum absorption wavelength values in the absence and presence of chloride ions at 4°C and 25°C were 555 and 533 nm, respectively. Absorption changes at 581 nm in the difference spectra plotted against chloride ion concentrations at (C) 4°C and (D) 25°C. (E) The data was fitted using the Hill equation (solid lines) to estimate the anion affinity at 4°C and 25°C. All fitting parameters were normalized as Δλmax and set to 1. Error bars represent standard deviation from three independent experiments. The titration experiments were performed with chloride ions and bromide ions at 4°C and 25°C. See also Supplemental Fig. 1 and Supplemental Table 2.

Figure 3. The structural comparison at two chloride-binding sites near the PSB and cytoplasmic regions. The 2Fo–DFc electron density map, contoured at 1.5 σ, near the PSB region of the (A) XFEL- and (B) synchrotron-derived CIR structures. Selected side-chains close to the ATR are shown as stick models. The chloride ions are shown as green spheres. (C) The chloride (Cl⁻-I) binding site near ATR linked covalently to Lys235, and (D) the chloride (Cl⁻-II) binding site near cytoplasmic region from the XFEL- and synchrotron-derived structure are shown as a stick model colored cyan and green, respectively. The two chloride ions located near the PSB and cytoplasmic regions are depicted as cyan and green spheres in the XFEL- and synchrotron-derived structures, respectively. The 2Fo–DFc electron density map, contoured at 1.5 σ, of Cl⁻-II and two water molecules (W514 and W515) near the cytoplasmic region of the (E) XFEL- and (F) synchrotron-derived CIR structures. The B-factor values of water molecules were reflected by the electron density map.

Figure 4. Channel cavities of the XFEL- and synchrotron-derived CIR structures. The cavities embedded in the XFEL-derived and synchrotron-derived CIRs with water molecules are shown (A, B) as cavity 1, 2, 3, and 4 marked by red, cyan, orange, and violet meshes, respectively. The volume (Å³) of individual cavities in XFEL-derived and synchrotron-derived CIRs are labeled. Superimposed structure of CIR derived from XFEL and synchrotron in (C) cavity 1, (D) cavity 2, (E) cavity 3, and (F) cavity 4 colored by slate and orange. Participating amino acid residues for each cavity are represented by a line model. Chloride ions and water molecules from the XFEL- and synchrotron-derived structures are depicted as blue, slate, red, and orange spheres, respectively.

Figure 5. The overall differences between the XFEL- and synchrotron-derived CIR structures by the DDMP analysis. (A) The difference distance matrix plot (DDMP) according to Cα atom deviations between the XFEL- and synchrotron-derived CIR structures. Red and blue dots indicate the relative movement closer or further, respectively, with color saturation indicating a difference of 0.5 Å or more. The XFEL- and synchrotron-derived CIRs differed slightly, as shown by the regions highlighted with black dotted boxes. (B) Blue dotted arrows connect these blocks to a ribbon diagram, indicating the position of the highlighted
regions within the structure such as helix C, helix E, helix G, the B-C loop, the C-D loop, and the C-terminal helix. (C) The direction of the relative movement of the transmembrane domains based on DDMP. Opposite motional directions are indicated by red and blue arrows in the extracellular (upper) and cytoplasmic (lower) regions.
Fig. 1

**A**

- Dark
- Light
- Dark

At 25°C
- 100mM NaCl
- + CCCP
- - CCCP
- + CCCP, + TPP^*

At 10°C
- 100mM NaCl
- + CCCP
- - CCCP
- + CCCP, + TPP^*

At 4°C
- 100mM NaCl
- + CCCP
- - CCCP
- + CCCP, + TPP^*

At -10°C
- 100mM NaCl
- + CCCP
- - CCCP
- + CCCP, + TPP^*

At -20°C
- 100mM NaCl
- + CCCP
- - CCCP
- + CCCP, + TPP^*

**B**

- Dark
- Light
- Dark

At 25°C
- 100mM NaBr
- + CCCP
- - CCCP
- + CCCP, + TPP^*

At 10°C
- 100mM NaBr
- + CCCP
- - CCCP
- + CCCP, + TPP^*

At 4°C
- 100mM NaBr
- + CCCP
- - CCCP
- + CCCP, + TPP^*

At -10°C
- 100mM NaBr
- + CCCP
- - CCCP
- + CCCP, + TPP^*

At -20°C
- 100mM NaBr
- + CCCP
- - CCCP
- + CCCP, + TPP^*

**ΔpH (0.1 unit/div.)**

**Time (s)**
Fig. 2

(A) Absorbance vs. Wavelength (nm) for 1M NaCl at 4°C.

(B) Absorbance vs. Wavelength (nm) for 1M NaCl at 25°C.

(C) Difference Absorbance vs. Wavelength (nm) for 1M NaCl at 4°C.

(D) Difference Absorbance vs. Wavelength (nm) for 1M NaCl at 25°C.

(E) Relative Difference Absorbance at 581 nm vs. Anion concentration (mM) for Cl⁻ and Br⁻ at 4°C and 25°C.
Fig. 4

A

Cavity 1 87.792 (Å³)

Cavity 1 86.052 (Å³)

Cavity 2 11.021 (Å³)

Cavity 2 11.021 (Å³)

Cavity 3 2.758 (Å³)

Cavity 3 2.626 (Å³)

Cavity 4 0.167 (Å³)

Cavity 4 0.292 (Å³)

B

C

D

E

F
### Fig. 5

#### A

| a.a.No. | 79~95 | 114~117 | 164~184 | 249~263 |
|---------|-------|---------|---------|---------|
| 1       | 27    | 54      | 81      | 108     |
| 108     | 135   | 162     | 189     | 216     |
| 216     | 243   | 270     | 27      | 54      |

#### B

- **BC loop**
- **Helix C**
- **Helix G**
- **C-ter Helix**
- **Helix E**
- **Cl-1**
- **Cl-2**
- **CD loop**

#### C

- **EC region**
- **N-ter**
- **B-C loop**
- **Helix D**
- **Helix C**
- **Helix F**
- **Helix G**
- **Helix A**
- **Helix B**
- **C-ter Helix**

### Annotations

- **a.a.** : Amino Acid Number
- **Å** : Angstrom
- **EC region**
- **CP region**
Non-cryogenic structure of a chloride pump provides crucial clues to temperature-dependent channel transport efficiency

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*J. Biol. Chem.* published online November 19, 2018

Access the most updated version of this article at doi: 10.1074/jbc.RA118.004038

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