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Structural and functional roles of the N- and C-terminal extended modules in Channelrhodopsin-1

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Abstract:

Channelrhodopsins have become a focus of interest because of its ability to control neural activity by light, used in a technology called optogenetics. The channelrhodopsin in the eukaryote *Chlamydomonas reinhardtii* (CrChR-1) is a light-gated cation channel responsible for motility changes upon photo-illumination and a member of the membrane-embedded retinal protein family. Recent crystal structure analysis revealed that CrChR-1 has unique extended modules both at its N and C-terminuscompared to other microbial retinal proteins. This study reports the first successful expression of a ChR-1 variant in *Escherichia coli* as a holoprotein: the ChR-1 variant lacking both the N and C-termini (CrChR-1_82-308). However, compared to ChR-1 having the extended modules (CrChR-1_1-357), truncation of the termini greatly altered the absorption maximum and photochemical properties, including the pK_a values of its charged residues around the chromophore, the reaction rates in the photocycle and the photo-induced ion channeling activity. The results of some experiments regarding ion transport activity suggest that CrChR-1_82-308 has a proton channeling activity even in the dark. On the basis of these results, we discuss the structural and functional roles of the N and C-terminal extended modules in CrChR-1.

1. Introduction

Many organisms utilize light as an energy source and/or as an environmental signal, which provides these biological systems with critical information. This is done by a variety of photoreceptor proteins which carry out a wide range of dissimilar biological functions such as photosynthesis, signal transduction and ion transportation. One of the largest groups of photoreceptors are the retinal proteins which possess vitamin-A aldehyde retinal as their chromophore which is bound via a protonated Schiff base linkage with a specific lysine residue within their 7 transmembrane alpha-helices.[1] Genomic analysis has revealed that these retinal proteins are widespread in many organisms, including prokaryotes (i.e., bacteria and archaea) (type-1) and eukaryotes (i.e., vertebrates and invertebrates) (type-2).[1-3] The first type-1 protein, named bacteriorhodopsin (BR), a proton pump, was identified from the archaeon *Halobacterium salinarum* by Oesterhelt and Stoeckenius in 1971.[4] In the 44 years since then, BR has been extensively studied by a number of methods, including spectroscopy, mutation analysis, computation and crystallography, a significant effort by many researchers. As a result, the proton transport mechanism that occurs in BR during its action has been elucidated,[3] and BR has become a model for the simplest and most essential features required in an active ion transporter. The first type-1 retinal protein discovered outside the archaea was a protein encoded by the *nop-1* gene of the fungus *Neurospora crassa* identified in 1999 and was named *Neurospora* rhodopsin[5] (NR). Although its biological function is still unclear, the structural and functional properties of NR could be characterized due to its successful expression in the methylotrophic yeast *Pichia pastoris* as a recombinant protein.[6] Furthermore, in 2000 the first eubacterial type-1 retinal protein, proteorhodopsin (PR), was discovered from the gamma-proteobacterium.[7] Using recombinant proteins expressed in the eubacterium *Escherichia coli*, it could be elucidated that PR is a light-driven proton pump.[7] The striking wide distribution of retinal proteins in nature suggests their biological significance for those organisms.

In 2002, two independent research groups discovered two novel type-1 retinal proteins that account for the phototactic behavior of the eukaryote *Chlamydomonas reinhardtii*.[8, 9] Both proteins, named channelrhodopsin-1 (CrChR-1) and channelrhodopsin-2 (CrChR-2), function as direct light-gated cation-selective ion channels, but differ in their saturating light intensities.[10] Although they can conduct Na^+,

K^+ and Ca^{2+} at a similar rate, the achieved conductance for those ions is much smaller than that for protons.[10] Thus, in nature, they are believed to function as proton channels to quickly depolarize the plasma membrane. In addition to their biological significance, ChRs have also become a focus of interest because of their ability to induce neural activation through light-dependent de-polarization,[11] in contrast to ion pumps, such as archearhodopsin-3 (AR3) and the chloride pump halorhodopsin (HR), which can induce a hyperpolarization by light used for neural silencing.[12, 13] By making use of these properties, type-1 ion transporting retinal proteins could brake new ground in a scientific field called "Optogenetics".[14]

In 2012, Kato, Nureki and coworkers determined the crystal structure at 2.3 Å resolution of a chimeric protein between CrChR-1 and CrChR-2 (named C1C2) using the lipidic cubic phase (LCP) method.[15] The structure clearly revealed information about the molecular architecture and ion transport pathway.[15] Interestingly, in contrast to other type-1 retinal proteins, such as BR and HR, the C1C2 structure showed unique extended modules both at the N- and C-termini (Figure 1a). Notably, some highly conserved cysteine residues in the N module form 3 disulphide bonds between protomers, which could stabilize their dimerization,[15] but are not strictly required for it.[16, 17] These extended modules might be the reason that researchers, to date, had to use eukaryotic cells, such as human embryonic kidney (HEK) 293, the yeast *Pichia pastoris* or Sf9 insect cells, for their characterization.[15, 18-20]

To further advance the characterization of this system, a recombinant protein expression system in *E. coli* would be indispensable, which would dramatically improve the expression yield and speed, and easy of genetic modification. Here, we prepared a variety of truncated variants of ChR-1 from *Chlamydomonas reinhardtii* (CrChR-1) (Figure 1b), which were then expressed those in *E. coli* and characterized. Using the information obtained the roles of the unique N- and C-terminal extended modules on the structure and function of ChR-1 are discussed.

2. Experimental

2.1. Gene preparation and protein expression. The genes encoding the CrChR-1 variants were amplified using PCR from the cDNA of *Chlamydomonas reinhardtii*. The primers were designed to have the restriction sites for *Nde*I at the 5' end and for *Xho*I at the 3' end where the stop codon was deleted during the amplification. The fragment was

digested by *Nde*I and *Xho*I enzymes, and was ligated to the *Nde*I and *XhoI* sites of the pET21c(+) vector (Novagen, Madison, WI). Consequently, the plasmid encodes truncated CrChR-1s with 6 histidines at the C-terminus as described previously.[21] The mutant genes of CrChR-1_82-308 were constructed by the QuikChange site-directed mutagenesis method as described previously.[22] All constructed plasmids were analyzed using an automated sequencer (ABI3100) to confirm the expected nucleotide sequences.

For protein expression, *E. coli* cells were grown in LB medium supplemented with ampicillin (final concentration of 50 μg/ml). Cells harboring the plasmid were grown in a shaking incubator at 37°C, followed by the addition of 1 mM IPTG and 10 μM all-*trans* retinal as described previously.[23] Cells were harvested at 5~10 hr post-induction by centrifugation at 4°C, resuspended in a buffer containing 10 mM citrate (pH 5.0) and 1 M NaCl, and then disrupted by sonication. Cell debris was removed by low speed centrifugation (5,000 × g, 10 min) at 4˚C. Crude membranes were collected by ultracentrifugation (100,000 × g,120 min) at 4˚C and were washed three times with a buffer containing 10 mM citrate (pH 5.0) and 1 M NaCl. *E. coli* DH5α cells were used as a host for DNA manipulation, and BL21(DE3) cells were used to express the genes.

2.2. Detection of protein expression. The crude membranes expressing CrChR-1 variants were dissolved in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer containing 5% 2-mercaptoethanol, and were subjected to 10%-acrylamide SDS-PAGE. Immunoblotting analysis was then performed using an anti-His-tag antibody (GE Healthcare, Uppsala, Sweden) by the standard method. To obtain the absorption spectra of the samples, the crude membranes were incubated under light (>420 nm for 10 min) in a buffer containing 10 mM citrate (pH 5.0) and 1 M NaCl with 50 mM hydroxylamine, a water-soluble reagent. The temperature was kept at 25˚C during the measurements.

2.3. Investigation of photochemical properties. pH-titration experiments were performed as described previously.[24] Briefly, *E. coli* cell membranes were resuspended in a buffer containing 50 mM Tris-HCl (pH 8.0) and 1 M NaCl using ultracentrifugation (100,000 × g, 120 min) at 4˚C. The pH values of the sample suspensions were adjusted by adding conentrated HCl, and were controlled using a SevenEasy pH-meter with a

9811 glass electrode (Mettler Toledo, Tokyo, Japan). Absorption spectra were measured in the dark using an UV2450 spectrophotometer with an ISR2200 integrating sphere (Shimazu, Kyoto, Japan). The apparatus and the procedure for the time-resolved strobe flash spectroscopy were essentially as described previously.[25] The temperature was kept at 25˚C during the measurements.

To measure light-induced pH changes, *E. coli* BL21(DE3) cells were suspended and washed 3 times in a solution containing 150 mM NaCl in order to remove the LB medium. After that, the cells were resuspended in the same solution, and kept on ice for 1 hr, after which they were used for the measurements. The samples were kept in the dark, and then were illuminated using a Xe lamp (Asahi Spectra, Co., Ltd., Tokyo, Japan) through a blue band-path filter (450 ± 10 nm) for 3 min. For comparison a similar experiment was performed using cells expressing *Gloeobacter* rhodopsin (GR), which were illuminated through a green band-path filter (550 ± 10 nm) for 3 min. The light-induced pH changes were monitored with a HORIBA F-72 pH meter in the presence or absence of 10 μM carbonyl cyanide m-chlorophenylhydrazone (CCCP, Sigma-Aldrich, St. Louis, MO). The temperature was kept at 25˚C during the measurements.

2.4. Effects of the expression of CrChR-1_82-308 on the cell growth and ion transport activity in the dark. *E. coli* cells which had either a vector or a plasmid encoding CrChR-227 were grown in LB medium containing 300 μM IPTG and 10 μM all-*trans* retinal with ampicillin (final concentration of 50 μg/ml). The cell growth was monitored by recording its optical density at 660 nm. For pH-jump experiments, *E. coli* BL21(DE3) cells were suspended in a solution containing 150 mM NaCl, kept on ice for 1 hr, and then a small amount of HCl was added to change the pH of the solution. The initial pH was ~ 6.4. The recovery of the pH was analyzed by a single exponential equation to determine the recovery rate constant. The holoprotein expression level of the mutants was investigated by visible absorption spectroscopy of the cell membrane, and it was almost the same as CrChR-1_82-308.

3. Results & Discussion

3.1. Expression of truncated CrChR-1s in *E. coli*. For some type-1 retinal proteins, such as sensory rhodopsin I (SRI) from *Halobacterium salinarum* or *Anabaena* sensory rhodopsin (ASR) from the cyanobacterium PCC 7120, protein expression levels in *H. salinarum* cells for SRI and *E. coli* cells for ASR, respectively, were greatly elevated by the truncation of the C-terminus.[26, 27] Previously, Hegemann and coworkers have tried to express both the full-length CrChR-1 and the truncated protein (CrChR-1_345) in *E. coli* and in *P. pastoris*,[20] however, in both cases, they were able to detect only the apoprotein, without the retinal chromophore.[20] On the basis of this background, we prepared 4 plasmids encoding further truncated CrChR-1s, which lack the N- and/or C-terminal modules, as shown in Figures 1a and 1b. The upper panel of Figure 1c shows SDS-PAGE patterns of the membranes by western blot analysis of the proteins using an anti-His-tag antibody. The expected molecular masses for CrChR1_327, CrChR-1_1-308, CrChR-1_82-327 and CrChR-1_82-308 are 37.9, 35.6, 28.4 and 26.3 kDa, respectively, which correspond well to the apparent sizes of the bands. These results imply that all truncated versions of CrChR-1s constructed in this study were successfully expressed in *E. coli* cells as apoproteins.

The lower panel of Figure 1c shows the colors of *E. coli* cells harboring the expression plasmids. The colors of the 3 truncated variants, CrChR-1_1-327, CrChR-1_1-308 and CrChR-1_82-327, were almost comparable to that of the negative control, "Vec", shown on the left, suggesting no (or slight) expression of the holoprotein. In contrast, the sample, shown on the right, containing the shortest truncated variant CrChR-1_82-308, had a bright orange color clearly indicating the expression of the holoprotein with the chromophore retinal. To estimate the absorption maximum (Figure 1d), the cell membranes were collected, resuspended in a buffer (pH 5.0), and reacted with hydroxylamine under illumination (> 420 nm), a chemical which is known to attack the protonated Schiff base in retinal proteins, and bleach the pigment, leading to formation of the retinal oxime.[28] The peak at 364 nm in the difference absorption spectrum represents the absorption of the formed retinal oxime, while the negative peak at 453 nm corresponded to the absorption of CrChR-1_82-308 having the protonated Schiff base. Using 33.6×10^3 M^{-1} cm^{-1} as the extinction coefficient of the retinal oxime,[29] the extinction coefficient of CrChR-1_82-308 could be estimated to be 31.1×10^3 M^{-1} cm^{-1}. These results clearly indicate that despite the removal of the N- and C-terminal extended modules of ChR-1 the holoprotein could be formed. However, the

absorption maximum of CrChR-1_82-308 was largely blue-shifted in comparison with CrChR-1_1-357 expressed in green monkey COS-1 cells (Table 1),[20] suggesting a structural alteration around the retinal chromophore due to the removal of the N- and C-terminal extended modules, or non-specific Schiff base formation of the retinal via any of the 9 remaining Lys residues, not restricted to Lys-296. Nevertheless, the visible color of the protein, which is an indication that we succeeded for the first time in expressing ChR in *E. coli* cells as a colorful recombinant protein, motivated us to perform further characterization.

3.2. Photochemical properties of CrChR-1_82-308. We then investigated the photochemical properties of CrChR-1_82-308. It has been reported that CrChR-1_1-357 expressed in COS-1 cells can be solubilized with n-dodecyl-β-D-maltoside (DDM), which is beneficial to reduce the baseline shift in the absorption spectra, and purified by immunoaffinity adsorption using an antibody.[20] However, CrChR-1_82-308 rapidly became yellow in the DDM solution due to the denaturation of the holoprotein, and, therefore, cell membranes expressing CrChR-1_82-308 had to be used for its photochemical characterization. We performed pH-titration experiments to estimate the pK_a value(s) of the charged residue(s) in CrChR-1, including the protonated Schiff base (Lys-296) and its putative counter ion (Glu-136). For this, the samples were initially suspended in a buffer containing 50 mM Tris-HCl (pH 8.0) and 1 M NaCl as described above. The pH was then adjusted to the desired value (pH 8.0 – 3.0) by the addition of HCl, followed by the measurement of the absorption spectrum in the dark (Figure 2a). The difference spectra clearly show a decrease in the absorption at ~360 nm, generally associated with holoproteins having a deprotonated Schiff base, with a concomitant increase in the absorption at ~440 nm (Figure 2b), around an isosbestic point at ~400 nm, which is likely to correspond to the protonation of the Schiff base with decreasing pH. The initial spectrum could be recovered, through the addition of NaOH to the sample returning the pH value to its initial value of about 8, suggesting the presence of a pH-dependent equilibrium between the forms having a deprotonated or a protonated Schiff base (Lys-296). The pK_a value was estimated to be ~6.8 by fitting analysis using the Henderson-Hasselbalch equation with a single pK_a, as shown by the solid line in Figure 2c, which is much smaller than that of CrChR-1_1-357 (> 8.0). It should also be noted that a further spectral red shift, corresponding to the change in the Schiff base

counter ion (Glu136) could not be observed for CrChR-1_82-308, suggesting that its pK_a value is lower than 3, which is also much smaller than that reported for CrChR-1_1-357 (~ 6.5). The strong reduction in pKa values (Table 1), and the blue shifted absorption maximum could indicate misfolding of CrChR-1_82-308 as reported for bacteriorhodopsin and sensory rhodopsin expressed in *E. coli*,[30] but could also be explained by structural change around the retinal chromophore in CrChR-1_82-308 compared to CrChR-1_1-357, induced by the removal of the N- and C-terminal modules. This might also explain the ability to express CrChR-1_82-308 as a holoprotein in *E. coli* cell membranes showing an orange color. If the prediction is correct, the pKa values of the charged residues are also much smaller than those of other type-1 retinal proteins, such as BR,[31] sensory rhodopsin I,[32, 33] sensory rhodopsin II[34, 35] and thermophilic rhodopsin,[36] suggesting a characteristic structure for CrChR-1_82-308 around the chromophore.

To estimate the photoreactions of CrChR-1_82-308, flash-photolysis experiments were performed. Figure 3a shows the light minus dark difference spectra of CrChR-1_82-308 monitored at 380, 450 and 520 nm. No signal was observed for the control (Vec), while a decrease in absorption was observed for CrChR-1_82-308, indicating a photoreaction. The depletion was completed within 1 ms, and was maintained for a long time (> 25 s), suggesting a fast formation (< 1 ms) and slow decay (> 25 s) of an intermediate. Similar experiments were then performed at varying monitor wavelengths. Because the recovery rate is extremely small, we used fresh samples, and several traces were averaged to improve the signal-to-noise ratio. Figure 3b shows flash-induced difference spectra of CrChR-1_82-308 over a spectral range from 380 to 650 nm of the monitor light. As can be seen, only a negative peak at ~450 nm was observed for CrChR-1_82-308, which is close to its absorption maximum (as shown in Figure 1d), whereas no peak could be observed for the control (Vec). These results suggest that: i) the absorption maximum of the formed intermediate of CrChR-1_82-308 is very close to that of the original state because the signal intensity is very small, and ii) the molar extinction coefficient of the intermediate is smaller than that of the original state. To determine the recovery rate, the absorbance after illumination was plotted against time, as shown in Figure 3c. The data was well fitted with a single exponential equation, and the half-time was estimated to be 4.3 hr, which is much longer than those for other ChRs, including CrChR-2 and a ChR-1 homolog

from *Chlamydomonas augustae* (CaChR-1) (20~25 s).[37, 38] Although the reason for the molecular origin of the long-lived state is unclear, its existence suggests that the truncation of the N- and C-terminal modules significantly altered the structure and/or structural changes of ChR-1. The putative kinetics of the photocycle are illustrated in Figure 3d and summarized in Table 1. Thus, these results imply that the N- and/or C-terminal regions are needed for the maintenance of the pK_a values of the charged residues and for the proper photochemical reactions of CrChR-1.

3.3. Light-induced proton transport activity of Cr**ChR-1_82-308.** To test whether the CrChR-1 variants transport protons in a light-dependent manner, we measured the light-induced pH changes of suspensions of *E. coli* cells expressing CrChR-1s (227, 246, 308, and 327). The experimental conditions are illustrated in the upper panel of Figure 4. The initial pH of the *E. coli* suspension was kept at ca. 6.4, which is below the cytosolic pH of *E. coli* cells (>7.0), and thus, a light-induced inward flux of protons should be observed by a passive proton channeling activity of CrChR-1, resulting in an increase in the external pH (ΔpH), as was indeed observed for another light-gated proton channel, AR3-T.[22] It should be noted that, in this experimental condition, CrChR-1_82-308 has 2 forms, one with a protonated and one with a deprotonated Schiff base (ratio of approximately 70:30) as judged by the pH titration experiments (Figure 2c). However, the deprotonated form of CrChR-1_82-308 does not absorb light at 450 nm which was used here (Figure 2c), and thus, the pH change would mainly be due to the protonated form of CrChR-1_82-308. In contrast, for an outward proton pump, the pH of the solvent is expected to decrease upon light illumination due to the light-driven active proton transport. When the proton-selective ionophore, CCCP, is added to the suspension, no (or less) pH change should be observed both for channels and pumps because the pH gradient between the inside and the outside of the cell membrane would diminish before the illumination with light.

Figure 4 (lines a, b, c) show the light-induced pH changes of suspensions of *E. coli* cells expressing the apoproteins CrChR-1_1-327, CrChR-1_1-308 or CrChR-1_82-327, respectively. As expected, almost no pH change was observed even in the absence of CCCP, confirming that chromophore binding is needed for the ion channeling function of ChR-1. However, the same results were obtained for cells expressing the holoprotein CrChR-1_82-308 (Figure 4, line d). As a positive control for

the experimental conditions, the cyanobacterial proton pump GR was used, which, as expected, actively transported protons toward the outside of the cells, leading to a pH decrease of the suspension (Figure 4, line e), and the signal intensity was largely decreased in the presence of CCCP as has been reported elsewhere.[39] Thus, these results indicate that the N- and/or C-terminal region are needed for the proton channeling activity of ChR-1. It should also be noted that the absorption maximum and photochemical activity can be strongly influenced by the choice of lipids or other changes in the membrane environment. Thus, to confirm that the results are not induced by the environment, but by the structure and structural changes of CrChR-1_82-308 itself, the electrophysiological experiments should be repeated in oocytes or HEK cells in future.

3.4. Estimation of ion transport activity of CrChR-1_82-308 in the dark. To investigate the activity of CrChR-1_82-308 in the dark, the effect of its expression on the growth of *E-coli* cells was investigated (Figure 5a). In order to express CrChR-1_82-308 constitutively, IPTG and all-trans retinal was added to the medium at the beginning of the cell culture. In contrast to the control experiment (vec.), almost no increase in optical density was observed for the cells having a plasmid encoding CrChR-1_82-308, indicating a toxic effect of CrChR-1_82-308 by possibly having a constitutive proton transport activity in the dark, which would lead to the inability to generate the necessary electrochemical potential across the cell membrane (Figure 5b). To confirm this, pH-jump experiments were performed (Figure 5c). As can be seen, after the addition of acid (HCl) a rapid increase in pH was observed, only for cells expressing ChR-1_82-308 (Figures 5c and 5d). To date, several residues important for the channeling function, such as R159 and D292, have been identified for the chimeric channelrhodopsin (C1C2).[15] To analyze the effects of mutations at those positions on the recovery of the pH, two mutants, R159A and D292A, were prepared, where the cells expressing R159A and D292A showed an orange or red color, respectively, indicating the expression of the holoprotein like for the wild-type ChR-1_82-308 (Figure 5c). The rapid recovery in pH was abolished by one of the mutations in the putative ion transport pathway (D292A) (Figures 5c and 5d), supporting the prediction that ChR-1_82-308 has indeed constitutive proton transport activity in the dark.

4. Conclusion

In this study, we prepared truncated CrChR-1 variants and expressed them in *E. coli* cell membranes. The shortest variant lacking both the N- and C-terminal extended modules of CrChR, CrChR-1_82-308, was successfully expressed in *E. coli* as a holoprotein showing a bright orange color. Its expression allowed us to investigate the structural and functional roles of the N- and C-terminal modules. CrChR-1_82-308 showed comparably low pK_a values of its charged residues (Glu-136 and Lys-296), and an extremely slow photocycle. The results of some experiments regarding ion transport activity suggest that CrChR-1_82-308 has a proton channeling activity even in the dark, and thus, the N- and/or C-terminal extended modules of ChR-1 are necessary for the maintenance of the proper photochemical properties and the light-gated ion channeling function.

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Table 1. Photochemical properties of CrChR-1_82-308 and CrChR-1_1-357.

Opsin type	λ_{max}	pK_a values	Life-time of the intermediate	Light-gated Channeling function
CrChR-1_82-308	~453 nm	6.8 (Lys-296) < 3 (Glu-136)	~ 4.3 hr	No
CrChR-1_1-357[a]	505 nm[a]	> 8 (Lys-296)[a] 6.5 (Glu-136)[a]	~20 s[b] 25 s[c]	Yes[a]

[a]Data from ref[20]

[b]Value determined for CrChR-2[37]

[c]Value determined for ChR-1 from *Chlamydomonas augustae* (CaChR-1)[38]

FIGURE LEGENDS

Figure 1. Truncated variants of CrChR-1 and their expression in *E. coli*. (a) Crystal structure of a chimeric protein between ChR-1 and ChR-2 (C1C2) from the eukaryote *C. reinhardtii* (PDB code: 3UG9).[15] (b) Schematics of the CrChR-1 variants studied; the full-length CrChR-1 (full-length), the C-terminal truncated variants (327 and 308), an N-terminal truncated variant (246) and an N/C terminal truncated variant (227). Colors blue, orange and magenta correspond to the colored regions shown in panel a. (c) Expression of His-tagged CrChR-1 variants in *E. coli*. The apo- and holo-proteins were detected using an anti-His-tag antibody (upper panel) and the retinal binding judged from the orange color of the cells (lower panel). The left lane (Vec) shows a sample from cells harboring the vector plasmid, pET21c(+). (d) Difference absorption spectrum of CrChR1_227 before and after reaction with hydroxylamine. "Vector" indicates membrane samples from cells harboring the vector plasmid, pET21c(+).

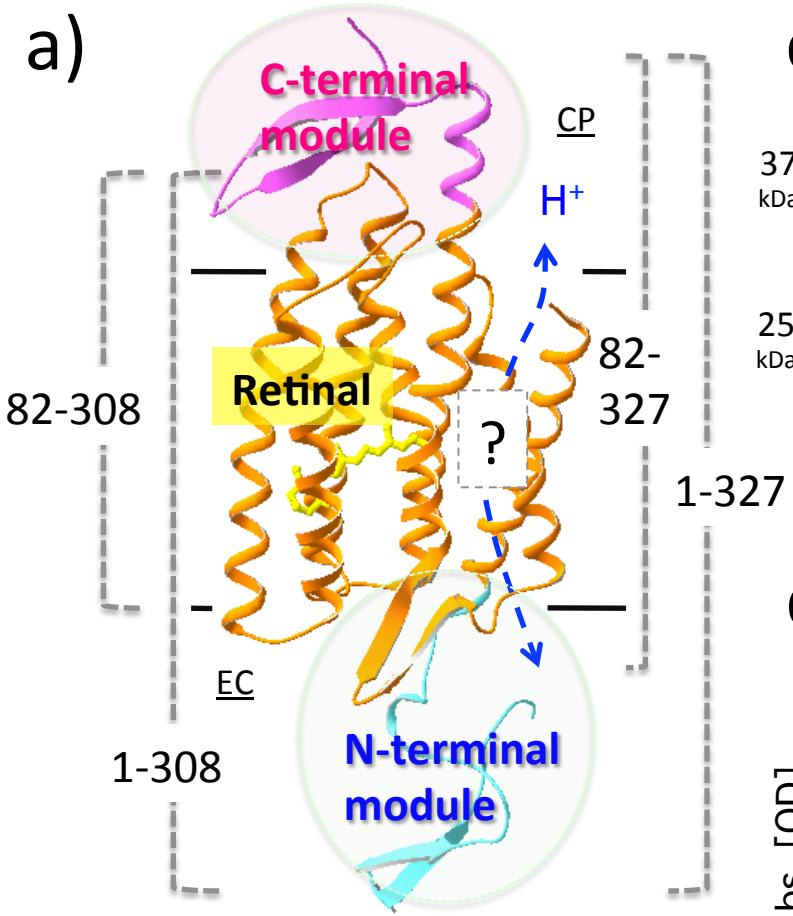
Figure 2. pH titration experiments for CrChR-1_82-308. (a) Absorption spectra of CrChR-1_82-308 at various pHs ranging from 3 to 8. (b) Difference absorption spectra obtained by subtracting the spectra from that at pH 8.0. (c) Estimation of the pK_a value; the titration curve was fitted by the Henderson-Hasselbalch equation with a single pK_a value of 6.8.

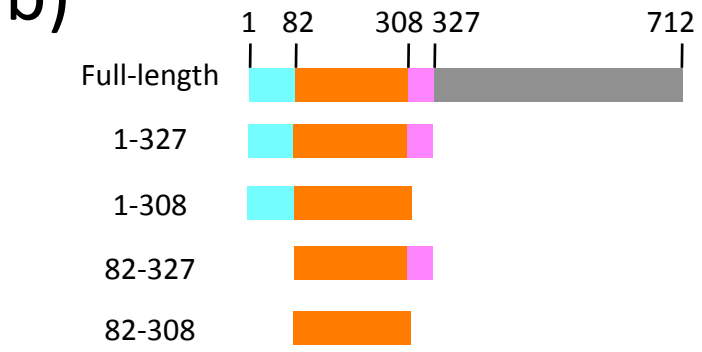
Figure 3. Flash-photolysis of CrChR-1_82-308. (a) Flash-induced absorbance changes monitored at 380, 450 and 520 nm. One division of the y-axis corresponds to 5 mOD. (b) Light minus dark difference spectra of *E. coli* cell membranes at varying wavelengths of light. (c) Recovery of the absorption monitored at 450 nm; error bars show standard deviations of 3 independent experiments. (d) Schematic illustration of the estimated photocycle kinetics for CrChR-1_82-308.

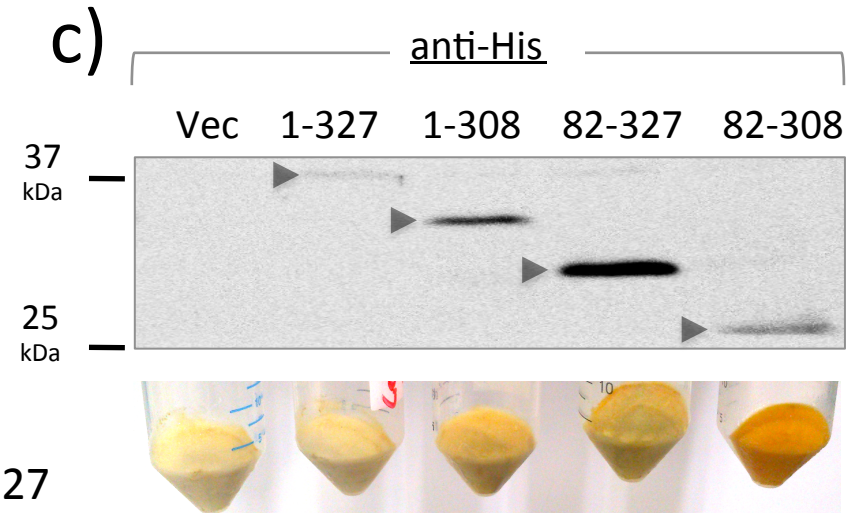
Figure 4. Light-induced pH change of *E. coli* suspension. The cells were suspended in 150 mM NaCl solution. The initial pH was ca. 6.4, where ~70% of ChR1_227 has a protonated Schiff base due to its higher pK_a value (6.8, see Figure 2). The cells expressing CrChR-1 variants were illuminated with blue light (450 ± 10 nm) for 3 min (a-d), while GR was illuminated with green light (550 ± 10 nm) for 3 min (e). Solid and dotted lines indicate the light-induced pH changes in the absence or presence of 10 μM CCCP, respectively. The data were expanded 5-fold (× 5) to observe small changes for CrChR-1_82-308 (d). The reproducibility of the data was confirmed in 3 independent experiments.

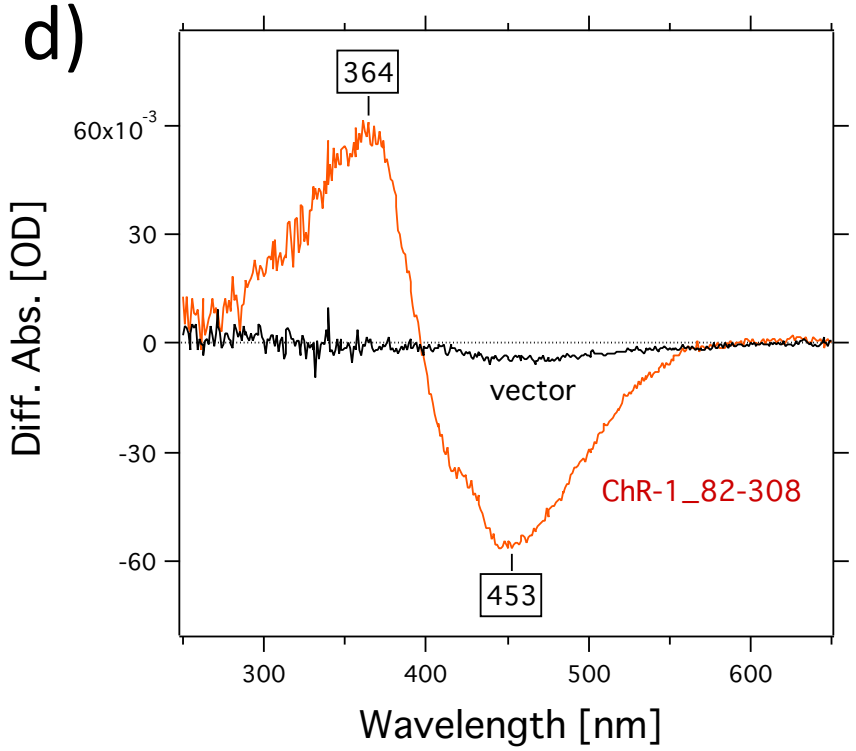
Figure 5. Estimation of ion transport activity of CrChR-1_82-308 in the dark. (a) The growth of cells having a vector plasmid (vec.) or a plasmid encoding CrChR-1_82-308 in LB medium containing 300 μM IPTG and 10 μM all-trans retinal in the dark. The cells were incubated in a shaker at 37˚C. (b) Schematics of our hypothesis. By addition of HCl into the medium, the proton should be transported into the cells if CrChR-1_82-308 works as a proton channel, resulting in partial disruption of membrane potential and increase in pH of the medium. (c) pH-jump experiments of *E. coli* cells expressing CrChR-1_82-308 and its mutants. The cells were suspended in 150 mM NaCl solution and the initial pH was ca. 6.4. Same amount of HCl was added in the

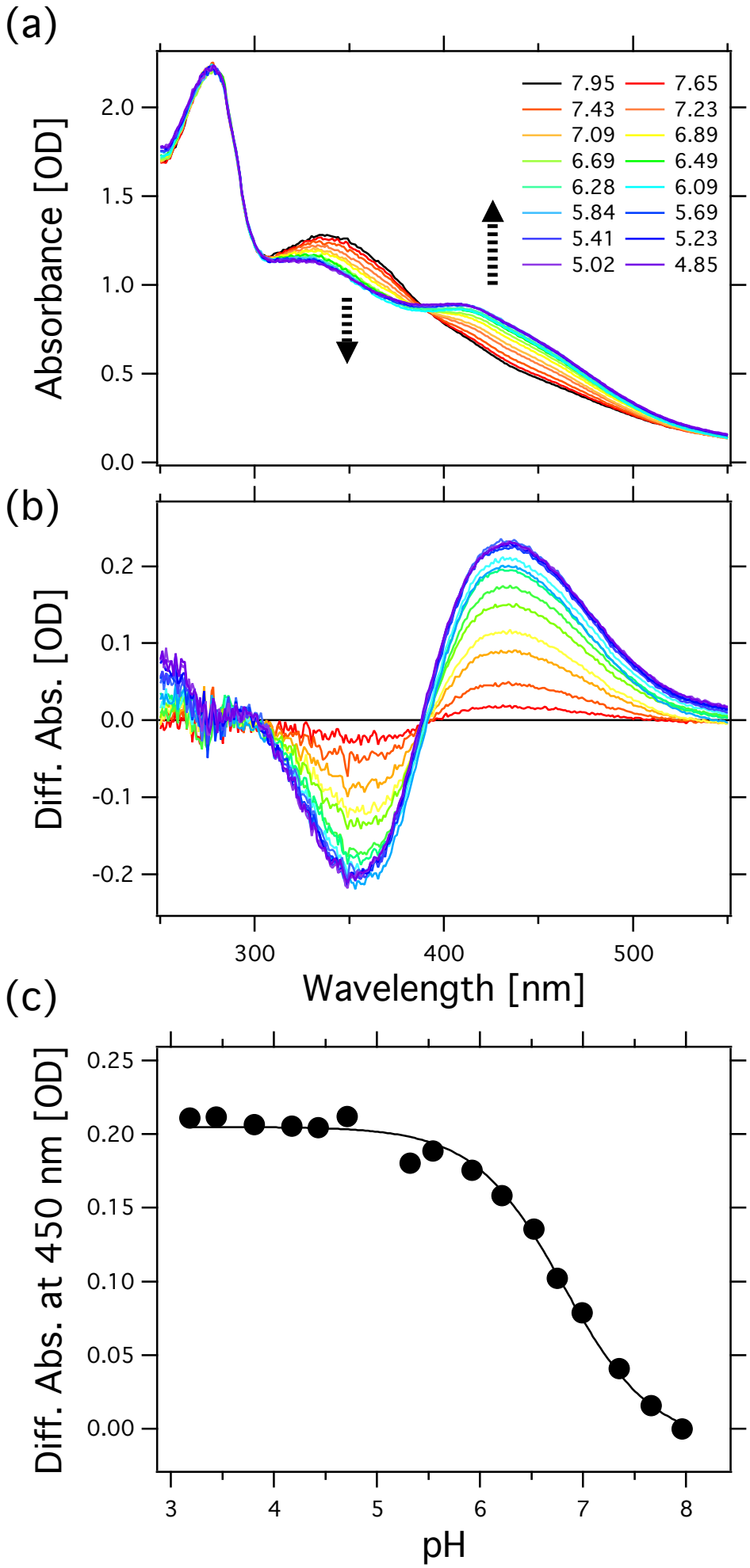
solution and the pH change was monitored for 5 minutes. (d) The recovery rate constants were quantitatively analyzed by a single exponential equation. The error bars indicate the standard deviation (SD) of the 3~7 independent experiments.

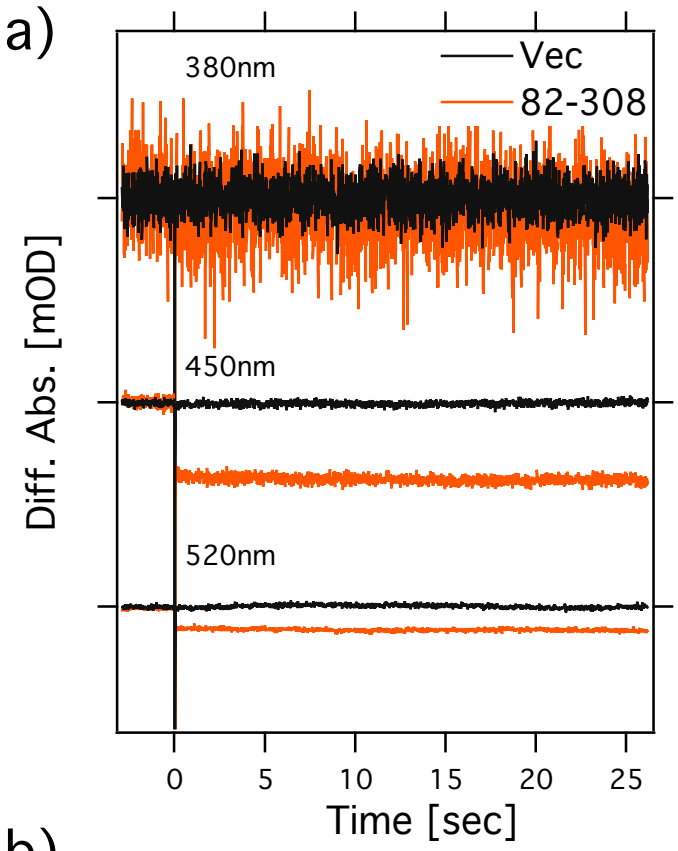


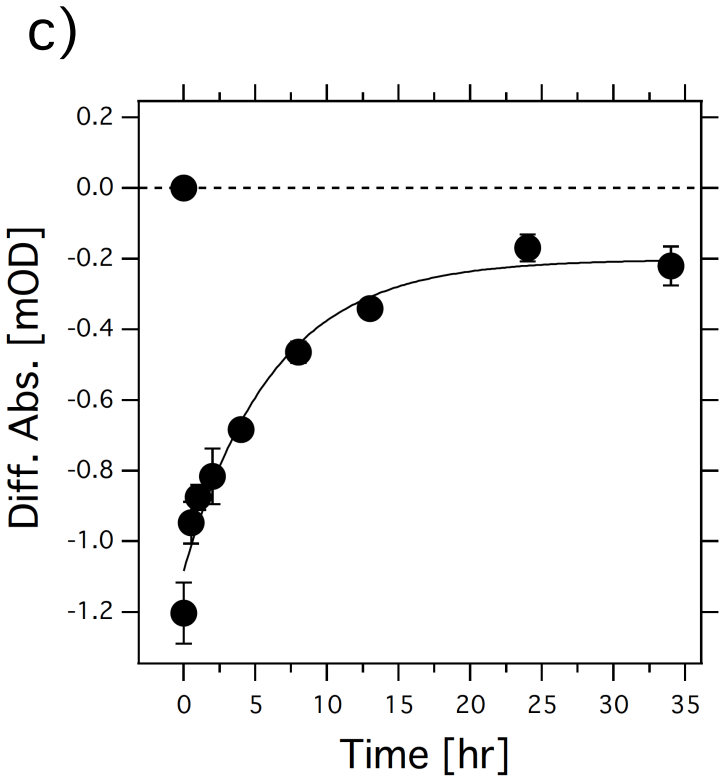


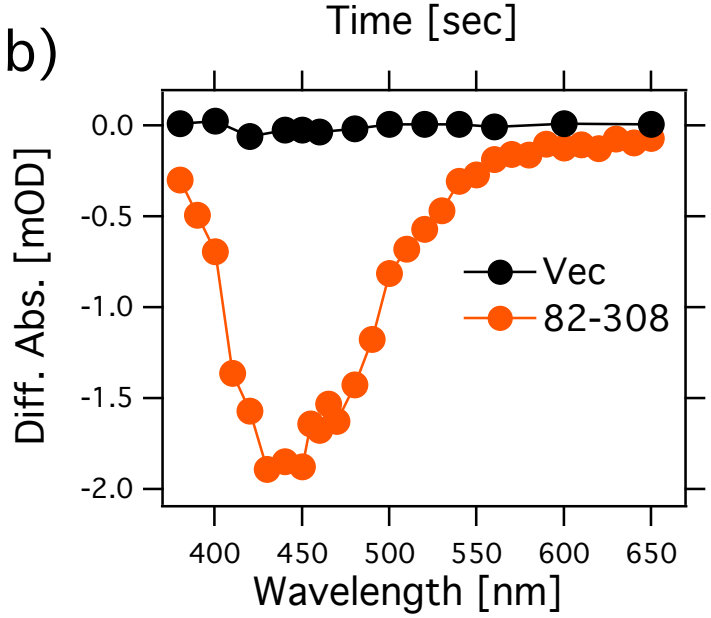


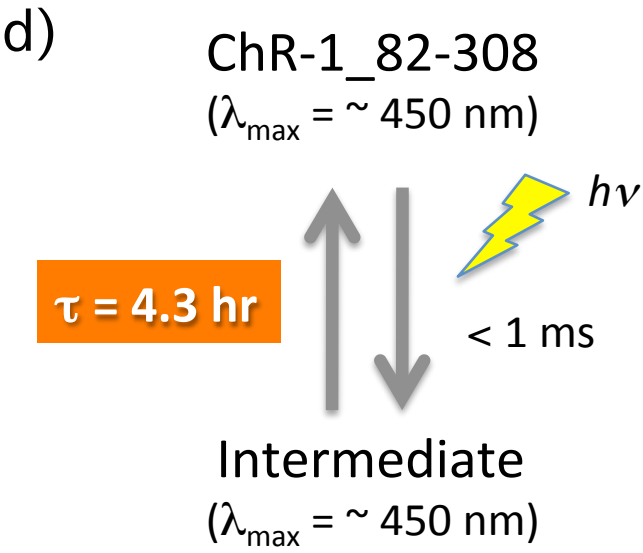


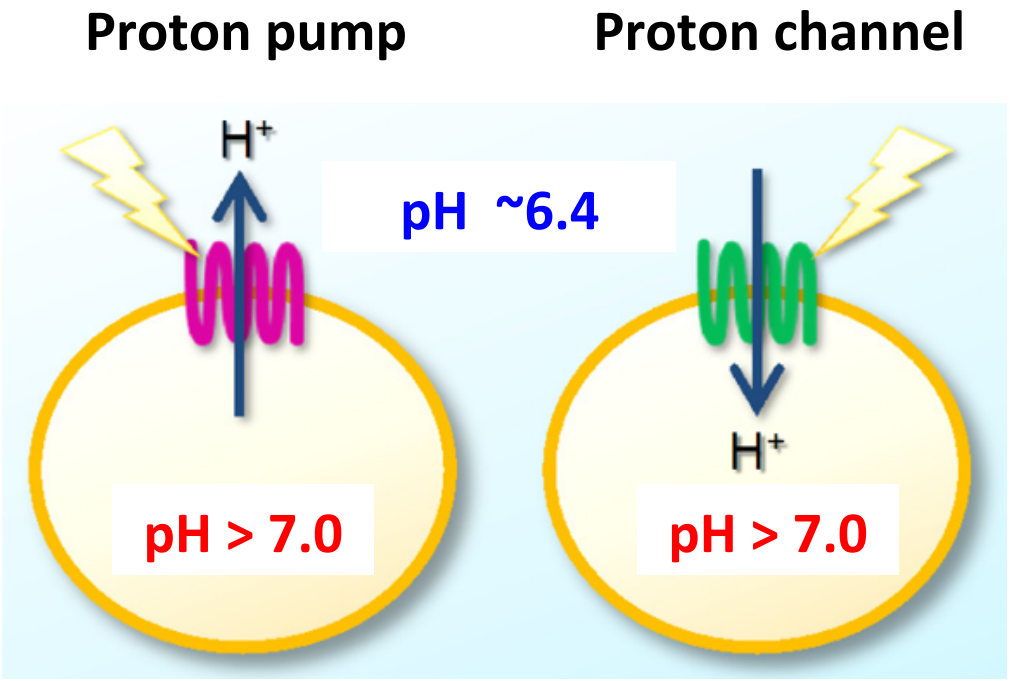


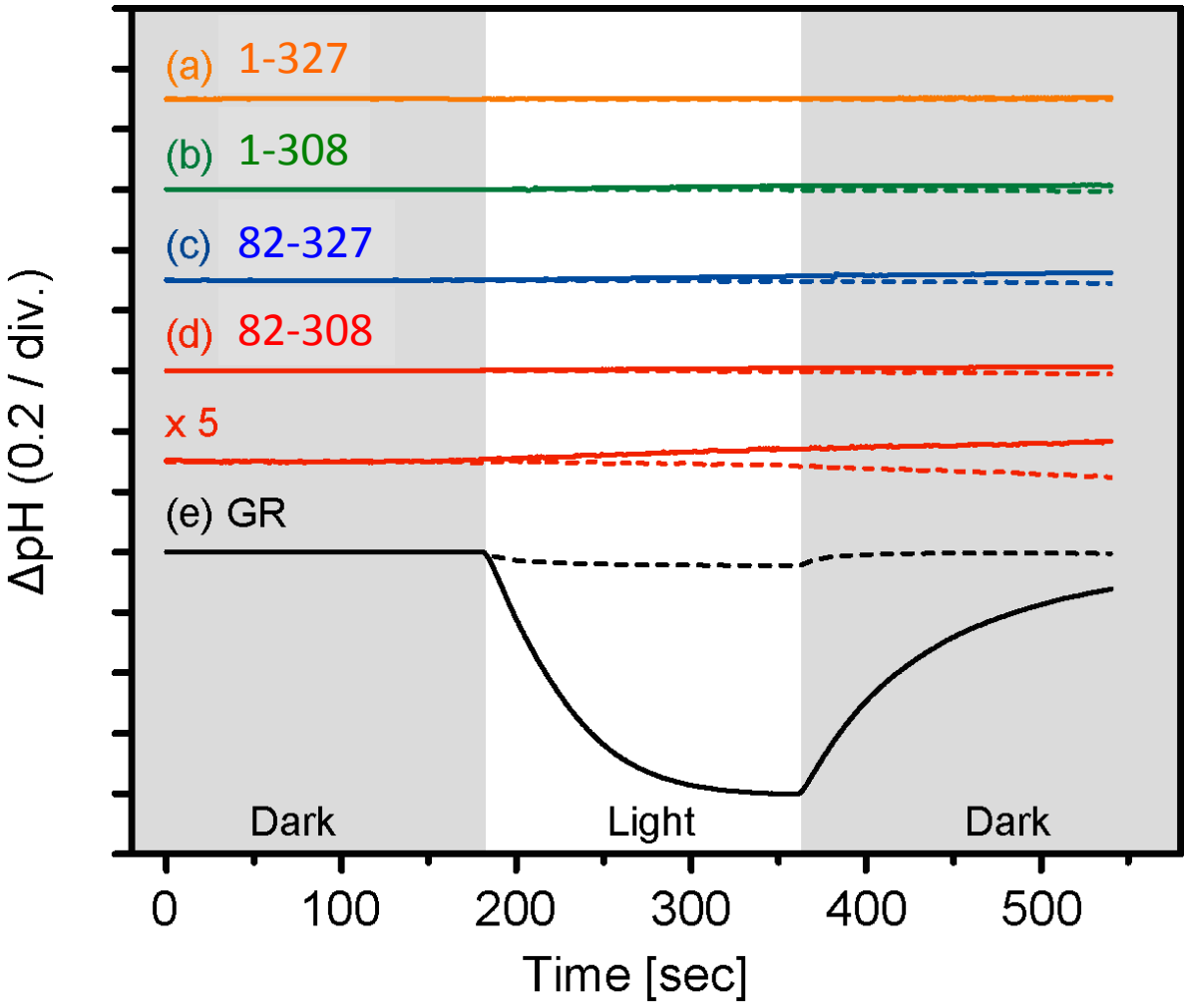


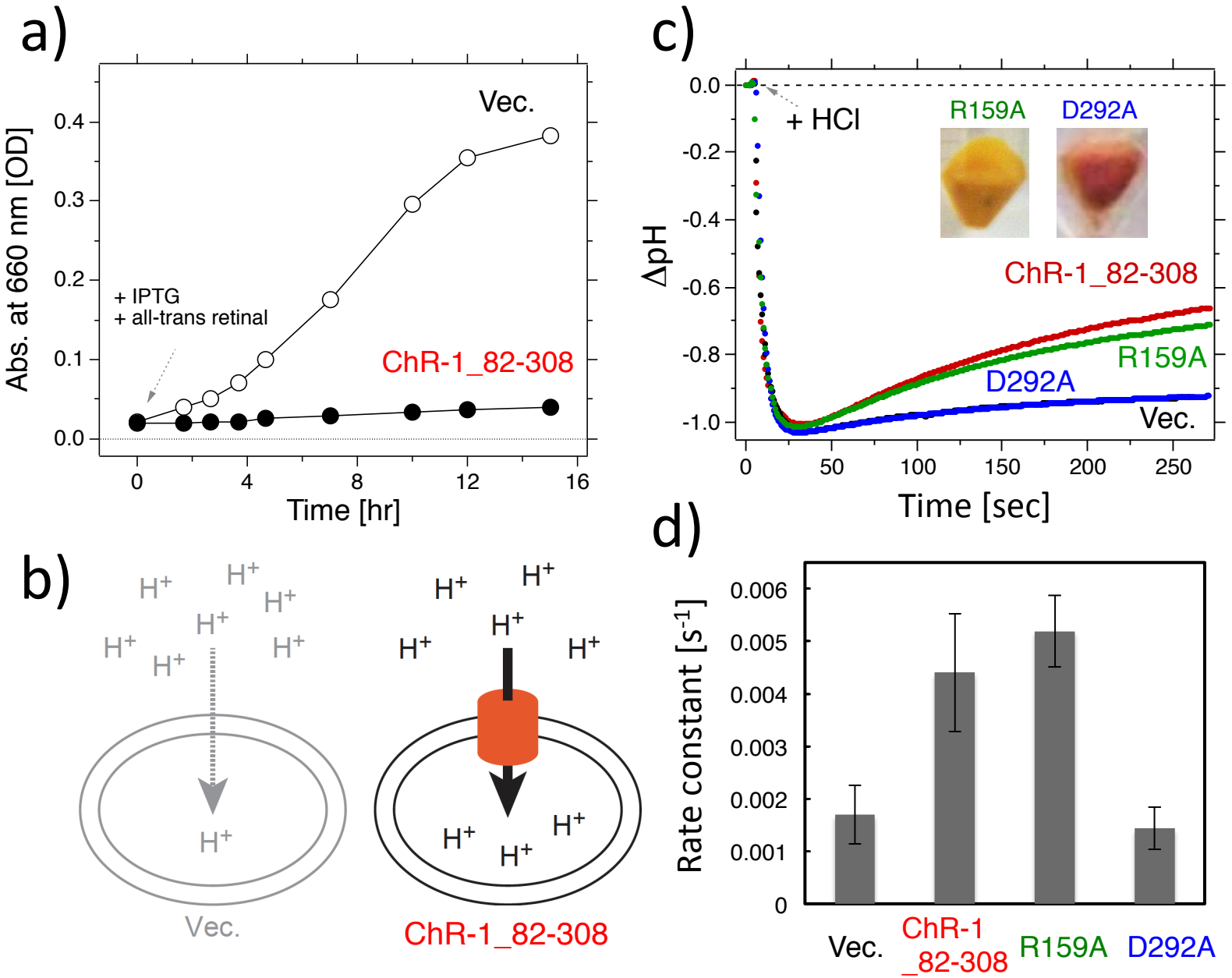


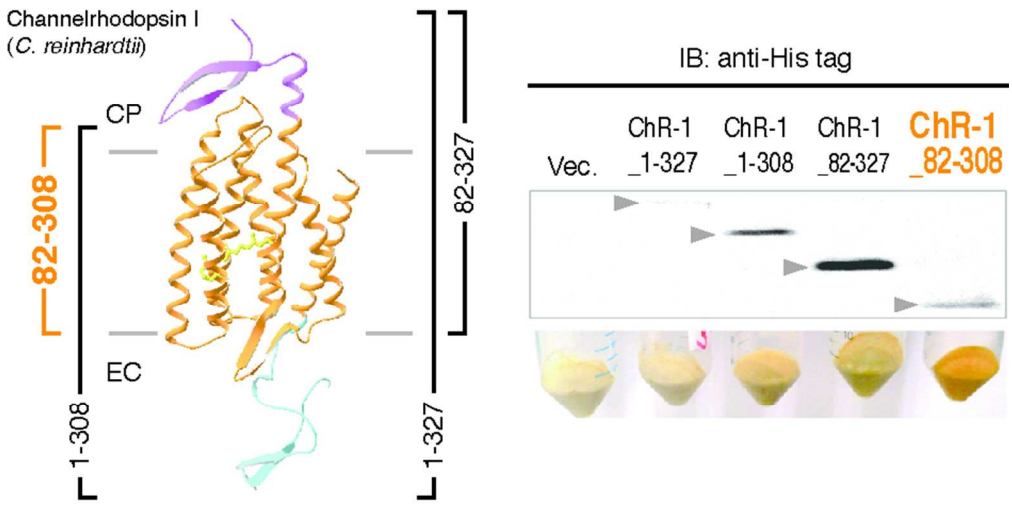












The extended modules of Channelrhodopsin-1 (ChR-1) are involved both in the maintenance of proper photochemical properties and in the function.
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