A Single Point Mutation Converts a Proton-pumping Rhodopsin into a Turn-on Fluorescent Sensor for Chloride

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Abstract

Gloeobacter Rhodopsin-based Fluorescent Sensor for Chloride (GR1)

The visualization of chloride in living cells with fluorescent sensors is linked to our ability to design hosts that can overcome the energetic penalty of desolvation to bind chloride in water. Fluorescent proteins can be used as biological supramolecular hosts to address this fundamental challenge. Here, we showcase the power of protein engineering to convert the fluorescent proton-pumping rhodopsin GR from *Gloeobacter violaceus* into GR1, a turn-on fluorescent sensor for chloride in detergent micelles and in live *Escherichia coli*. This non-natural function was unlocked by mutating D121, which serves as the counterion to the protonated retinylidene Schiff base chromophore. Substitution from aspartate to valine at this position (D121V) creates a binding site for chloride. The addition of chloride tunes the pK_a of the chromophore towards the protonated, fluorescent state to generate a pH-dependent response. Moreover, ion pumping assays combined with bulk fluorescence and single cell fluorescence microscopy experiments with *E. coli*, expressing a GR1 fusion with cyan fluorescent protein, show that GR1 does not pump ions nor sense membrane potential but instead provides a reversible, ratiometric readout of chloride. This discovery sets the stage to use natural and laboratory-guided evolution to build a family of rhodopsin fluorescent chloride sensors for cellular applications and learn how proteins can evolve and adapt to bind anions in water.

Introduction

Chloride is an essential inorganic ion for life.^[1,2] Beyond its role as an electrolyte, chloride mobilization across cellular membranes is known to be involved in a wide range of homeostatic processes including, but not limited to, pH regulation, fluid excretion, and electrical activity.^[2–4] The growing importance of chloride in normal physiology and disease is further evident by recent discoveries of new transporters and biological roles, which have been accelerated through the application of fluorescent sensors for chloride.^[5–9] The fluorescence imaging of chloride in living cells is linked to our ability to build hosts that can recognize chloride in water, a fundamental challenge in the field of supramolecular chemistry.^[10–12] Advances along these lines could contribute to our general understanding of aqueous anion recognition and to the design of new fluorescent sensors for chloride.

A key limitation for the recognition of chloride in water is the inherent energetic penalty for desolvation.^[10,12–15] To overcome this, successful examples of fluorescent molecular and polymer hosts integrate Nature's design principles and combine cooperative, non-covalent interactions, including hydrogen bonding, ion-pairing, and van der Waals with the hydrophobic effect.^[4,11,16–20] However, synthetic hosts that allow for the fluorescence imaging of chloride in living cells remain rare. Quinolinium and acridinium-based fluorophores bear a positively charged pyridinium cation that directly interacts with chloride, resulting in collisional fluorescence quenching.^[11,18,21,22] These molecular fluorescent sensors are pH-independent, and additional bioconjugation strategies have enabled improved cellular uptake, organelle targeting, and ratiometric fluorescent outputs.^[4,23–28]

As a complementary approach, we use and evolve Nature to build protein-based hosts for chloride. Even though there are only twenty proteinogenic amino acids, protein sequence space is vast and can be further enriched through protein engineering methods.^[29] By selecting for a desired function or property, laboratory-guided evolution allows for this sequence space to be quickly sampled and filtered.^[29] For our goal, this approach affords a diverse pool of hosts that can further evolved and applied for the fluorescence imaging of chloride in living cells. Along these lines, the green fluorescent protein family (GFP, PF01353) has been widely explored and engineered to build fluorescent sensors for chloride.^[4,30,31] Indeed, pioneering work from Wachter and Remington established that variants of the green fluorescent protein (avGFP) from the jellyfish *Aequorea victoria* are sensitive to chloride.^[32–34] Of these, the yellow fluorescent protein avYFP-H148Q is well characterized, and the chloride binding pocket is lined with hydrophobic residues and four polar amino acids, including two glutamines, arginine, and tyrosine, that can hydrogen bond with the chloride ion.^[34] The latter tyrosine residue connects the binding of chloride through a π - π interaction with the 4-(p-hydroxybenzylidene)imidazolidin-5-one chromophore.^[34] Because of this close proximity, chloride binding increases the pK_a of the chromophore and shifts the equilibrium from the fluorescent phenolate state towards the non-fluorescent phenol state, generating a turn-off fluorescence response.^[34,35] The E²GFP variant of avGFP is also sensitive to chloride but operates via static fluorescence quenching with no change in the chromophore

equilibrium.^[36] This sensing mechanism could be attributed to a different chloride binding pocket; the chloride ion forms an electrostatic interaction with the imidazoline ring of the chromophore and three hydrogen bonds: one with a tyrosine residue and two with the peptide backbone connecting the chromophore to valine and glutamine residues.^[36] Both avYFP-H148Q and E²GFP, and variants thereof, have been applied for the fluorescence imaging of chloride.^[31,35,37–41] Furthermore, fusion with other fluorescent proteins have enabled ratiometric fluorescent outputs that account for variability in protein expression or pH dependence in living cells.^[42–47]

Relative to these literature precedents, we have identified low sequence identity homologues that are also sensitive to chloride.^[48,49] The chloride binding pocket in the naturally occurring yellow fluorescent protein from the jellyfish *Phialidium* sp. (phiYFP) is identical to that of avYFP-H148Q.^[34,50] Similarly, chloride binding increases the pK_a of the chromophore but also generates an excitation ratiometric, turn-on fluorescence response, likely through an excited state proton transfer process.^[48] In addition, through a structure-guided search, we identified that the yellow fluorescent protein IanYFP from the cephalochordate *Branchiostoma lanceolatum* and its engineered monomer mNeonGreen also bind chloride.^[51] In mNeonGreen, chloride forms five hydrogen bonds with histidine, arginine, serine, threonine, and tyrosine residues near the chromophore.^[51] Based on this, we recently reported that chloride binding decreases the pK_a of the chromophore and shifts the equilibrium from the non-fluorescent phenol state to the highly fluorescent phenolate state, generating a turn-on fluorescence response.^[49] Furthermore, we attributed this unique sensing mechanism to a non-coordinating arginine residue, instead of a tyrosine residue, above the chromophore. Together, these homologues highlight how differences in the amino acid residues, within and outside of the chloride binding pocket and even in the chromophore, can influence sensor properties. This is a key advantage of sampling and enriching the vast protein sequence space to build hosts for chloride.

Building off of the green fluorescent protein family, we envisioned that microbial rhodopsins (Bac_rhodopsin, PF01036) could also serve as scaffolds to engineer fluorescent sensors for chloride. Microbial rhodopsins are light-activated, transmembrane ion pumps with seven α -helices and a covalently bound retinylidene Schiff base chromophore (SBC) — generated from the condensation of all-*trans*-retinal and a lysine residue (Figure 1A).^[52–56] The SBC and its protonation state are stabilized by a key counterion position, which plays a role in the ion pumping pathway and selectivity.^[57] In chloride-pumping rhodopsins (CPRs), this counterion position corresponds to a threonine or asparagine, such that the chloride ion serves as the counterion to the protonated SB.^[52,54,57–63] This is observed in the X-ray crystal structures of representative CPRs from *Halobacterium salinarum* (HR) and *Nonlabens marinus* S1-08^T (CIR) (Figures 1B–1C).^[58,61,62] The chloride binding pocket also consists of polar amino acids, including serine and threonine, and highly ordered water molecules that can form hydrogen bonding interactions with the chloride ion.^[61,62] However, the homologous counterion position in proton-pumping rhodopsins (PPRs) corresponds to a negatively charged aspartate, which accepts the proton from the protonated SBC (Figure 1D).^[54,60,64] Interestingly, the well-studied PPR bacteriorhodopsin (BR) from *Halobacterium salinarum* can be converted into a CPR by substituting the aspartate counterion position (D85) with

serine, threonine, or asparagine.^[65] Absorption spectroscopy indicates that these variants bind chloride and other anions including bromide, iodide, and nitrate, but with varying pumping activities.^[65–68] Of the three variants, BR D85S has been crystallized with bromide (Figure 1E).^[69] Similar to CPRs, the serine residue at the counterion position directly interacts with the bromide ion, which forms a hydrogen bond with the protonated SBC.^[69] The residues R82, mediated by a highly ordered water molecule, and D212 form two hydrogen bonding interactions with bromide, thus adapting to accommodate this non-natural function.^[69] Outside of these contexts, when illuminated with near-infrared light, PPRs can also access fluorescent states in the photocycle.^[70–73] Because of this property, engineered PPRs have been repurposed as fluorescent proton sensors to provide a readout of the membrane potential in living cells.^[71,72,74–76] On a molecular level, the protonation state of the SBC controls the on and off fluorescent states, which can be influenced by the counterion position.^[71,72]

Figure 1. (A) Microbial rhodopsins are light-activated, transmembrane ion pumps with a covalently bound retinylidene Schiff base chromophore (SBC), formed from all-*trans*-retinal (yellow sticks) and a conserved lysine residue (Lys, black sticks). The counterion position (black sticks) stabilizes the protonated SBC (PSBC) and controls ion selectivity (PDB ID: 6NWD). Comparison of (B) the chloride-pumping rhodopsin (CPR) from *Halobacterium salinarum* (HR) with chloride (PDB ID: 5AHY), (C) the CPR from *Nonlabens marinus* S1-08^T (CIR) with chloride (PDB ID: 5G28), (D) the proton-pumping rhodopsin (PPR) from *Halobacterium salinarum* (BR, PDB ID: 1KGB), (E) the engineered PPR BR D85S with bromide (PDB ID: 1MGY), and (F) the fluorescent PPR from *Gloeobacter violaceus* (wtGR, PDB ID: 6NWD) selected for this study. For each structure, the residues corresponding to the anion binding pocket in the CPRs and BR D85S are shown as light gray sticks with the oxygen (red) and nitrogen (blue) atoms. Hydrogen bonds are shown as black dashed lines. Each residue is labeled with the position number and the single letter amino acid code. The retinal (RET) is shown as sticks in yellow covalently bound to conserved lysine residue, generating the SBC. The counterion position to the SBC is labeled in bold. The water molecules, chloride ions, and bromide ion are shown as spheres in red, green, and purple, respectively.

Motivated by these studies, we hypothesized that chloride could replace the aspartate as the counterion in a fluorescent PPR, form an electrostatic interaction with the protonated SBC, and tune the optical properties to generate a fluorescent sensor for chloride. To test this, we selected the fluorescent PPR from the cyanobacterium *Gloeobacter violaceus* (GR) because it has been spectroscopically and structurally characterized, can readily be expressed in *Escherichia coli*, and remains fluorescent even after the introduction of multiple mutations (Figure 1F).^[77–89] We have developed a workflow to rapidly evolve, screen, and discover chloride-sensitive variants of GR. Based on this screen, we highlight how a single point mutation at the SB counterion position from aspartate to valine, a non-natural substitution, converts wild-type (*wt*) GR into GR1, a pH-dependent, turn-on fluorescent sensor for chloride. *In vitro* data indicate that chloride binding to GR1, but not *wt*GR, increases the pK_a of the SBC, shifting the equilibrium towards the protonated, fluorescent state, generating the turn-on response. Ion pumping assays combined with bulk fluorescence measurements and single cell fluorescence imaging of live *E. coli* demonstrate that a GR1 does not pump ions nor sense membrane potential but instead is a reversible sensor for chloride.

Results and Discussion

Fluorescence library screening and identification of GR sensors for chloride

The fluorescence of wild-type (*wt*) GR was evaluated in live *E. coli* at pH 5, 6, and 7 in the absence and presence of 400 mM sodium chloride. To account for any variability in protein expression, *wt*GR was fused to a chloride-insensitive cyan fluorescent protein (CFP) to generate the *wt*GR-CFP construct resulting in a ratiometric output for normalization (Figures S1–S3).^[88] Upon excitation at 530 nm, the rhodopsin-based emission of *wt*GR-CFP is centered at 710 nm and does not change in the presence of chloride (Figures 2, S3). Next, site-saturation mutagenesis (SSM) was carried out at position 121 to sample all possible amino acid substitutions that could give rise to a chloride-sensitive variant. To our surprise, mutation from aspartate to valine (GR1-CFP) results in a *ca.* 2.2-fold turn-on fluorescence response with 400 mM sodium chloride at pH 5 (Figures 2, S4–S5). In addition, substitution with leucine (*ca.* 1.2-fold), threonine (*ca.* 1.3-fold), asparagine (*ca.* 1.3-fold), or isoleucine (*ca.* 1.5-fold) result in a turn-on fluorescence response, albeit to a lesser extent (Figures S4, S6–S9). No chloride-sensitive variants were identified in the library screen at pH 6 or pH 7. However, upon rescreening, GR1-CFP shows a *ca.* 1.2-fold turn-on fluorescence response at pH 6 (Figure 2). Given these results, the CFP tag was removed from the *wt*GR and GR1 constructs, and comparable results were observed in live *E. coli* (Figure S10). Interestingly, asparagine and threonine are enriched at this counterion position in a subset of CPRs, whereas, the hydrophobic substitutions, such as leucine, isoleucine, and valine, have yet to be identified at this position in the rhodopsin family (sequence identity > 23%, Figure S4C).

Figure 2. Site-saturation mutagenesis at the D121 counterion position converts *wtGR*-CFP into the chloride-sensitive variant GR1-CFP. Bars represent the integrated emission response of the rhodopsin (F_{GR}) normalized by the integrated emission of CFP (F_{CFP}) in the absence (black bars) and presence of 400 mM sodium chloride (gray bars). Spectra were acquired with live *Escherichia coli* expressing *wtGR*-CFP or GR1-CFP in 50 mM sodium acetate buffer at pH 5, 50 mM sodium phosphate buffer at pH 6, and 50 mM sodium phosphate buffer at pH 7. Excitation for the rhodopsin was provided at 530 nm for the rhodopsin, and the emission was collected and integrated from 600–800 nm (F_{GR}). Excitation for CFP was provided at 390 nm, and the emission was collected and integrated from 425–560 nm. The average of three biological replicates with standard error of the mean is reported.

Spectroscopic characterization of purified *wtGR* and GR1

Next, the *in vitro* properties of purified *wtGR* and GR1 in detergent micelles were compared in acetate buffer at pH 5, supplemented with sodium gluconate for protein stability.^[67,90,91] Consistent with previously reported absorbance spectra, *wtGR* has one absorption maximum at 550 nm that corresponds to the covalently bound SBC (Figure S13).^[78,79,84,85,87,89] Upon excitation at 530 nm, *wtGR* has a broad emission band with a maximum centered at 630 nm and a shoulder at 670 nm ($\Phi_{apo} = 2.0 \times 10^{-3}$, Figures 3A, S14).^[82] The *wtGR* absorption and emission maxima do not shift upon the addition of sodium chloride (Figure S13). Instead, the emission intensity fluctuates by ~8% with no clear indication of binding ($\Phi_{Cl} = 2.4 \times 10^{-3}$, Figures 3A, S14). As expected, the absorption maximum of GR1 is red-shifted to 565 nm and does not change upon the addition of sodium chloride (Figure S15).^[88] However, with excitation at 530 nm, GR1 displays a *ca.* 1.3-fold turn-on fluorescence response that saturates with 400 mM sodium chloride at 655 nm ($\Phi_{apo} = 3.0 \times 10^{-3}$, $\Phi_{Cl} = 3.9 \times 10^{-3}$) with no shift in the emission maximum, suggesting an excited state process (Figures 3B, S14–S15). This titration data can be fitted to a single site binding model for chloride resulting in an apparent dissociation constant (K_d) of 80 ± 1 mM (Figure S15).

Figure 3. Characterization of purified *wtGR* and GR1 in detergent micelles shows that GR1 has a chloride binding site and is also sensitive to bromide, iodide, and nitrate. Fluorescence spectra of ~3 μ M (A) *wtGR* and (B) GR1 in the presence of 0 (bold), 12.5, 25, 50, 100, 200, 300, and 400 (red) mM sodium chloride. Insets: Integrated emission response (y-axis) to sodium chloride. (C) Integrated emission response of ~3 μ M GR1 to 0 mM (F_i , Ctrl) or 400 mM (F_f) sodium chloride, bromide, iodide, nitrate, or dihydrogen phosphate. All spectra were acquired in 50 mM sodium acetate buffer containing 600 mM sodium gluconate at pH 5. Excitation for the rhodopsin was provided at 530 nm, and the emission was collected and integrated from 560–800 nm. The average of three technical replicates with standard error of the mean is reported.

Moreover, a turn-on fluorescence response is also observed with 400 mM of sodium bromide (ca. 1.3-fold) and iodide (ca. 1.4-fold), but quenching is observed with sodium nitrate (~17%) and no change is observed with sodium hydrogen phosphate (Figure 3C, S16). As described above, these anion preferences are also found in naturally occurring CPRs and engineered variants of the PPR BR, which suggests that the anion binding site in GR1 could be adjacent to the SBC. To determine how the binding of chloride generates the turn-on fluorescence response, we next measured the pK_a of the SBC with fluorescence spectroscopy. The pK_a for apo *wtGR* is 5.0 ± 0.2 and, as expected, does not significantly change upon the addition of 400 mM sodium chloride ($pK_a = 4.7 \pm 0.2$,

Figure S17). These pK_a values are consistent with that previously reported for *wtGR* in the presence of 300 mM sodium chloride using absorption spectroscopy.^[85] However, the pK_a for GR1 increases from 4.1 ± 0.1 to 5.1 ± 0.3 with 400 mM sodium chloride (Figure S18). This clearly indicates that the binding of chloride shifts the equilibrium towards the protonated, fluorescent SBC resulting in the observed turn-on fluorescence response. Even though *in vitro* purification of GR1 using detergent micelles is artificial and does not recapitulate the response observed in the library screening, our data demonstrates that mutation of the SBC counterion residue creates a chloride binding site, generating a turn-on fluorescent sensor. We speculate that, like BR D85S, GR1 adapts to this new function. While the hydrophobic valine side chain cannot hydrogen bond with the chloride ion, it could interact through van der Waals forces. It is also likely that chloride interacts with the protonated SBC along with nearby hydrogen-bond donating residues or water molecules (Figures 1E–1F). These additional interactions combined with the desolvation of the chloride ion could contribute favorably to the overall driving force for chloride binding. Moreover, the gain from the desolvation of GR1 would likely be minimal since it is a hydrophobic, transmembrane protein.^[13–15,92]

Application and validation of GR1-CFP in live *E. coli*

With *in vitro* characterization in hand, we turned our attention to validating GR1-CFP as a ratiometric fluorescent sensor for chloride in living cells using bulk and single cell fluorescence measurements. For this proof-of-concept, *E. coli* cells were selected due to their tolerance to pH 5 buffer.^[93] Consistent with the library screening, cells expressing *wtGR*-CFP show a measurable level of fluorescence that does not change with increasing concentrations of sodium chloride (Figures 4A, S19). In contrast, the fluorescence of cells expressing GR1-CFP increases, in a dose-dependent manner, by *ca.* 2.5-fold with 400 mM sodium chloride with no interference from sodium acetate (Figures 4B, S20–S21).

Figure 4. Bulk fluorescence measurements show that GR1-CFP is a ratiometric, turn-on fluorescent sensor that dynamically responds to changes in sodium chloride concentrations in live *E. coli* cells. Normalized fluorescence spectra of (A) wtGR-CFP and (B) GR1-CFP in the presence of 0 (bold), 12.5 25, 50, 100, 200, and 400 (red) mM sodium chloride. Inset: Integrated emission response of the rhodopsin (F_{GR}) over the integrated emission of CFP (F_{CFP}) (y-axis) to sodium chloride. (C) Normalized integrated emission response (F_{GR} / F_{CFP}) of GR1-CFP in the presence of buffer (Ctrl, F_i , white bar) and 400 mM (F_f) sodium chloride (black bars). Buffer treated cells were re-equilibrated with buffer only (Ctrl, F_i , gray bar), and sodium chloride treated cells were re-equilibrated with 400 mM sodium chloride, sodium acetate buffer, or sodium gluconate (F_f , gray bars). All spectra were acquired with *E. coli* expressing wtGR-CFP or GR1-CFP in 50 mM sodium acetate buffer at pH 5. Excitation for the rhodopsin was provided at 530 nm, and the emission was collected and integrated from 600–800 nm (F_{GR}). Excitation for CFP was provided at 390 nm, and the emission was collected and integrated from 425–560 nm (F_{CFP}). The average of three biological replicates with standard error of the mean is reported.

Similar to the *in vitro* measurements, a single site binding model can also be used to fit this titration data, but an improved affinity for chloride ($K_d = 42 \pm 1$ mM) is observed in cells (Figure S20). This result, although surprising, is in line with the fact that GR1-CFP maintains proper folding when expressed in live and intact membranes and has a larger dynamic range compared to purified GR1. To test the reversibility of the turn-on response

for GR1-CFP, cells were equilibrated in buffer or 400 mM sodium chloride followed by re-equilibration in sodium chloride, sodium acetate buffer, or sodium gluconate (Figure 4C). With this washout experiment, the fluorescence response was restored to baseline levels, indicating that GR1-CFP can sense dynamic changes in extracellular chloride that can readily equilibrate with the periplasmic space.^[93] Finally, GR1-CFP was applied at the single cell level using confocal fluorescence microscopy. As can be seen in Figure 5, GR1 localizes to the cell membrane, whereas CFP is diffusely localized in the cytosol. Cells expressing GR1-CFP immobilized on agarose pads supplemented with 400 mM sodium chloride display a ca. 1.8-fold turn-on response. Under the same conditions, *wt*GR-CFP does not show a response (Figures 5, S22).

Figure 5. Molecular imaging shows that GR1-CFP is a fluorescent chloride sensor at the single cell level in live *E. coli*. Representative confocal fluorescence microscopy images of *E. coli* expressing (A) *wt*GR-CFP and (C) GR1-CFP immobilized on 1.5% agarose pads containing 0 mM and 400 mM sodium chloride in 50 mM sodium acetate buffer at pH 5. Images are shown for the rhodopsin in red (left) and an overlay of the CFP in cyan with the transmitted light image (right). Scale bar = 2 μm. Single cell analysis of the normalized emission response to 400 mM sodium chloride for (B) *wt*GR-CFP (for 0 mM chloride n = 2829 regions of interest (ROIs); for 400 mM chloride n = 3818 ROIs) and (D) GR1-CFP (for 0 mM chloride n = 1890 ROIs; for 400 mM chloride n = 2913 ROIs). The median fluorescence intensity of the rhodopsin (F_{GR}) was normalized to the median fluorescence intensity of CFP (F_{CFP}). Boxplots represent the analysis for all cells in the fields of view with the lower and upper quartile data enclosed by the gray box. The median values are indicated by the red line, and the minimum and maximum values for each data set are indicated by the lines extending below and above the gray box. Data points that fall outside of these parameters are considered outliers and are shown as open circles. At least four different fields were analyzed for each biological replicate (n = 3).

Finally, since *wt*GR is a light-activated outward proton pump, ion pumping assays were conducted in 400 mM sodium chloride. White light LED illumination of *E. coli* expressing *wt*GR-CFP results in a time-dependent decrease in the extracellular pH (Figure S23). Upon removal of LED illumination, the extracellular pH is restored to baseline levels.

Moreover, treatment with the protonophore carbonyl cyanide 3-chlorophenylhydrazone (30 μ M CCCP) serves as a control to abolish the proton gradient and equalize the membrane potential, thus validating our experimental setup.^[94] Under the same conditions, GR1-CFP does not pump protons; these observations are in line with dye-based proton-pumping assays.^[88] We can also conclude that GR1-CFP is not a light-activated chloride pump because the extracellular pH does not change as observed in other CPRs and engineered PPRs.^[65,94–97] Furthermore, since PPRs can also be used as fluorescent indicators of membrane potential, chloride titrations were carried out at pH 5 in the presence of CCCP.^[71] Even though CCCP treatment uncouples the membrane potential, as independently confirmed with a commercially available dye, the fluorescence response of both *wt*GR-CFP and GR1-CFP are not affected (Figures S24–S25).^[98] Taken together, our data show that *wt*GR-CFP can be converted into a non-pumping, ratiometric fluorescent sensor for chloride that is independent of membrane potential; thus, allowing for the reversible detection of chloride in exogenously supplemented live *E. coli*. Our discovery establishes that rhodopsins can be a new platform to build fluorescent sensors for studying chloride in living cells and provides an opportunity at the intersection of protein engineering and molecular recognition to create biological supramolecular hosts for chloride.

Conclusion

From a molecular perspective, naturally occurring CPRs combine the hydrophobic effect with multivalent, cooperative interactions to achieve anion recognition in water. Drawing on these design principles, we showcase how protein engineering can be used to create a chloride binding site in the cyanobacterial proton-pump *Gloeobacter violaceus* rhodopsin (*wt*GR), resulting in a non-pumping, pH-dependent, turn-on fluorescent sensor for chloride (GR1) in detergent micelles and live *E. coli*. This non-natural function was unlocked by substituting the aspartate counterion to the protonated SBC with valine at position 121, which to date has not been identified in the rhodopsin family. *In vitro* characterization shows that chloride binding tunes the pK_a of the SBC towards the protonated, fluorescent state to generate a pH-dependent response to not only chloride, but also bromide, iodide, and nitrate. Based on these spectroscopic data, it is likely that chloride forms an electrostatic interaction with the protonated SBC in the excited state. Future investigations with time-resolved spectroscopy will be used to confirm this mechanism.^[72,73] Moreover, bulk and single cell fluorescence measurements of *E. coli* expressing the GR1-CFP fusion show that GR1 does not sense membrane potential but instead provides a reversible, ratiometric readout of chloride in living cells. Looking forward, we will utilize directed evolution to improve the dynamic range, sensitivity, and operational pH of GR1. We envision that rhodopsins and other pigment-containing proteins can serve as scaffolds to create functional tools to study chloride in living systems and, more broadly, serve as biological supramolecular hosts to advance our fundamental understanding of anion recognition in aqueous systems.

Methods

General. All reagents and chemicals were purchased from Sigma-Aldrich, Thermo Fisher Scientific, or VWR and were used as received unless otherwise stated.

Design and cloning of plasmids, site-saturation mutagenesis library, and point mutations. The DNA sequence encoding the *Gloeobacter violaceus* rhodopsin (GR, UniProt ID: Q7NP59) was used as previously described but with T121.^[88] This gene was synthesized and cloned into the pET-21a(+) vector between the Nde1 and Not1 restriction sites (GenScript, Figure S1A). For the GR T121-CFP construct, the DNA sequence encoding the cyan fluorescent protein from *Aequorea victoria* (CFP, UniProt ID: P42212) was used as previously described.^[88] This gene synthesized and cloned into the GR T121 pET-21a(+) construct between the Not1 and Xho1 restriction sites (GenScript, Figure S1B).

The site-saturation mutagenesis (SSM) library was generated using the GR T121-CFP construct as previously described.^[48,99] The forward primers were 37 bases long with the degenerate bases NDT, VHG, or TGG at position 121, and the reverse primer was 34 bases long that contained 18 overlapping bases with the forward primer (Sigma-Aldrich, Table S1). A solution of the three forward primers were combined in a 12:9:1 ratio. The forward and reverse primers were each diluted to a final concentration of 10 μ M with autoclaved water. The polymerase chain reaction (PCR) was carried out with the Phusion High Fidelity PCR Kit (New England Biolabs) according to the manufacturer's instructions with 1 μ L of template plasmid from a 10 ng/ μ L stock solution, 1.5 μ L of the forward and reverse primers from 10 μ M stock solutions, 0.5 μ L of dNTPs from a 10 mM stock solution, 0.5 μ L of dimethyl sulfoxide (DMSO), 5 μ L of Phusion GC buffer, 0.25 μ L of Phusion DNA polymerase, and 9.75 μ L of autoclave water to a final volume of 25 μ L. The PCR conditions are outlined in Table S2. After the PCR, the reaction mixture was treated with 1 μ L Dpn1 (New England Biolabs) for 2 h at 37 °C to remove the template DNA. The PCR product was then purified with agarose gel electrophoresis and extracted with the Zymoclean Gel DNA Recovery Kit (Zymo Research) according to the manufacturer's instructions. Ten microliters of the purified PCR product was ligated using the Gibson Assembly Master Mix (New England Biolabs), followed by cleanup with the DNA Clean & Concentrator Kit (Zymo Research) according manufacturers' instructions. One microliter of the resulting DNA was used to transform *E. cloni* EXPRESS BL21(DE3) Competent Cells (Lucigen) by electroporation (Bio-Rad Laboratories). The constructs for wild-type (wt) GR (D121) and the GR1 variant (V121) were prepared with the pET-21a(+) GR T121 construct. The forward primers were 37 bases long with the mutation site, and the same reverse primer described above was used (Table S1). The PCR conditions and downstream processing steps were carried out as described above (Table S3).

Following electroporation, *E. cloni* were plated on Luria Broth (LB) agar plates containing 100 μ g/mL ampicillin. To verify the sequences of the point mutants, colonies were picked into 5 mL of LB containing 100 μ g/mL ampicillin in 14 mL culture tubes (Corning) and incubated overnight (New Brunswick Innova 42R Shaker) at 37 °C with shaking at 250 rpm. The following day, cells were collected by centrifugation at 3,000g for 5 min (Allegra

X-14R, Beckman Coulter) and stored at -20 °C. The plasmid DNA was isolated with the QIAprep Spin Miniprep Kit (Qiagen) and sequenced to identify the correct clone (Eurofins Scientific).

SSM library expression, screening, and validation. The methods described here were adapted from a previously reported procedure.^[88] Eighty-eight single colonies of *E. coli* transformed with the SSM library were picked into 300 µL of LB containing 100 µg/mL ampicillin in a 96-well deep well plate (Greiner Bio-One) and sealed with an Easy App microporous film (USA Scientific). The 96-well deep well plate was incubated overnight at 37 °C with shaking at 230 rpm. The following day, each well of a 96-well deep well plate was filled with 950 µL of LB containing 100 µg/mL ampicillin and inoculated with 50 µL of the overnight culture using a Biomek NX^P liquid handler (Beckman Coulter). After 2 h and 15 min of incubation at 30 °C with shaking at 230 rpm, protein expression was induced with the addition of 50 µL of LB containing 100 µg/mL ampicillin, 105 mM isopropyl beta-D-thiogalactopyranoside (IPTG, Gold Biotechnology) and 2.1 mM all-*trans*-retinal (ATR, Sigma-Aldrich) to each well for a final concentration of 500 µM IPTG and 10 µM ATR. Protein expression was carried out at 30 °C with shaking at 230 rpm. After 4 h, the cells were harvested by centrifugation at 4,000g for 5 min (Allegra X-14R, Beckman Coulter). Using the liquid handler, the cell pellets were resuspended in 500 µL of 50 mM sodium acetate buffer at pH 5, 50 mM sodium phosphate buffer at pH 6, or 50 mM sodium phosphate buffer at pH 7, and 175 µL of the cell suspension was transferred to a clear 96-well microtiter plate (Caplugs) for plate reader measurements. Excitation for the rhodopsin was provided at 530 nm with a 10-nm bandwidth, and the emission was collected from 580–760 nm with a 20-nm bandwidth, a 10-nm step size, 25 flashes, and gain of 100 (Spark 20M, Tecan). Excitation for CFP was provided at 425 nm with a 10-nm bandwidth, and the emission was collected from 470–510 nm with a 20-nm bandwidth, a 10-nm step size, 25 flashes, and gain of 55. Following these emission scans, 25 µL of a 3.2 M sodium chloride stock solution in water was added to each well using a multichannel pipette (Eppendorf) for a final concentration of 400 mM sodium chloride. Then, the 96-well plate was shaken for 5 s and processed for plate reader measurements as described above.

Variants that showed an increase in fluorescence intensity in the presence of sodium chloride were streaked onto fresh LB agar plates containing 100 µg/mL ampicillin to isolate single colonies for rescreening. Eight single colonies of each variant were picked into 5 mL of LB containing 100 µg/mL ampicillin in 14 mL culture tubes and incubated overnight at 37 °C with shaking at 250 rpm. The following day, the overnight cultures were diluted 1:20 (v/v) into 3 mL of LB containing 100 µg/mL ampicillin in 14 mL culture tubes and incubated at 30 °C with shaking at 250 rpm. After 2.5 h, protein expression was induced with 15 µL of 100 mM IPTG and 15 µL of 2 mM ATR for a final concentration of 500 µM IPTG and 10 µM ATR and incubated at 30 °C for 4 h with shaking at 250 rpm. For a negative control, three single colonies of GR1-CFP were grown as described above but not induced (Figure S2). The cell pellets were collected by centrifugation at 2,500g for 5 min (5810 R, Eppendorf) and resuspended in 200 µL of 50 mM sodium acetate buffer at pH 5. For the plate reader rescreen, 25 µL of the cell suspension for each biological replicate was transferred to two separate wells of a 96-well microtiter plate

containing 175 μ L of 50 mM sodium acetate buffer at pH 5 or buffer containing 457 mM sodium chloride for a final concentration of 400 mM sodium chloride. Excitation scans were acquired to determine the optimal excitation wavelength for the rhodopsin emission at 710 nm with a 20-nm bandwidth. Excitation spectra were collected from 500–650 nm with a 10-nm bandwidth, a 5-nm step size, 30 flashes, and gain of 100 (Spark 10M, Tecan). Consistent with the SSM library screening methods, the rhodopsin was excited at 530 nm with a 10-nm bandwidth, and the emission was collected from 600–800 nm with a 20-nm bandwidth, a 5-nm step size, 30 flashes, and gain of 100. The CFP was excited at 390 nm with a 10-nm bandwidth, and the emission was collected from 425–560 nm with a 20-nm bandwidth, a 5-nm step size, 30 flashes, and gain of 55. The rhodopsin and CFP emissions were integrated from 600–800 nm (F_{GR}) and 425–560 nm (F_{CFP}), respectively, using the *trapz* function in MATLAB R2017a (MathWorks). For each variant, the average emission response of eight biological replicates with standard error of the mean is reported (Figures S4, S6–S9).

Rhodopsin family sequence alignment. The amino acid sequence for *wtGR* (UniProt ID: Q7NP59, residues 45–270) was used to search the UniProt Knowledgebase for homologous proteins in the rhodopsin family using the HMMER webserver v2.41.1 (www.ebi.ac.uk/Tools/hmmer).^[100,101] The expectation value (E) threshold was set to 0.01 to filter and obtain 6,906 protein sequences that are similar to *wtGR*. Next, we excluded protein sequences containing consecutive gaps, in the alignment, longer than 20% of the protein length (> 45 consecutive gaps) to reduce the noise from partial sequences. The resulting 3,285 protein sequences were aligned using the Clustal Omega software v1.2.4. The amino acid diversity at the counterion position in *wtGR* (D121) was analyzed and is presented in Figure S4C.

***wtGR* and GR1 chloride titrations in live *E. coli*.** Three 3 mL cultures of *E. coli* containing the *wtGR* and GR1 only constructs were expressed at 30 °C as described above. After 4 h, the expression cultures were collected by centrifugation 3,000g for 5 min (Allegra X-14R, Beckman Coulter) and resuspended in 200 μ L of 50 mM sodium acetate buffer at pH 5. In a 96-well plate, 175 μ L of the cell suspension was diluted with 25 μ L of 0 M or 3.2 M sodium chloride in water to a final concentration of 0 mM and 400 mM sodium chloride. Excitation was provided at 530 nm with a 10-nm bandwidth, and the emission was collected from 600–760 nm with a 20-nm bandwidth, a 5 nm-step size, 25 flashes and a gain of 100 (Spark 10M, Tecan). The average of three technical replicates with standard error of the mean is reported (Figure S10).

***wtGR*-CFP and GR1-CFP pH profiles in live *E. coli*.** Three single colonies of *E. coli* containing the *wtGR*-CFP and GR1-CFP plasmids were picked into 5 mL of LB containing 100 μ g/mL ampicillin and incubated overnight at 37 °C as described above. The next day, the overnight cultures were diluted 1:20 (v/v) into 25 mL of LB containing 100 μ g/mL ampicillin in 125 mL baffled flasks and incubated at 30 °C with shaking at 250 rpm. After 2.5 h, protein expression was induced with 119 μ L of 105 mM IPTG and 20.8 μ L of 12 mM ATR to a final concentration of 500 μ M IPTG and 10 μ M ATR and incubated at 30 °C with shaking at 250 rpm. After 4 h, 6 mL of each expression culture was collected by centrifugation 2,500g for 5 min (5810 R, Eppendorf), washed 3 mL of 50 mM sodium

acetate buffer at pH 5, 50 mM sodium phosphate buffer at pH 6, or 50 mM sodium phosphate buffer at pH 7, and resuspended in 400 μ L of the corresponding buffer. In a 96-well plate, 25 μ L of the cell suspension was diluted with 175 μ L of the corresponding buffer containing 0 mM or 457 mM sodium chloride to a final concentration of 0 mM and 400 mM sodium chloride. Excitation scans were acquired for the rhodopsin emission at 710 nm with a 20-nm bandwidth. Excitation spectra were collected from 500–650 nm with a 10-nm bandwidth, a 5-nm step size, 30 flashes, and gain of 100. The rhodopsin was excited at 530 nm with a 10-nm bandwidth, and the emission was collected from 600–800 nm with a 20-nm bandwidth, a 5-nm step size, 30 flashes, and gain of 100. The CFP was excited at 390 nm with a 10-nm bandwidth, and the emission was collected from 425–560 nm with a 20-nm bandwidth, a 5-nm step size, 30 flashes, and gain of 55 (Spark 10M, Tecan). The rhodopsin and CFP emissions were integrated from 600–800 nm (F_{GR}) and 425–560 nm (F_{CFP}), respectively, using the *trapz* function in MATLAB R2017a (MathWorks). The average of three biological replicates with standard error of the mean is reported (Figures 2, S3, S5).

Large-scale protein expression and purification. For large-scale protein expression, *E. coli* were transformed as described above with plasmids encoding *wtGR* and GR1. Single colonies were picked into 50 mL 2xYT media containing 50 μ g/mL ampicillin in 250 mL baffled flasks and incubated overnight at 37 °C with shaking at 230 rpm. The following day, cultures were diluted 1:40 (v/v) into 1 L of 2xYT containing 50 μ g/mL ampicillin in 2.8 L baffled flasks and incubated at 37 °C until the OD₆₀₀ reached ~0.6–0.8. The flasks were then cooled on ice for 30 min. Following this, protein expression was induced with the addition of 952 μ L of 105 mM IPTG and 833 μ L of 12 mM ATR to a final concentration of 100 μ M IPTG and 10 μ M ATR and incubated overnight at 18 °C with shaking at 230 rpm. The following day, the cells were collected by centrifugation at 3,000g (5810 R, Eppendorf) for 30 min at 4 °C and stored at -20 °C until further use.

The following steps were carried out at 4 °C. The frozen cell pellet was thawed and resuspended in a 1:5 (w/v) of pre-chilled 20 mM Tris buffer at pH 7.5 containing 200 mM sodium chloride, 5 mM magnesium chloride, 30 μ g/mL deoxyribonuclease I, and two cOmplete Protease Inhibitor Tablets (Roche) and homogenized with stirring in a foiled beaker. Cell lysis was carried out with sonication at 40% amplitude, 15 s pulse on, and 45 s pulse off for 10 min (Q500, QSonica). The cell debris was collected by centrifugation at 3,000g for 30 min (5810 R, Eppendorf). The membrane fraction was isolated by ultracentrifugation at 159,200g (Sorvall WX 80+, Thermo Fisher Scientific) for 1 h, resuspended in 15 mL of 20 mM Tris buffer at pH 7.5 containing 200 mM sodium chloride and 10% glycerol, homogenized using a tissue grinder (Wheaton), and stored at -80 °C until further use.

The following day, the isolated cell membrane was thawed on ice for 30 min and solubilized with vigorous stirring in 35 mL of 20 mM Tris buffer at pH 7.5 containing 300 mM sodium chloride, 1% n-Dodecyl- β -D-Maltopyranoside (DDM, w/v, Anatrace), and one cOmplete Protease Inhibitor Tablet. Insoluble debris was collected by ultracentrifugation at 159,200g (Sorvall WX 80+, Thermo Fisher Scientific) for 20 min, and the supernatant was loaded onto a 90 mL sample loop (Bio-Rad Laboratories). Protein purification was

carried out with the NGC Quest 10 Chromatography System (Bio-Rad Laboratories). A 5-mL nickel nitrilotriacetic acid (Ni-NTA) affinity column (HisTrap, GE Healthcare) was equilibrated with 5 column volumes (CV) of 20 mM Tris running buffer at pH 7.5 containing 300 mM sodium chloride, 25 mM imidazole, and 0.02% DDM (w/v). The protein sample was loaded onto the Ni-NTA column with a 5 mL/min flow rate, eluted with a 0–100% gradient of the running buffer and the 20 mM Tris elution buffer at pH 7.5 containing 300 mM sodium chloride, 500 mM imidazole, and 0.02% DDM (w/v) over 20 CV, and washed with 5 CV of the elution buffer. Fractions with an absorbance at 570 nm were combined and loaded onto a HiPrep 26/10 desalting column (GE Healthcare) pre-equilibrated with 50 mM sodium acetate buffer at pH 5 containing 0.05% DDM (w/v). Protein fractions were pooled and concentrated using an Amicon Ultra-15 Centrifugal Filter Unit with a 10 kDa molecular weight cut-off (MilliporeSigma), aliquoted, flash frozen in powdered dry ice, and stored at -80 °C for assays described below.

SDS-PAGE and Coomassie staining. The purity of purified *wtGR* and GR1 was evaluated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using a previously reported procedure with the modification that samples were heated in Laemmli sample buffer at 37 °C for 10 min (Figure S11).^[48]

General spectroscopic materials and methods. Absorbance and fluorescence spectra were collected using the Agilent Cary 7000 Spectrophotometer and the Horiba Fluorolog-3, respectively, as previously reported.^[48] Measurements were acquired with a 0.2-cm x 1-cm quartz cuvette (0.4 mL, Hellma USA).

Extinction coefficient and quantum yield determination with purified proteins. Aliquots of *wtGR* and GR1 in 50 mM sodium acetate buffer with 0.05% DDM at pH 5 were first diluted to ~5 μ M with 50 mM sodium acetate buffer at pH 5 containing 600 mM sodium gluconate. Protein concentrations were determined using the Beer-Lambert Law by dividing the *wtGR* and GR1 absorbance values at 550 nm and 565 nm, respectively, by their molar extinction coefficients and the pathlength of 1 cm. The molar extinction coefficients were determined using a previously reported procedure.^[102] Briefly, a 2.7 M hydroxylamine stock solution was prepared fresh in deionized water and 148 μ L of this solution was added to 252 μ L of the diluted protein stock solution for a final concentration of 1 M hydroxylamine and ~3 μ M protein in a 0.4 mL quartz cuvette to displace the covalently bound SBC. Absorbance spectra were collected from 300–700 nm with a 2-nm bandwidth and a 5-nm step size every 10 min for 12 h. The change in absorbance of free ATR at 360 nm (Δ Abs_{ATR}) was plotted versus the change in absorbance of the rhodopsin at 550 nm for *wtGR* or 565 nm for GR1 (Δ Abs_{GR}) over time and fitted to a linear fit model: $y = mx + b$, where the m is the slope variable and is equal to Δ Abs_{GR} / Δ Abs_{ATR}. The molar extinction coefficients for *wtGR* and GR1 (ϵ_{GR}) were determined using the following equation: $\epsilon_{GR} = \Delta$ Abs_{GR} / Δ Abs_{ATR} * ϵ_{ATR} where the molar extinction coefficient for ATR (ϵ_{ATR}) in ethanol is 43,400 M⁻¹ x cm⁻¹.^[103] The average of three technical replicates with standard error of the mean is reported (Figure S12).

The *wtGR* and GR1 quantum yield experiments were carried out in the absence and presence of 400 mM sodium chloride with respect to rhodamine 101 inner salt in methanol

($\Phi = 1.0$) as a reference.^[104] Serial dilutions of *wt*GR and GR1 were prepared in 50 mM sodium acetate buffer at pH 5 containing 600 mM sodium gluconate, and serial dilutions of rhodamine 101 inner salt were prepared in methanol. Each sample was transferred to a 96-well half area plate (Greiner Bio-One) for plate reader measurements. Absorbance spectra were collected from 400–750 nm with a 5-nm step size, and the absorbance values were kept between 0 and 0.3. Excitation was provided at 530 nm with a 10-nm bandwidth, and the emission was collected from 565–800 nm with a 20-nm bandwidth, a 5-nm step size, 30 flashes, and gain of 58 (Spark 10M, Tecan). The emission of the rhodopsins and rhodamine 101 inner salt were integrated from 565–800 nm using the *trapz* function in MATLAB. A standard curve was generated for each sample by plotting the absorbance value at 565 nm versus the integrated emission using the linear fit model: $y = mx + b$, where m is the slope variable ($R^2 > 0.99$). The quantum yields for *wt*GR and GR1 were calculated using the equation: $F_{GR} = (m_{GR} / m_{ref})(\eta_{GR}^2 / \eta_{ref}^2)$ where η is the refractive index values for water ($\eta = 1.770$) and methanol ($\eta = 1.765$) (Figure S14).^[105,106] The average of three technical replicates with standard error of the mean is reported (Figure S14).

Chloride titrations with purified protein. Aliquots of purified *wt*GR and GR1 in 50 mM sodium acetate buffer at pH 5 with 0.05% DDM were diluted into 50 M sodium acetate buffer at pH 5 containing 1.2 M sodium gluconate to a final concentration of ~6 μ M protein and 600 mM sodium gluconate. The protein stock solution was further diluted 1:1 (v/v) into 50 mM sodium acetate buffer at pH 5 containing 600 mM sodium gluconate and 0, 25, 50, 100, 200, 400, 600, and 800 mM sodium chloride for a final concentration of ~3 μ M protein and 0, 12.5, 25, 50, 100, 200, 300, and 400 mM sodium chloride. Excitation spectra were collected from 475–650 nm with a 14-nm bandpass and a 5-nm step size for the rhodopsin emission at 710 nm with a 14-nm bandpass. The excitation for *wt*GR and GR1 was provided at 530 nm with a 14-nm bandpass, and the emission spectra were collected from 560–800 nm with a 14-nm bandpass and a 5-nm step size. The rhodopsin emission was integrated from 560–800 nm using the *trapz* function in MATLAB. The integrated emission response of GR1 defined as $F = (F_{obs} - F_{min}) / (F_{max} - F_{min})$ was plotted versus the $[Cl^-]$ in Kaleidagraph v4.5 (Synergy Software) to determine the apparent dissociation constant (K_d). The K_d was calculated using the following equation: $F = [Cl^-] / (K_d + [Cl^-])$, where F_{obs} is the observed fluorescence and F_{min} and F_{max} are the fluorescence intensities at 0 mM and 400 mM sodium chloride, respectively. The average of three technical replicates with standard error of the mean is reported (Figures 3, S13, S15).

Anion selectivity with purified proteins. Aliquots of purified GR1 in 50 mM sodium acetate buffer at pH 5 with 0.05% DDM were diluted into 50 M sodium acetate buffer at pH 5 containing 1.2 M sodium gluconate to a final concentration of ~6 μ M protein and 600 mM sodium gluconate. The protein stock solution was further diluted 1:1 (v/v) into 50 mM sodium acetate buffer at pH 5 containing 600 mM sodium gluconate and/or 800 mM sodium chloride, sodium bromide, sodium iodide, sodium nitrate, sodium dihydrogen phosphate, or sodium hydrogen sulfate to a final concentration of ~3 μ M protein, 0 mM, and 400 mM of the anion. The excitation for GR1 was provided at 530 nm with a 14-nm bandpass, and the emission was collected from 560–800 nm with a 14-nm bandpass and

a 5-nm step size. The rhodopsin emission was integrated from 560–800 nm using the *trapz* function in MATLAB. The average emission response for three technical replicates with standard error of the mean is reported (Figures 3C, S16).

Determination of the Schiff base pK_a with purified proteins. Aliquots of purified wtGR and GR1 in 50 mM sodium acetate buffer at pH 5 with 0.05% DDM were diluted into a mixture of six-buffers to a final concentration of ~ 3 μ M protein. The mixture of six buffers contained 5 mM bicine, 5 mM N-cyclohexyl-3-aminopropanesulfonic acid, 5 mM N-cyclohexyl-2-aminoethanesulfonic acid, 5 mM 2-(N-morpholino)ethanesulfonic acid, 5 mM 3-(N-morpholino)propanesulfonic acid, and 5 mM sodium citrate, and 600 mM sodium gluconate starting at pH 4.^[102] The pH was adjusted then by adding 1 μ L of 1 M NaOH to 1 mL of the protein solution. Excitation was provided at 530 nm with a 14-nm bandpass, the emission was collected from 560–800 nm with a 14-nm bandpass and a 5-nm step size. The rhodopsin emission was integrated from 560–800 nm using the *trapz* function in MATLAB. The relative emission response was plotted versus pH in Kaleidagraph v4.5 and fitted to the Henderson-Hasselbach equation to determine the SBC pK_a . The average integrated emission for three technical replicates with standard error of the mean is reported (Figures S17–S18).

General protein expression protocol and plate reader settings for assays with live *E. coli*. *E. coli* were transformed with plasmids encoding wtGR-CFP and GR1-CFP as described above. Single colonies were picked into 5 mL of LB containing 100 μ g/mL ampicillin in 14 mL culture tubes and incubated overnight at 37 °C with shaking at 250 rpm. The next day, overnight cultures were diluted 1:20 (v/v) in either 3 mL of LB containing 50 μ g/mL ampicillin in 14 mL culture tubes or 25 mL LB containing 50 μ g/mL ampicillin in 125 mL baffled flasks. The cultures were incubated at 30 °C with shaking at 250 rpm. After 2.5 h, protein expression was induced with the addition of 15 μ L of 100 mM IPTG and 15 μ L of 2 mM ATR in 3 mL LB or 119 μ L of 105 mM IPTG and 20.8 μ L of 12 mM ATR in 25 mL LB to a final concentration of 500 μ M IPTG and 10 μ M ATR. Protein expression was carried out at 30 °C for 4 h with shaking at 250 rpm and processed for plate reader assays. For the rhodopsin, excitation was provided at 530 nm with a 10-nm bandwidth, and the emission was collected from 600–800 nm with a 20-nm bandwidth, a 5 nm-step size, 30 flashes and a gain of 100 (Spark 10M, Tecan). Excitation for CFP was provided at 390 nm with a 10-nm bandwidth, and the emission collected from 425–560 nm with a 20-nm bandwidth, a 5-nm step size, 30 flashes, and a gain of 55. For each variant, the rhodopsin emission was integrated from 600–800 nm (F_{GR}), and the CFP emission was integrated from 425–560 nm (F_{CFP}) using the *trapz* function in MATLAB. The normalized emission is reported as F_{GR}/F_{CFP} .

Chloride titration assays in live *E. coli*. Three-25 mL cultures of *E. coli* containing the wtGR-CFP and GR1-CFP plasmids were expressed at 30 °C as described above. After 4 h, 6 mL of each expression culture was collected by centrifugation 2,500g for 5 min (5810 R, Eppendorf), washed with 3 mL of 50 mM sodium acetate buffer at pH 5 and resuspended in 400 μ L of 50 mM sodium acetate buffer at pH 5. In a 96-well plate, 25 μ L of the cell suspension was diluted with 175 μ L of 50 mM sodium acetate buffer at pH 5 containing 0, 14.3, 28.5, 57, 114.3, 228.5, or 457 mM sodium chloride to a final

concentration of 0, 12.5, 25, 50, 100, 200, and 400 mM sodium chloride. The plate reader measurements were carried out as described above. The GR1-CFP apparent K_d for chloride were determined as described above. The average of three technical replicates with standard error of the mean is reported (Figures 4, S19–S20).

This assay was repeated in sodium phosphate buffer to test the effects of sodium acetate. Three 3 mL cultures of *E. coli* containing the GR1-CFP plasmid were expressed at 30 °C as described above. After 4 h, the cells were collected by centrifugation 2,500g for 5 min (5810 R, Eppendorf), washed with 3 mL of 50 mM sodium phosphate buffer at pH 5, and resuspended in 200 μ L of 50 mM sodium phosphate buffer at pH 5. In a 96-well microtiter plate, 25 μ L of the cell suspensions were diluted into 175 μ L of 50 mM sodium phosphate buffer at pH 5 containing 0 mM, 457 mM sodium chloride, or 457 mM sodium acetate to a final concentration of 0 mM, 400 mM sodium chloride, or 400 mM sodium acetate. The plate reader measurements were carried out as described above. The average of three biological replicates with standard error of the mean is reported (Figure S21).

To test the reversibility of the chloride response, three 25 mL cultures of *E. coli* containing the GR1-CFP plasmid were expressed at 30 °C as described above. After 4 h, 6 mL of each expression culture was collected by centrifugation at 2,500g for 5 min (5810 R, Eppendorf), washed with 3 mL of 50 mM sodium acetate buffer at pH 5, and resuspended in 400 μ L of buffer. In four 2 mL centrifuge tubes (USA Scientific), 75 μ L of the cell suspensions were diluted into 525 μ L of 50 mM sodium acetate buffer at pH 5 or buffer containing 457 mM sodium chloride to a final concentration of 0 mM and 400 mM sodium chloride. The cell suspensions were incubated for 10 min at room temperature with shaking at 300 rpm (ThermoMixer C, Eppendorf). Then, 200 μ L of the cell suspensions were transferred to a 96-well microtiter plate for plate reader measurements. The remaining cells were collected by centrifugation at 500g for 15 min (5424 R, Eppendorf), resuspended in 400 μ L of 50 mM sodium acetate buffer at pH 5 or buffer containing 400 mM sodium chloride or 400 mM sodium gluconate, and incubated for 10 min at room temperature with shaking at 300 rpm. The plate reader measurements were repeated with 200 μ L of the resuspended cells in a 96-well microtiter plate. The average of three biological replicates with standard error of the mean is reported (Figure 4C).

Live *E. coli* imaging and analysis. Three 3 mL cultures of *E. coli* containing the wtGR-CFP and GR1-CFP plasmids were expressed at 30 °C as described above. After 4 h, the cells were collected by centrifugation at 2,500g for 5 min (5810 R, Eppendorf) and resuspended in 100 μ L of 50 mM sodium acetate buffer at pH 5. Fifty microliters of each cell suspension was further diluted into 350 μ L of 50 mM sodium acetate buffer at pH 5, and 3 μ L of this solution was transferred to a 1.5% agarose pad and inverted onto a 35 mm imaging dish with a No. 1.5 20-mm glass coverslip (MatTek). The agarose pads (1.5%, w/v, Gold Biotechnology) were prepared in 50 mM sodium acetate buffer at pH 5 containing 0 mM or 400 mM sodium chloride. The agarose was solidified to an even thickness between two microscope slides and cut into 3 mm² pads.

Images were acquired using a confocal laser scanning microscope equipped with a single motorized pinhole, a cooled GaAsP photomultiplier, a multi-alkali photomultiplier, a motorized volume phase holographic transmission diffraction grating, a motorized adjustable slit, an external fluorescence light source, and a LED lamp with an integrated external transmitted light photomultiplier detector (FV3000RS, Olympus). A UPLSAPO 100X silicone immersion objective with a numerical aperture of 1.35 and working distance of 0.2 mm was used for imaging. Z-stacks were acquired for each sample. First, the rhodopsin was excited at 561 nm (20 mW), and the emission was collected from 630–730 nm. Then, the CFP was excited at 405 nm (50 mW), and the emission collected from 405–505 nm. Images were analyzed using the Fiji Is Just ImageJ (Fiji) v2.0 software.^[107] Default threshold settings were applied to the CFP maximum intensity Z-projection to create masks. From the masks, regions of interest (ROIs) with pixels greater than 3 and circularity between 0 and 1 were selected using the Analyze Particles function in Fiji. Overlapping cells that could not be identified as single cells were grouped as one ROI. For each Z-stack, the ROIs were transferred to the maximum intensity Z-projection of both the rhodopsin and CFP channels, and the fluorescence median intensity was measured. For each biological replicate ($n = 3$), at least four different fields of cells were sampled. The median fluorescence intensity of the rhodopsin (F_{GR}) was normalized to the median fluorescence intensity of CFP (F_{CFP}) (Figures 5, S22).

Proton and chloride-pumping assays in live *E. coli*. Proton and chloride pumping activities were measured using modified literature procedures.^[94] Three-25 mL cultures of *E. coli* containing the *wtGR*-CFP and *GR1*-CFP plasmids were expressed at 30 °C as described above. After 4 h, the cells were collected by centrifugation at 3,000g (Allegra X-14R, Beckman Coulter) for 5 min at 4 °C, washed 3 times with 10 mL of 400 mM sodium chloride, and resuspended in 4 mL of 400 mM sodium chloride to a final $OD_{600} \sim 0.8$. The cells were incubated in the dark for 1 h at 4 °C and then illuminated with a 90-W BR40 LED light bulb (EcoSmart, Home Depot) for 2 min. Light induced pH changes were measured using a pH electrode (Mettler Toledo) every 10 s for 300 s. The summation of the change in pH (ΔpH) was calculated for each time point. Pumping assays were also carried out with cells treated with carbonyl cyanide 3-chlorophenylhydrazone (CCCP, Sigma-Aldrich). A stock solution of 10 mM CCCP was prepared in DMSO and 12 μ L of the stock solution was added to the 4 mL cell resuspension to a final concentration of 30 μ M CCCP. The average ΔpH for three biological replicates with standard error of the mean is reported (Figure S23).

Chloride titrations in live *E. coli* with CCCP. To validate that the *GR1*-CFP turn-on response to chloride is independent of the membrane potential, chloride titrations were carried out with cells treated with 30 μ M CCCP. Three-3 mL cultures of *E. coli* containing the *wtGR*-CFP and *GR1*-CFP plasmids were expressed at 30 °C as described above. After 4 h, each expression culture was collected by centrifugation 2,500g for 5 min (5810 R, Eppendorf) and resuspended in 400 μ L of 50 mM sodium acetate buffer at pH 5. In a 96-well plate, 10 μ L of the cell suspension was diluted into 175 μ L of 50 mM sodium acetate buffer at pH 5 containing 0, 14.3, 28.5, 57, 114.3, 228.5, or 457 mM sodium chloride to a final concentration of 0, 12.5, 25, 50, 100, 200, and 400 mM sodium chloride. Each well was further diluted with 15 μ L of 3.2% DMSO (v/v) in buffer as the control or

400 μ M CCCP in buffer for a final concentration of 0 μ M and 30 μ M CCCP. The 96-well microtiter plate was incubated at 30 °C for 30 min with shaking for 1 min every 5 min in the plate reader. The plate reader measurements were carried using the settings described above. The average of three biological replicates with standard error of the mean is reported (Figure S24).

Membrane potential measurements with DiOC₂(3). To confirm that CCCP treatment at pH 5 uncouples the membrane potential, experiments were carried out with the ratiometric dye 3-3'-diethyloxacarbocyanine iodide (DiOC₂(3), Alfa Aesar).^[98] Three-5 mL cultures of *E. cloni* containing the GR1-CFP plasmid were incubated overnight at 37 °C with shaking at 250 rpm. The following day, 1 mL of the overnight cultures were harvested by centrifugation at 2,500g for 5 min (5810 R, Eppendorf) and resuspended with 1 mL of 50 mM sodium acetate buffer at pH 5. A portion of the cell resuspension (119 μ L) was transferred to a 5-mL Falcon Round-Bottom Polystyrene Tube (Corning) and further diluted into 875 μ L of sodium acetate buffer at pH 5 containing 0 mM, 457 mM sodium chloride, or 457 mM sodium gluconate to a final concentration of 0 mM, 400 mM sodium chloride, or 400 mM sodium gluconate. The samples were then treated with 1 μ L of DMSO or 1 μ L of a 30 mM CCCP stock solution in DMSO and 4 μ L of an 11 mM DiOC₂(3) stock solution in DMSO for a final concentration of 0 μ M or 30 μ M CCCP and 22 μ M DiOC₂(3). The samples were incubated in the dark at room temperature for 30 min before flow cytometry measurements (BD LSRFortessa). Cells were excited with the 488 nm laser (50 mW power). The green and red emissions were collected using the FITC emission at 530 nm with a 30-nm bandpass and the PerCP-Cy5-5 emission at 710 nm with a 50-nm bandpass, respectively. For each sample 50,000 events were recorded. Unstained and untreated *E. cloni* in 50 mM sodium acetate buffer at pH 5 were used as negative controls to gate cell populations using the software FlowJo v10.8. For each treatment, the average median emission intensity of three biological replicates with standard error of the mean is reported (Figure S25).

Supporting Information

Nucleotide sequences and primers, polymerase chain reaction conditions, Coomassie stained SDS-PAGE gels, absorbance and fluorescence spectra, saturation curves, quantum yields, fluorescence microscopy analysis, proton and chloride pumping assays, flow cytometry scatter and fluorescence plots, and membrane potential assays are provided in the Supporting Information.

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J.N.T, J.L., C.H., F.F., and S.C.D designed and performed research and analyzed data. S.S.A and G.M. contributed technical expertise for protein purification. Q.Z. and F.M. generated the multiple sequence alignment of the rhodopsin family. J.N.T. and S.C.D. wrote the manuscript.

Funding

F.M. acknowledges support from the National Science Foundation (MCB-1943442) and the National Institute of General Medical Sciences of the National Institutes of Health (R35GM133631). G.M. acknowledges support from the Welch Foundation (AT-1935-20170325) and the National Institute of General Medical Sciences of the National Institutes of Health (R35GM128704). S.C.D. acknowledges support from startup funds provided by The University of Texas at Dallas startup, the Welch Foundation (AT-1918-20170325), and the National Institute of General Medical Sciences of the National Institutes of Health (R35GM128923). This work is the sole responsibility of the authors and does represent the views of the funding agencies.

Notes

The authors have no competing financial interests.

Acknowledgements

We thank members of the Dodani and Meloni labs, Dr. Alexander R. Lippert, Dr. John W. Sibert, and Dr. Ronald A. Smaldone for helpful discussions. We also acknowledge Dr. Jacob Henderson (Flow Cytometry Core at UT Dallas) and Dr. Ved Prakash (Olympus Discovery Center at UT Dallas) for expert technical assistance with method development and data analysis.

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