Crystal Structure of Cruxrhodopsin-3 from Haloarcula vallismortis

Siu Kit Chan¹, Tomomi Kitajima-Ihara¹, Ryudoh Fujii¹, Toshiaki Gotoh¹, Midori Murakami¹, Kunio Ihara², Tsutomu Kouyama¹,³

¹ Department of Physics, Graduate School of Science, Nagoya University, Nagoya, Japan, ² Center for Gene Research, Nagoya University, Nagoya, Japan, ³ RIKEN Harima Institute/SPring-8, Mikazuki, Sayo, Hyogo, Japan

Abstract

Cruxrhodopsin-3 (cR3), a retinylidene protein found in the claret membrane of Haloarcula vallismortis, functions as a light-driven proton pump. In this study, the membrane fusion method was applied to crystallize cR3 into a crystal belonging to space group P321. Diffraction data at 2.1 Å resolution show that cR3 forms a trimeric assembly with bacterioruberin bound to the crevice between neighboring subunits. Although the structure of the proton-release pathway is conserved among proton-pumping archaeal rhodopsins, cR3 possesses the following peculiar structural features: 1) The DE loop is long enough to interact with a neighboring subunit, strengthening the trimeric assembly; 2) Three positive charges are distributed at the cytoplasmic end of helix F, affecting the higher order structure of cR3; 3) The cytoplasmic vicinity of retinal is more rigid in cR3 than in bacteriorhodopsin, affecting the early reaction step in the proton-pumping cycle; 4) The cytoplasmic part of helix E is greatly bent, influencing the proton uptake process. Meanwhile, it was observed that the photobleaching of retinal, which scarcely occurred in the membrane state, became significant when the trimeric assembly of cR3 was dissociated into monomers in the presence of an excess amount of detergent. On the basis of these observations, we discuss structural factors affecting the photostabilities of ion-pumping rhodopsins.

Introduction

Since the light-driven chloride ion pump halorhodopsin from Natronomonas pharaonis (pHR) was discovered to have the ability to silence the electrical activity of neurons [1], a number of ion-pumping rhodopsins have proved useful for optical control of the neuron activity [2,3]. Among them, the light-driven proton pump archaeorhodopsin-3 (Arch) was reported to be most powerful in optically silencing neurons [4]. Meanwhile most microbial rhodopsins expressed in neural cells are rapidly inactivated under illumination. Although light-induced inactivation is less significant in Arch and its homologs, little is known about what structural factor determines the protein stability under illumination. To date, several proton-pumping rhodopsins [5–14] and two anion-pumping rhodopsins [15,16] have been crystallized. Structural comparison of these proteins enabled the discussion about a common structural motif that is relevant to the ion-pumping activity [17]. However, it is still difficult to elucidate which structural factors affect the protein stability of ion-pumping rhodopsins in neuron cells. Against this background, it is important to accumulate more structural data of microbial rhodopsins.

Cruxrhodopsin (cR) is a member of the archaeal rhodopsin family and it functions as a light-driven proton pump. The presence of cruxrhodopsin-1 and -2 in Haloarcula argentinensis and Haloarcula mukohataei, respectively, was first reported by Mukohata and colleagues [18,19]. A homologous protein, cruxrhodopsin-3 (cR3), was isolated from Haloarcula vallismortis [20]. Sequence identity among these homologs is high (>90%) [21]. But the amino acid sequences of all members of the cR family are distinct from those of the archael proton-pumping rhodopsins with known structure (i.e., sequence identities with bacteriorhodopsin (bR) are 40–54%) (Fig.1). Since cR works as a powerful neuron silencer [3], its structural information would provide a clue in engineering a novel neuron silencer with a more suitable structural and spectral property.

In the present study, we investigated spectroscopic and structural properties of cR3. This target protein was expressed in a bR-deficient mutant strain of Halobacterium salinarum (MPK-109) and crystallized by the membrane fusion method [22]. Diffraction data showed that cR3 forms a trimeric assembly with bacterioruberin bound to the crevice between neighboring subunits, as previously observed in 3D crystals of archaeorhodopsin-2 (aR2), deltarhodopsin-3 (dR3) and pharaonis halorhodopsin (pHR) [11,12,16]. Although the structure of the proton-release pathway is highly conserved among the proton-pumping rhodopsins, cR3 possesses the following peculiar structural features: i) The cytoplasmic vicinity of retinal is more rigid in cR3 than in bR; ii)
The cytoplasmic end of helix E is greatly bent so that a large cavity is created between helices E and F; iii) The DE loop interacts with a neighboring subunit to strengthen the trimeric assembly; iv) Three positive charges are distributed along helix F. Meanwhile, it was observed that the decay kinetics of some photoreaction states of cR3 were significantly different from those reported for bR. For example, the decay rate of the K state was ten times slower in cR3 than in bR. It was also shown that in the solubilized state cR3 exhibited a much higher photostability than observed for bR. By comparing the structural and absorption kinetics data of cR3 with those of other ion-pumping rhodopsins, we discuss structural factors affecting the photoreaction kinetics of rhodopsins.

Results
Spectroscopic properties of cR3
When the cells of *H. salinarum* that were transformed with the cop3 gene were repeatedly washed with distilled water, claret membrane vesicles containing cR3 as the major protein were isolated (Fig. S1). The absorption spectrum of purified claret membrane has three significant peaks at 475, 505, and 541 nm, which are attributable to the vibronic bands of bacterioruberin (Fig. 2A). The visible absorption band of retinal was recognized as a shoulder at the longer wavelength. A high content of bacterioruberin in claret membrane made it difficult to analyze the spectroscopic property of retinal in cR3. Meanwhile, a limited amount of bacterioruberin was incorporated into a trigonal crystal of cR3 (Fig. 2B). From the two absorption spectra of the light-adapted crystal that were measured with polarized light, it was shown that the absorption dipole moment of retinal is tilted largely from the c axis of the crystal, whereas the absorbance of bacterioruberin is significant only when the polarization plane of the measuring light is in parallel to the c axis. It was estimated that the retinal in cR3 has a broad absorption band with an absorption peak at ~560 nm.

Photoreaction of cR3
The difference spectrum associated with the light adaptation of cR3 exhibited a positive peak at 585 nm and a negative peak at 500 nm (Fig. 2A). Since this difference spectrum is similar to the
of the K state of bR. The P2 component, which is characterized by the trans isomer of bR (bR570) is described by the reaction scheme: K → M → N → O → bR570. It has been shown from absorption kinetics data of the purple membrane of H. salinarum that the photoreaction cycle of the trans isomer of bR [bR570] is described by the reaction scheme: K → L → M → N → O → bR570, where the L/M state represents a rapid, dynamic equilibrium between L (L2) and M (M1) [24,25]. It might be expected that the proton-pumping cycle of cR3 is described by the same reaction scheme. In fact, the formation/decay rates of the individual reaction states of cR3 were considerably different from those observed for bR. Fig. 3A shows the absorption changes observed when cR3 in a membrane suspension was excited with light pulses at 532 nm. In the investigated time region, the absorption kinetics of cR3 were fitted with four exponential components (Fig. S2). At pH 8, the difference spectrum associated with the L state of cR3 (Fig. S2). The L state, which occurs between the K state and the L/M state in the photocyte of bR, is not detected for kinetic reason. The L/M state becomes undetectable at high pH, where its decay rate is likely to be faster than the decay rate of the K state.

Crystal packing

Figure 4 shows the protein packing in the P321 crystal of cR3. The side view of the unit cell shows that the P321 crystal is composed of membranous layers. In each membranous layer, cR3 trimers are arranged on a honeycomb lattice such that neighboring trimers have opposite orientations. Along the c-axis, the membranous layers are piled up straightly. This crystal packing is similar to that seen in the P321 crystal of aR2 [10]. But the cell dimension of the cR3 crystal (a = 106.2 Å and c = 55.2 Å) is noticeably larger than that of the aR2 crystal (a = 98.2 Å and c = 56.2 Å). The problem of crystal twinning, which was reported to be serious in the crystallization of aR2, was overcome when the P321 crystal of cR3 was grown at pH 4.

The structural model of cR3 at the highest resolution was constructed using diffraction data from the crystal soaked at pH 5, though essentially the same crystal structure was observed at pH 4. At these low pH levels, Glu5 and Glu72 on the extracellular surface are close to Glu239 and Asp166, respectively, on the cytoplasmic surface of a neighboring subunit in a different membranous layer (Fig. S3). It would be expected that when these acidic residues are deprotonated at a higher pH, the electrostatic repulsive forces between them become strong enough to destroy the crystal structure. In fact, the cell dimension along the c axis increased significantly (by 5 Å) when the P321 crystal was soaked in a post-crystallization solution at or above pH 6 (Table 1). It was observed that pH-induced conformational change and/or disorder in the BC loop and the C-terminal polypeptide was accompanied by a remarkable expansion of the inter-membrane space (Fig. S3). Nonetheless, the honeycomb structure of the cR3 trimers was little affected by the pH increase. It should be mentioned that no high-quality crystal of cR3 grew at neutral pH. So, it is possible that the crystal structure observed after the crystal soaking at neutral pH represents a quasi-stable state.

The structure of the individual protein

The polypeptide chain of cR3 is folded into seven transmembrane helices (helix A through G), a β-sheet at the BC loop, and a short amphiphilic helix at the C-terminal region (Fig. 4). The retinal chromophore is bound to the ε-amino group of Lys220 in helix G and it adopts the all-trans configuration At pH 5, the β-sheet at the BC loop is tilted towards the DE loop, so that the main chain of the BC loop (at Ala70) is hydrogen-bonded to the main-chain of the DE loop (at Ala127). The C-terminal amphiphilic helix, which fills the open space between the AB and EF loops, is fixed by interactions with residues [Arg36, Arg38, Tyr41, Asp166, and Thr171] from helices B, E and F. Upon the crystal soaking at
pH 6, the BC loop and the C-terminal polypeptide underwent large structural alterations and became disordered, whereas the structure of the protein inside scarcely changed (Fig. 3S). In the investigated pH range (pH 4–7), two glutamates (Glu198 and Glu208) in the proton-release channel formed a paired structure (Fig. 5B). The close distance between these residues (2.4 Å) is explainable by a low-barrier hydrogen bond [27].

The long DE loop extends towards a neighboring subunit to strengthen the trimeric structure; i.e., two residues in the DE loop interact with the main chain of the N-terminal polypeptide (at Pro2) and helix B (at Leu60 and Gly61) of the neighboring subunit (Fig. 4E). Inter-subunit hydrogen bonds are also seen on the cytoplasmic side; i.e., two residues (Asn104 and Arg105) in helix D interact with residues (Ser35, Gln38 and Lys39) in helix A of the neighboring subunit. The trimeric assembly of cR3 is further strengthened by the carotenoid bacterioruberin, which binds to the crevice between neighboring subunits (Fig. 4F). One terminal end of bacterioruberin is fixed by residues from helices A and B of one subunit and helices D and E of a neighbor subunit, while the other terminal is excluded from the inter-subunit crevice. This binding mode is similar to that observed in the trimeric assembly of aR2 [10].

Compared with bR, peculiar features of cR3 are seen in the following regions. i) The side chain of Leu149 makes contact with the indole ring of Trp186, reducing the motional freedom of Trp186 (Fig. 6A). ii) Tyr81 OH is hydrogen-bonded to Thr124 OH, enlarging a cavity between Tyr81 and Glu198 (Fig. 5S). iii) The cytoplasmic end of helix E is greatly bent, creating a large cavity near the cytoplasmic surface between helices E and F (Fig. 6C). iv) Three positive charges (Arg172, Lys176, and Arg179) are distributed along helix F (Fig. 6C). v) Ser82 OH in helix C is hydrogen-bonded to Asn54 ND2 (Fig. 1). vi) The indole nitrogen of Trp192 in helix F is hydrogen-bonded to the main chain of helix G (at Glu208) (Fig. 1).

A particularly interesting property of cR3 is a long DE loop that interacts with a neighboring subunit (Fig. 4E). This peculiar structure would confer cR3 with a higher ability to form a trimeric assembly. To investigate this possibility, we measured the photo-stability of cR3 in the solubilized state. When an aqueous suspension of claret membrane was exposed to strong orange light (>570 nm) from a xenon lamp, no significant absorption change was induced (Fig. 7). This result indicates that as long as cR3 is embedded in the membrane, the retinal chromophore is protected from photobleaching. Meanwhile, the light-induced bleaching of retinal became significant upon solubilization of cR3 with an excess amount of detergent (nonylglucoside). It is important to point out that the rate of this photobleaching becomes higher with the increasing detergent concentration. This dependence can be explained by supposing that the photo-stability of cR3 decreases when the native protein-lipid interactions and/or...
the trimeric structure are destroyed by an excess amount of
detergent. In the case of bR, the photobleaching was already
significant at a low detergent concentration (13 mM). At this low
detergent concentration, where the photostability of cR3 was five
times higher than observed for bR, a considerable fraction of cR3
seems to maintain a trimeric assembly and/or native protein-lipid
interactions.

To investigate whether cR3 is able to maintain a trimeric
assembly in the presence of detergent, we performed blue-native
PAGE (Fig. S6). The result shows that the trimeric assembly of
cR3 is maintained at a low detergent concentration (13 mM
nonlygoside), whereas it is dissociated into monomers at much
greater detergent concentrations.

Discussion

The trimeric assemblies of microbial rhodopsins

The present result shows that cR3 forms a trimeric structure
with bacterioruberin bound to the crevice between neighboring
subunits. Besides cR3, five microbial rhodopsins (bR, aR2, dR3,
pHR, and halorhodopsin from H. salinarum) have been shown to
form trimeric assemblies under crystallization conditions [5–
7,10,11,15,16]. As long as we discuss their structures found in the
3D crystals grown by the membrane fusion method, there is no
difference in the architecture of the trimeric assembly (Fig. S7).
Especially the membrane-embedded region (i.e., the seven
transmembrane helices) is superimposed well. [Comparison of
the cR3 trimer with the aR2, bR, and dR3 trimers shows RMSD
of 0.93 Å (over 618 residues), 0.98 Å (over 591 residues), and
0.56 Å (over 558 residues), respectively]. These trimeric assemblies
have a common architecture: 1) helices B, C and D are aligned in
parallel to the 3-fold rotation axis, whereas helices A, E, F and G
are largely tilted from this axis; 2) the extracellular half of helix E
makes contact with helix B of a neighboring subunit, whereas these
two helices are separated on the cytoplasmic side; 3) the crevice
created between neighboring subunits is filled by some lipid
component. It is possible that because a flexible lipid exists in the
inter-subunit crevice, the cytoplasmic half of the protein can
undergo a large structural change during the proton-pumping
cycle.

In the trimeric assemblies of aR2, cR3, dR3, and pHR,
bacterioruberin was observed to bind to the inter-subunit crevice
[10,11,16]. Nonetheless, the residues surrounding bacterioruberin
are not necessarily conserved among these proteins. It appears that
the binding of bacterioruberin to the inter-subunit crevice is not
very specific. A recent study has shown that although the trimeric
assembly of pHR is strengthened in the presence of bacterior-
uberin, pHR can form a trimeric assembly even in the absence of
bacterioruberin [28]. We can’t exclude the possibility that the cell
membrane of Haloarcula vallismortis contains a different type of
carotenoid than bacterioruberin, which binds strongly to the
trimeric assembly of cR3. It has been reported that xanthorho-
dopsin, a light-driven proton pump found in halophilic eubacteria,
utilizes salinixanthin as an antenna molecule for efficient capture
of solar energy [12,29]. It is interesting to ask whether the same
energetic role is possessed by the postulated carotenoid that is
bound to cR3 in Haloarcula vallismortis.
A recent study has shown that Gloeobacter rhodopsin, a eubacterial proton pump, forms a trimeric assembly in the presence of dodecylmaltoside [30]. It seems possible that most ion-pumping rhodopsins can form a trimeric assembly under the physiological conditions. Meanwhile, the proton-pumping activity of bR has been shown to be high even in the monomeric state [31]. An interesting question is: what merit is gained by formation of the trimeric assembly. It has been shown that the thermal stability of bR under illumination is much lower in the monomeric form than in the trimeric form [32]. One possible physiological role of the trimeric assembly is to prevent undesirable photoreactions leading to inactivation of the protein. It is noteworthy that cR3 possesses a long DE loop that interacts with a neighboring subunit (Fig. 4E). This peculiar structure would confer cR3 with a higher ability to from a trimeric assembly.

Higher order structure of cR3

Another interesting structural feature of cR3 is the three positive charges (Arg172, Lys176, and Arg179) distributed at the cytoplasmic end of helix F (Fig. 6C). It would be expected that this cluster of positive charges affects inter-trimer interactions; i.e., cR3 trimers may not form a 2D hexagonal lattice as observed in the purple membrane of Halobacterium salinarum. In fact, cR3-rich claret membranes are isolated as vesicles (not planar sheets) at a low ionic strength. The cryo-electron micrographs as shown in Figure S1 suggested that cR3 trimers are arranged on a polyhedral lattice with a diameter of 50–200 nm.

Previous studies have shown that under a special crystallization condition bR is able to form an icosahedral assembly with a diameter of 50 nm [33], and that successive fusion of such vesicular assemblies yields the P622 crystal of bR [22]. It seems possible that the local lattice structure in the polyhedral assembly of cR3 is similar to the honeycomb lattice seen in the P622 crystal of bR. In this case, it would be expected that cR3 trimer is arranged in such an orientation that helix F faces to a large opening space in the honeycomb lattice.

Table 1. Data collection and final refinement statistics.

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Refinement

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Rmsd

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1 R cryst = |Σhkl||Fl| - |Σhkl||Fhkl| |
2 Rfree = |Σhkl||Fl| - |Σhkl||Fhkl| |

doi:10.1371/journal.pone.0108362.t001

Structural conservation of the proton-release pathway

The structure of the proton-release channel is well conserved among archaeal proton pumps. The conformations of ionizable residues (Arg86 cR3, Asp89 cR3, Glu198 cR3, Glu208 cR3 and Lys220 cR3) in cR3 are nearly identical to those seen in bR, aR2, and dR3 (Fig. S5). A noticeable difference is seen in the orientation of the tyrosine in helix C (Tyr83 bR, Tyr81 cR3); i.e., Tyr81 cR3 OH is hydrogen-bonded to the OH group of Thr124 cR3, whereas Tyr83 bR OH is hydrogen-bonded to the indole nitrogen atom of Trp193 bR. This difference is accompanied by a slight alteration in the water distribution; i.e., two water molecules are inserted between Tyr81 cR3 and Glu198 cR3, while only one water molecule exists between Tyr83 bR and Glu194 bR.

Figure S1 suggested that cR3 trimers are arranged on a polyhedral lattice with a diameter of 50–200 nm. Previous studies have shown that under a special crystallization condition bR is able to form an icosahedral assembly with a diameter of 50 nm [33], and that successive fusion of such vesicular assemblies yields the P622 crystal of bR [22]. It seems possible that the local lattice structure in the polyhedral assembly of cR3 is similar to the honeycomb lattice seen in the P622 crystal of bR. In this case, it would be expected that cR3 trimer is arranged in such an orientation that helix F faces to a large opening space in the honeycomb lattice.
Irrespective of these minor differences, the paired structure of two glutamates (Glu198 and Glu208 in cR3) in the proton-release channel is well conserved among aR2, bR, cR3, and dR3. It has been reported that although this paired structure is formed in the neutral purple form of bR, it is broken in an alkaline pink form (at pH = 10) or an acidic blue form (at pH = 3.5) [34]. In some 3D crystals (e.g., the C2 crystal [6]) of bR, Glu194 was reported to adopt a similar conformation as observed in the alkaline purple form. It is possible that the pKa value of the alkaline transition is dependent on the protein-lipid interactions. In the P321 crystal of cR3, the neutral purple form with the paired structure of Glu198cR3 and Glu208cR3 was stable in the investigated pH range (pH 4–7).

It has been reported that in the bR-vesicles reconstituted with egg lecithin, the neutral purple form of bR is stable in a narrow pH range; i.e., an acidic blue form with λmax at 600 nm is generated below pH 4, while an alkaline red form with λmax at 400 nm appears above pH 6.5 [35]. In the envelope vesicles of Halobacterium salinarum, on the other hand, the neutral purple form of bR exists stably in a wide pH range (pH 3–pH 9). These observations suggest that the pKa value of the purple-to-red transition is dependent on the lipid-protein interactions. This dependence should be kept in mind in the discussion of the proton-pumping activities of microbial rhodopsins expressed in neuron membranes.

Structural factors affecting the photoreaction kinetics of archaeal proton pumps

When the proton-pumping cycle of cR3 is compared with that of bR, the most significant difference is seen in the decay rate of the K state. It is noteworthy that the kinetics of the early reaction step of the photocycle of cR3 is rather similar to that of aR2; namely, the K state decays so slowly that the L state is undetectable for kinetics reasons (Fig S2). It would be expected that the decay rate of the K state correlates with the structure around the retinal chromophore. Although most residues in the retinal-binding pocket are conserved among microbial rhodopsins, Met145 in bR is replaced by leucine (Leu149) in cR3 or by phenylalanine (Phe150) in aR2. This replacement is accompanied by a noticeable shift in the position of the tryptophan residue (Trp187bR, Trp186cR3, Trp182aR2) that makes contact with the C15 methyl of retinal (Fig. 6A). Owing to the side chain of the K state.
Leu149\textsuperscript{cR3} (or Phe150\textsuperscript{aR2}), the indole ring of this tryptophan is pushed towards helix G. This implies that the motional freedom of this indole is more restricted in cR3 or aR2 than in bR. Previous crystallographic studies of the K and L states of bR have shown that the K-to-L transition is accompanied by a horizontal movement of Trp182\textsuperscript{bR} towards Met145\textsuperscript{bR} and a rotation of the side chain of Leu93\textsuperscript{bR} [36,37]. It is possible that the conformational relaxation of retinal from a twisted 13-cis configuration to a planar 13-cis configuration, which takes place in the K-to-L transition, is inhibited when the structure around the C13 methyl group is made rigid by the influence of the side chain of Leu149\textsuperscript{cR3} or Phe150\textsuperscript{aR2} (Fig. 6B). In cR3, the relaxation of retinal from a twisted 13-cis configuration to a planar 13-cis configuration, which takes place in the K-to-L transition, is inhibited when the structure around the C13 methyl group is made rigid by the influence of the side chain of Leu149\textsuperscript{cR3} or Phe150\textsuperscript{aR2}.

Another significant difference in the photoreaction kinetics between cR3 and bR is seen in the M-to-N transition; namely, this transition occurs much faster in cR3 than in bR. This difference is attributable to a structural difference in the proton-pumping pathway. The cytoplasmic end of helix F is greatly bent so that the cavity is created between helices E and F (Fig. 6C). This cavity is large enough to accommodate a water molecule. (The number/occupancy of water molecules in this cavity may depend on the temperature.) Previous structural analyses of bR have suggested that the cytoplasmic half of helix F is tilted outwards upon formation of the N state [39–42]. A similar structural change has been shown to take place during the proton-pumping cycle of \textit{H. salinarum} in complex with azide [43]. When its long-living N/M state is generated at high pH, the cytoplasmic half of helix F is largely deformed and a linear water cluster is formed between the retinal Schiff base and Lys215\textsuperscript{pHR}. It has been postulated that a similar linear water cluster is transiently generated during the proton-pumping cycle of any archaebial proton pump [17]. Because the unphotolyzed state of cR3 already contains a water molecule in the vicinity of Arg179 (the counterpart of Lys215\textsuperscript{pHR}), it would be expected that the formation of a linear water cluster in the cytoplasmic inter-helical space (i.e., the formation of the N state) takes place more rapidly in cR3 than in bR. It should be pointed out that in the \textit{P321} crystal of cR3, the M state decays very slowly (t~100 ms at 24°C). This elongated lifetime of M is explained by supposing that the opening of the cytoplasmic half is inhibited by the protein-protein interactions, as previously reported for the M-to-N transition of bR [44].

**Materials and Methods**

**Expression and purification of cR3**

Cruxrhodopsin-3 was expressed in a bR-deficient strain of \textit{Halobacterium salinarum} (MPK409) according to a method used for preparation of dR3 [11]. Briefly, a \textit{bop} gene fragment in the vector pMPK85 [45] was cloned into the vector pUC18 and, after introducing a \textit{NdeI} site at the start codon of the \textit{bop} gene, the 700 bp \textit{NdeI}-\textit{NotI} fragment was substituted with the synthetic adaptor 5’-CATATGTCGGAGAGATCTGAGCCGGCGCCGC-3’. The \textit{BamHI} fragment in the modified vector was cloned into the \textit{BamHI} site of pMPK85, producing a vector pKI72. Using \textit{Haloarcula vallismortis} genome as a template, the cruxropsin-3 (\textit{cop3}) gene fragment was amplified using the polymerase chain reaction with the two primers CATATGGCTAGGGAGAGATCTGAGCCGGCGCCGC. The \textit{acrucruxrhodopsin} vector pKI72 was cloned into the \textit{BamHI} site of pMPK85, producing a vector pK172. Using \textit{Halorubrum sp. Atl} as a template, the \textit{cop3} gene fragment was amplified using the polymerase chain reaction with the two primers CATATGGCTAGGGAGAGATCTGAGCCGGCGCCGC. The \textit{acrucruxrhodopsin} vector pKI72 was cloned into the \textit{BamHI} site of pMPK85, producing a vector pK172.

**Preparation of bR and aR2**

Purple membrane containing bR was isolated from \textit{Halobacterium salinarum} as described previously [48]. Clayton membrane containing aR2 was isolated from \textit{Halorubrum sp. Atl} and purified according to a procedure as previously described [11].

**Blue native polyacrylamide gel electrophoresis**

For estimation of the molecular weight of the lipid-protein complex in the presence of detergent, we performed blue native polyacrylamide gel electrophoresis according to the reported procedure [49] with slight modifications. In this study, a low concentration of the detergent (i.e., 6 mM nonylglucoside) was added to an acrylamide separating gel.
controlled experimental setup with a digital oscilloscope and a frequency-doubled Nd:YAG laser [50]. The absorption kinetics measured at various wavelengths were analyzed using the singular value decomposition method [51].

The absorption spectrum of cR3 crystal was measured by a micro-spectrophotometer as described previously [22]. Briefly, the measuring light from Shimadzu double monochromator was passed through a pin hole with a diameter of 0.03 mm and a polarizer and focused to a single crystal of cR3 adhered to the lower glass of the crystallization kit.

Electron microscopy
Cryogenic electron microscopy was performed as described previously [53]. Briefly, an aqueous suspension of cR3-rich claret membrane was mounted on a carbon-coated grid and, after removal of excess water, the sample was flash-cooled with liquid propane at its melting temperature. Cryo-electron micrographs were recorded with a CCD camera (Gatan SC200D) installed in a JEM2010 (Jeol) electron microscope.

Crystallization of cR3
A high-quality crystal of cR3 was grown at pH 4 by the membrane fusion method [22]. A mixture solution containing claret membrane (3 mg/ml), 5 mg/ml nonylglucoside, 1 M ammonium sulfate, 0.08 M sodium chloride, 0.04 M sodium azide and 0.04 M sodium citrate (pH 4) was slowly concentrated by the sitting-drop vapor diffusion method, using 0.5 ml of 2.2–2.8 M ammonium sulfate and ~0.1 M sodium citrate (pH 4) as a reservoir solution. Incubation at 15°C for ~1 month yielded trigonal crystals with a typical size of 50 × 50 × 50 μm3. For X-ray diffraction measurements, a single crystal was picked up and soaked in a post-crystallization solution containing 2.2 M ammonium sulfate, 0.1 M pH buffer (HEPES or citrate) and 30% trehalose for ~10 minutes; subsequently the crystal was flash-cooled in dim light with liquid propane at its melting temperature.

Data collection, scaling and refinement
X-ray diffraction data were collected on beamline BL38B1 at SPring-8, where a crystal kept at 100 K was exposed to a monochromatic X-ray beam at a wavelength of 1.0 Å with an X-ray flux rate of ~2 × 10¹³ photons/mm²/sec. Diffraction data were collected with an oscillation range of 1° and an X-ray flux of ~1 × 10¹³ photons/mm² per image. Indexing and integration of diffraction spots were carried out using MOSFILM 6.1 [54]. The scaling of data was accomplished using SCALA in the CCP4 program suite [55]. Structural analysis was performed with CNS [56] and XIA2View [57]. Firstly the structure of bR (pdb entry: 1HW6) [58] was modified using Swiss-Model [59], by which non-conserved residues between bR and cR3 were automatically conserved.

Supporting Information
Figure S1 Cryo-electron micrograph of cR3-rich claret membrane.
(PDF)
Figure S2 Flash-induced absorption changes in cR3, bR, and aR2.
(PDF)
Figure S3 pH dependence of the structure of cR3 in the cytoplasmic and extracellular surface regions
(PDF)
Figure S4 Trimeric structure of cR3 in complex with bacterioruberin.
(PDF)
Figure S5 The proton-release pathway in proton-pumping archaeal rhodopsins.
(PDF)
Figure S6 Blue-native polyacrylamide gel electrophoresis of cR3.
(PDF)
Figure S7 The trimeric assemblies of ion-pumping archaeal rhodopsins.
(PDF)

Acknowledgments
We thank the staff of BL38B1 of SPring-8 (Harima, Japan) for technical assistance during data collection.

Database
The coordinates and structural factors of cR3 at pH 5 and 6 have been deposited in the Protein Data Bank with accession number 4L35 and 4JR8, respectively.

Author Contributions
Conceived and designed the experiments: TK SKC KI. Performed the experiments: SKC TKI RF KI TK. Analyzed the data: TK SKC. Contributed reagents/materials/analysis tools: TG MM. Wrote the paper: TK SKC.

References


