

Coral anthozoan-specific opsins employ a novel chloride counterion for spectral tuning

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This study makes the **fundamental** discovery of the first natural animal rhodopsin that uses a chloride ion instead of an amino acid side chain as a counterion. Using a combination of biochemical and spectroscopic experiments together with QM/MM simulations, the authors identify the spectral tuning mechanism in the dark state and in the photoproduct state. The methods are sound and the results are **convincing**. This work will be of interest to biologists working on visual proteins and it also raises new questions about how environmental factors might affect coral opsins.

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Abstract

Animal opsins are G protein coupled receptors that have evolved to sense light by covalently binding a retinal chromophore via a protonated (positively charged) Schiff base. A negatively charged amino acid in the opsin, acting as a counterion, stabilises the proton on the Schiff base, which is essential for sensitivity to visible light. In this study, we investigate the spectroscopic properties of a unique class of opsins from a reef-building coral belonging to the anthozoan-specific opsin II group (ASO-II opsins), which intriguingly lack a counterion residue at any of established sites. Our findings reveal that, unlike other known animal opsins, the protonated state of the Schiff base in visible light-sensitive ASO-II opsins is highly dependent on exogenously supplied chloride ions (Cl^-). By using structural modelling and QM/MM calculations to interpret spectroscopy data, we conclude that, in the dark state, ASO-II opsins employ environmental Cl^- as their native counterion, while a nearby polar residue, Glu292 in its protonated neutral form, facilitates Cl^- binding. In contrast, Glu292 plays a crucial role in maintaining the protonation state of the Schiff base in the light-activated protein, serving as the counterion in the photoproduct. Furthermore, Glu292 is involved in G

protein activation of the ASO-II opsin, suggesting that this novel counterion system coordinates multiple functional properties.

Introduction

Animals sense light by using opsins, photosensitive proteins belonging to the large family of G protein-coupled receptors (GPCRs). Opsins have a seven-transmembrane helix structure and bind to a retinal chromophore to form a light-sensitive pigment. Based on their phylogenetic relationships (Koyanagi and Terakita, 2014 [↗](#)), eumetazoan animal opsins can be classified into eight groups with distinctive properties: vertebrate visual/non-visual opsins, opn3/TMT opsins, invertebrate Go-coupled opsins, cnidarian Gs-coupled opsins (cnidopsins), neuropsins (opn5), Gq-coupled visual pigments/melanopsins (opn4), peropsins, and retinochrome/RGR. Such diversity possibly underlies the diversification of light-dependent physiologies in animals. But, furthermore, this diversity also provides a range of potential optogenetic tools to manipulate intracellular G protein-mediated signaling (Koyanagi and Terakita, 2014 [↗](#)).

Reef-building corals and sea anemones belong to the subphylum Anthozoa, which together with the subphylum Medusozoa constitute the phylum Cnidaria. Cnidarian animals possess multiple opsins categorised as part of the cnidarian Gs-coupled opsin group (cnidopsins), which regulate light-dependent processes (Koyanagi et al., 2008 [↗](#)). For example, a member of this group is expressed in the ciliary type visual cells of the box jellyfish lens eyes (Koyanagi et al., 2008 [↗](#); Kozmik et al., 2008 [↗](#)). Beyond these Gs-coupled cnidopsins, anthozoan animals have opsins phylogenetically distant from the other known eight groups, as well as opsins found exclusively in anthozoans (Feuda et al., 2012 [↗](#); Suga et al., 2008 [↗](#)). These anthozoan-specific opsins (ASO) can be further classified into two groups, ASO-I and ASO-II (Gornik et al., 2020 [↗](#); Hering and Mayer, 2014 [↗](#); Mason et al., 2023 [↗](#); Picciani et al., 2018 [↗](#); Ramirez et al., 2016 [↗](#)). Gornik et al. (2021) proposed that both ASO-I and ASO-II were present in the last common ancestor of Anthozoa and Medusozoa, but were lost secondarily in the Medusozoa lineage (Gornik et al., 2020 [↗](#)). While it has been reported that both ASO-I and ASO-II are expressed in multiple tissues of sea anemones (Gornik et al., 2020 [↗](#); Suga et al., 2008 [↗](#)) and corals (Levy et al., 2021 [↗](#)), there is still a limited understanding of their molecular characteristics and physiological functions.

The members of the ASO-II group are not only phylogenetically unique but also display interesting features in their amino acid sequences. For instance, several of these opsins lack an amino acid residue conserved among typical opsins that is crucial for absorption of visible light (Gornik et al., 2020 [↗](#); Mason et al., 2023 [↗](#)). While free retinal in solution has its absorption maximum (λ_{max}) in the ultraviolet (UV), this shifts to visible light when retinal is bound to a lysine residue in the transmembrane bundle of the opsin (usually at Lys296, numbering according to the bovine rhodopsin sequence) through a protonated Schiff base to form the pigment. Such protonated Schiff base is necessary to achieve sensitivity to visible light in opsin-based pigments (Pitt et al., 1955 [↗](#)). However, the proton on the positively charged Schiff base is energetically unstable in the hydrophobic transmembrane environment. To stabilise this proton, a negatively charged residue, glutamic or aspartic acid, is situated near the Schiff base to act as a counterion. This counterion is essential for opsin-based pigments to absorb visible light, and the residues serving as the counterion are highly conserved across opsins (Terakita, 2005 [↗](#); Terakita et al., 2012 [↗](#)). To date, three experimentally confirmed sites for the counterion have been identified in animal opsins: 94 in helix 2 (Gerrard et al., 2018 [↗](#)), 113 in helix 3 (Nathans, 1990 [↗](#); Sakai et al., 2022 [↗](#); Sakmar et al., 1989 [↗](#); Zhukovsky and Oprian, 1989 [↗](#)) and 181 in extracellular loop 2 (Nagata et al., 2019 [↗](#); Terakita et al., 2004 [↗](#), 2000 [↗](#)). Remarkably, some opsins belonging to the ASO-II group lack glutamic or aspartic acid at any of these established counterion positions (Gornik et al., 2020 [↗](#); Mason et al., 2023 [↗](#)). This absence raises the question of whether these opsins can absorb visible light, and if so, by what mechanism.

In this study, we investigate the spectroscopic properties of opsins in the ASO-II group isolated from the reef-building coral *Acropora tenuis*. Absorption spectra reveal that this group includes opsins sensitive to both UV and visible light. We then focus on a particular visible light-sensitive opsin within the ASO-II group (Antho2a) by spectroscopically analysing the protonated and deprotonated states of the Schiff base in the wild type and in single point mutants. By interpreting the spectroscopy data in the light of hybrid quantum mechanics/molecular mechanics (QM/MM) simulations, we demonstrate that a chloride anion (Cl^-) serves as a counterion to the retinylidene Schiff base in animal opsins, specifically in visible light-sensitive opsins of the ASO-II group.

Results

Identification of *Acropora tenuis* opsins

We identified 17 opsins from the *Acropora tenuis* genome and transcriptome datasets by homology search, which included eight opsins in Gs-coupled cnidopsin group, one opsin in the ASO-I group, and eight opsins in the ASO-II group (**Fig. S1** [↗](#); **Fig. 1A** [↗](#)). Full-length cDNAs of seven out of the eight opsins in the ASO-II group were isolated and cloned from adult or larval tissues of the coral (highlighted by bold letters in **Fig. 1A** [↗](#)). We failed to amplify one opsin in the ASO-II group (gene model ID: aten_s0263.g14) by RT-PCR possibly because of its little mRNA expression level. Amino acid sequence alignment shows that all the seven *A. tenuis* opsins in the ASO-II group lack a glutamic or aspartic acid at the established counterion positions 94, 113 or 181 (**Fig. S2** [↗](#)). These opsins also have no E(D)RY motif at the cytoplasmic end of helix 3, which is conserved throughout most class A GPCRs (Hofmann et al., 2009 [↗](#)).

Absorption spectra of *Acropora tenuis* opsins in the ASO-II group

We expressed seven members of the ASO-II group in COS-1 cells and purified their recombinant pigments in detergent-solubilised conditions. We successfully obtained the absorption spectra of three (Antho2a, Antho2c and Antho2e) out of the seven members, which showed that Antho2a and Antho2c are visible light-sensitive opsins having with λ_{max} at 503 nm and 450 nm, respectively, whereas Antho2e is a UV-sensitive opsin with λ_{max} at ~ 360 nm (**Fig. 1B** [↗](#)). We have previously reported that one opsin in the ASO-II group, acropsin 4 of the coral *Acropora millepora*, induces a light-dependent elevation of intracellular Ca^{2+} levels (Mason et al., 2023 [↗](#)). Here, we showed that Antho2a, Antho2c and Antho2e evoked a similar light-dependent increase of Ca^{2+} levels in HEK293S cells (**Fig. 1C** [↗](#)).

Search for the counterion in *Acropora tenuis* opsins of the ASO-II group

Antho2a and Antho2c form visible light-sensitive pigments in the dark (**Fig. 1B** [↗](#)) despite the lack of a negatively charged counterion at any of the established positions (**Fig. S2** [↗](#)). To investigate how the protonated Schiff base is stabilised in these opsins, we studied in more detail Antho2a ($\lambda_{\text{max}} = 503$ nm), as it could be expressed well in cultured cells and was stable in detergent-solubilised conditions (**Fig. 1B** [↗](#)).

(a) Contribution of Glu292 to the absorbance spectra of the dark state and photoproduct of Antho2a

First, we searched for potential counterions at positions different from known established amino acid sites (91, 113, and 181) in the Antho2a sequence. Using the crystal structure of bovine rhodopsin (PDB ID: 1U19) as a template, we identified glutamic or aspartic acids located within 5 Å of the Schiff base in Antho2a and other members in the ASO-II group. Notably, all *A. tenuis* opsins

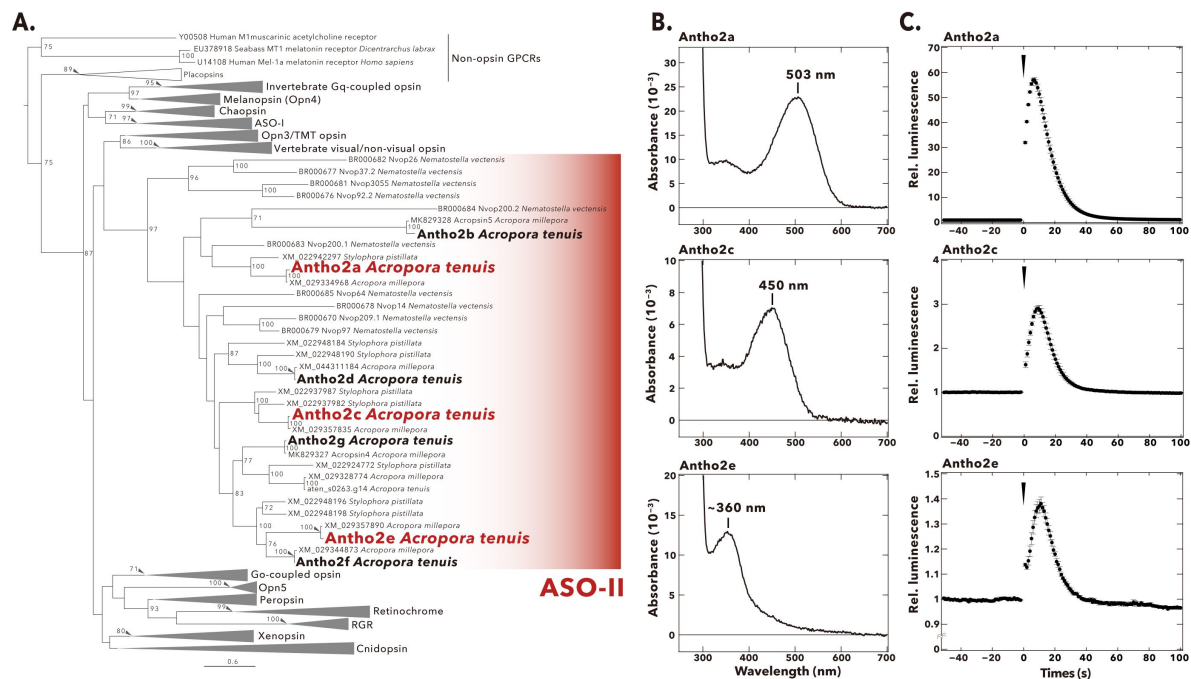


Fig. 1.

Phylogenetic tree, absorption spectra, and light-induced Ca²⁺ responses of *Acropora tenuis* opsins belonging to the ASO-II group.

(A) Maximum-likelihood (ML) tree of animal opsins including *A. tenuis* opsins in the ASO-II group. Seven opsins in the ASO-II group that were identified and cloned from *A. tenuis* in this study are shown in bold and the three members for which we obtained absorption spectra are highlighted in red. Numbers at the nodes represent support values of each ML branch estimated by 1000 bootstrap samplings ($\geq 70\%$ are indicated). Scale bar = 0.6 substitutions per site. All branches and support values are provided in Fig. S1. (B) Absorption spectra in the dark of three *A. tenuis* opsins in the ASO-II group (Antho2a, Antho2c and Antho2e). The absorption spectra were measured at 0°C in 140 mM NaCl at pH 6.5. The number in each graph shows the $\lambda_{\max}$ value. (C) Results of the aequorin-based bioluminescent reporter assay for monitoring light-induced changes in Ca²⁺ in HEK293S cells expressing the same three opsins in the ASO-II group as in B. In each graph, luminescence values were normalised to the baseline. Black circles with error bars indicate the means $\pm$ SEMs (n = 3) of the measured relative luminescence. Black arrowheads at time 0 indicate the timing of one-minute irradiation with green (495 nm; for Antho2a and Antho2c) or UV (395 nm; for Antho2e) light.

in this group contain a conserved glutamic/aspartic acid at position 292 (**Fig. S2**), positioned just one helix turn away from the retinal-binding residue Lys296. To determine whether Glu292 could function as the counterion in Antho2a, we mutated Glu292 to alanine and measured the absorption spectra. The absorption spectrum of the E292A mutant in the dark was nearly identical to that of wild type (**Fig. 2A, B**, curve 1), exhibiting a clear absorbance in the visible light region with only a slightly red-shifted λ_{max} (505 nm) at 140 mM NaCl and pH 6.5. This shows that a negative charge other than Glu292 may serve as a counterion in the dark state of wild type Antho2a.

We next investigated the spectroscopic properties of the photoproduct of wild type Antho2a and the E292A mutant. Upon irradiation of wild type Antho2a with orange light, the λ_{max} shifted from 503 nm in the dark to 476 nm in the photoproduct (**Fig. 2A**, curve 2; **Fig. S3A**, curves 2 and 3). This shift is due to the photoisomerisation of the 11-*cis* retinal chromophore to its all-*trans* form, converting almost 100% of the dark state to the photoproduct (**Fig. S3B**). The photoproduct remained stable for at least 5 minutes (**Fig. S3A**, curve 2 and curve 3), but it exhibited a gradual decay (**Fig. S3C, D**), indicating that purified Antho2a is neither strictly bleach resistant nor bistable (see also **Fig. S3** legend). In contrast to the dark state, the photoproduct of the E292A mutant displayed two distinct absorption peaks in UV and visible light regions, at ~370 nm and 476 nm respectively (**Fig. 2B**, curve 2). This suggests that the E292A mutation causes UV-light absorption due to a deprotonated Schiff base in the photoproduct. Additionally, altering the pH modified the ratio of absorbance between the ~370 nm and 476 nm peaks in the E292A mutant (**Fig. S4B**, curves 2), with the UV-peak to the visible light-peak ratio increasing at higher pH levels (pH 7.5, **Fig. S4B**, curve 2). Conversely, the wild type did not exhibit an increase in UV absorbance under similar high pH condition (pH 7.5, **Fig. S4A**, curve 2). These results indicate that the Schiff base in the photoproduct of the Antho2a E292A mutant has a lower acid dissociation constant (pK_a) than that of the wild type, suggesting that Glu292 acts as the counterion in the photoproduct of Antho2a.

We then further explored the nature of the counterion in the dark state of Antho2a. Previous studies have shown that in the bovine rhodopsin E113A and E113Q mutants, as well as in the retinochrome E181Q mutant (referred to as “counterion-less” mutants), halide ions like Cl^- can act as “surrogate” counterions to stabilise the proton on the Schiff base. Consequently, these counterion-less mutants can still absorb visible light in the presence of Cl^- (Nathans, 1990; Sakmar et al., 1991; Terakita et al., 2000). To assess the potential role of Cl^- as a surrogate counterion in the dark state of the Antho2a E292A mutant, we performed spectroscopic analyses under Cl^- -depleted conditions (**Fig. 2C, D**). We observed that the λ_{max} of the E292A mutant shifted to the UV region (**Fig. 2D**, curve 1). Unexpectedly, a similar shift in absorption to the UV region was also observed in the wild type under the Cl^- -depleted condition (**Fig. 2C**, curve 1). These results indicate that, in the absence of Cl^- , the Schiff base in both the wild type and the E292A dark states becomes deprotonated. The subsequent addition of Cl^- (final concentration: 140 mM NaCl) restored clear absorbance in the visible light region (**Fig. 2C, D**, curves 2), showing that Cl^- facilitates the protonation of the Schiff base of the dark state even in the wild type. In contrast, the photoproduct of the wild type exhibited no significant change in the ratio of UV to visible-light absorption peaks at pH 6.5 across NaCl concentrations from 0.28 mM to 800 mM (**Fig. S5**). The wild type consistently absorbed visible light under these NaCl conditions (curves 2 in **Fig. S5A, B**), suggesting that Cl^- has little impact on the Schiff base pK_a in the photoproduct of wild type Antho2a. However, the photoproduct of the E292A mutant exhibited a pH-dependent shift in the ratio of UV to visible-light absorption between pH 4.8 and pH 7.6, even at 800 mM NaCl, where the dark state predominantly absorbed visible light (**Fig. S5C**). This further supports that Glu292 serves as the counterion in the photoproduct of Antho2a.

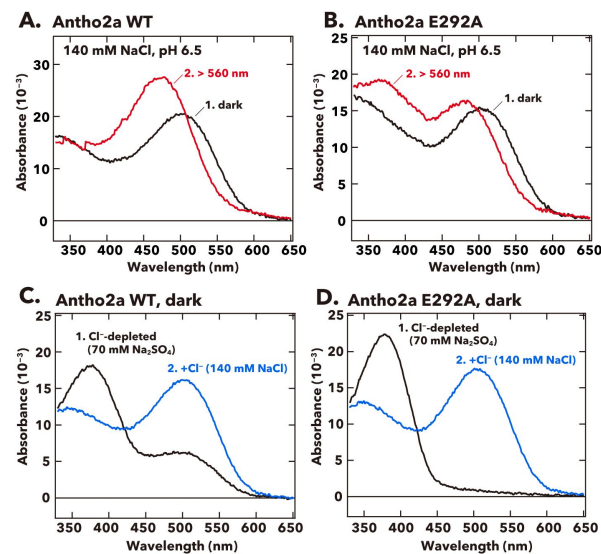


Fig. 2.

Absorption spectra of wild type and the E292A mutant of *A. tenuis* Antho2a.

(A, B) Absorption spectra of the dark state (curve 1, black) and the photoproduct (curve 2, red) of the wild type (Antho2a WT, A) and the E292A mutant (Antho2a E292A, B) at 140 mM NaCl and pH 6.5. The samples were kept at 0°C during the spectroscopic measurements. (C, D) Absorption spectra of the dark state of Antho2a WT (C) and Antho2a E292A (D) prepared in Cl⁻-depleted conditions, before (curve 1, black) and after (curve 2, blue) adding Cl⁻ (see Materials and Methods for details). In the Cl⁻-depleted condition, the pigments were solubilised in 70 mM Na₂SO₄, which reportedly does not access to the Cl⁻ binding site in the chicken red-sensitive cone visual pigment iodopsin (Shichida et al., 1990 [\[1\]](#)), to moderate protein denaturation.

(b) Effect of halide anions on λ_{max} values of the dark state of Antho2a

To obtain a further evidence supporting the Cl^- counterion in the dark state of Antho2a, we examined the impact of different halide anions on the absorption spectrum in the dark state of Antho2a, as observed in the bovine rhodopsin counterion-less mutant (Nathans, 1990 [↗](#); Sakmar et al., 1991 [↗](#)). Antho2a readily absorbed visible light in the presence of bromide ion (Br^-) and iodide ion (I^-) as well as Cl^- and the λ_{max} of wild type Antho2a shifted depending on the halide solutions (503 nm in 140 mM NaCl; 506 nm in 140 mM NaBr; 511 nm in 140 mM NaI solutions; **Fig. S6A** [↗](#)). The E292A mutant showed a similar shift in λ_{max} (505 nm in 140 mM NaCl; 507 nm in 140 mM NaBr; 517 nm in 140 mM NaI solutions; **Fig. S6B** [↗](#)).

(c) Effect of Cl^- concentration on the pK_a of the protonated Schiff base of Antho2a

To further investigate the influence of Cl^- on the protonation state of the Schiff base in the dark state of Antho2a, we estimated the pK_a of the Schiff base by measuring the pH dependent-changes in the absorption spectra of Antho2a at different Cl^- concentrations. The pH-dependent equilibrium between the visible (protonated Schiff base) and UV (deprotonated Schiff base) forms revealed that their ratio changes with Cl^- concentration (**Fig. S7A–E** [↗](#)). A plot of the changes in absorbance at λ_{max} against pH (**Fig. 3A** [↗](#)) shows that in wild type Antho2a, the pK_a of the protonated Schiff base increases with higher Cl^- concentrations (7.3 at 0.28 mM NaCl, 8.0 at 2.8 mM, 8.8 at 28 mM, 8.8 at 140 mM, and 9.0 at 500 mM). We failed to determine the pK_a at 0 mM NaCl, as the observed λ_{max} in acidic conditions (pH < 6.5) were shorter than expected in Antho2a (503 nm), suggesting that a normal pigment was not produced under these conditions (**Fig. S8A** [↗](#)). Similarly, the Cl^- concentration also affected the pK_a of the protonated Schiff base in the E292A mutant (6.1 at 2.8 mM NaCl, 6.8 at 28 mM, 7.7 at 140 mM, and 8.9 at 500 mM) (**Fig. 3B** [↗](#); **Fig. S9** [↗](#)). At 0 mM NaCl, the E292A mutant showed no visible light absorption, even under the most acidic conditions (pH 4.7), preventing the determination of its pK_a (**Fig. S8B** [↗](#)). Notably, at low Cl^- concentrations (2.8 mM NaCl), the wild type exhibited a higher pK_a than the E292A mutant (8.0 and 6.1, respectively). However, at 500 mM NaCl, the pK_a of the E292A mutant and wild type were comparable (9.0 and 8.9, respectively; **Fig. 3A, B** [↗](#)). These results suggest that Cl^- , rather than Glu292, serves as the counterion in the dark state of Antho2a, while Glu292 facilitates the protonation of the Schiff base by the Cl^- counterion.

(d) Binding affinity of Cl^- to wild type Antho2a and the E292A mutant

To evaluate the Cl^- binding affinities of both wild type Antho2a and the E292A mutant, we measured changes in their absorption spectra by gradually increasing Cl^- concentrations at pH 6.5 and estimated the Cl^- dissociation coefficients (K_d). The relative absorbance in the visible region increased with higher Cl^- concentrations both in the wild type and the E292A mutant (**Fig. S10A, B** [↗](#)). By fitting the Hill equation to the experimental data (**Fig. 3C** [↗](#)), the dissociation constants (K_d) of Cl^- were determined to be 0.079 ± 0.010 mM for the wild type Antho2a and 12.7 ± 0.519 mM for the E292A mutant. This significant increase in the K_d value for the E292A mutant suggests that the Cl^- binding affinity is considerably reduced due to the mutation. Consequently, we suggest that while Glu292 does not act as a direct counterion, it plays a crucial role in facilitating Cl^- binding to Antho2a.

Structural modelling and QM/MM calculations of the dark state of Antho2a

To gain a deeper understanding of the environment surrounding the retinylidene Schiff base in the dark state of Antho2a, we performed QM/MM-based structural modelling of both the wild type Antho2a (with Glu292 either neutral or negatively charged) and the E292A mutant. The QM/MM

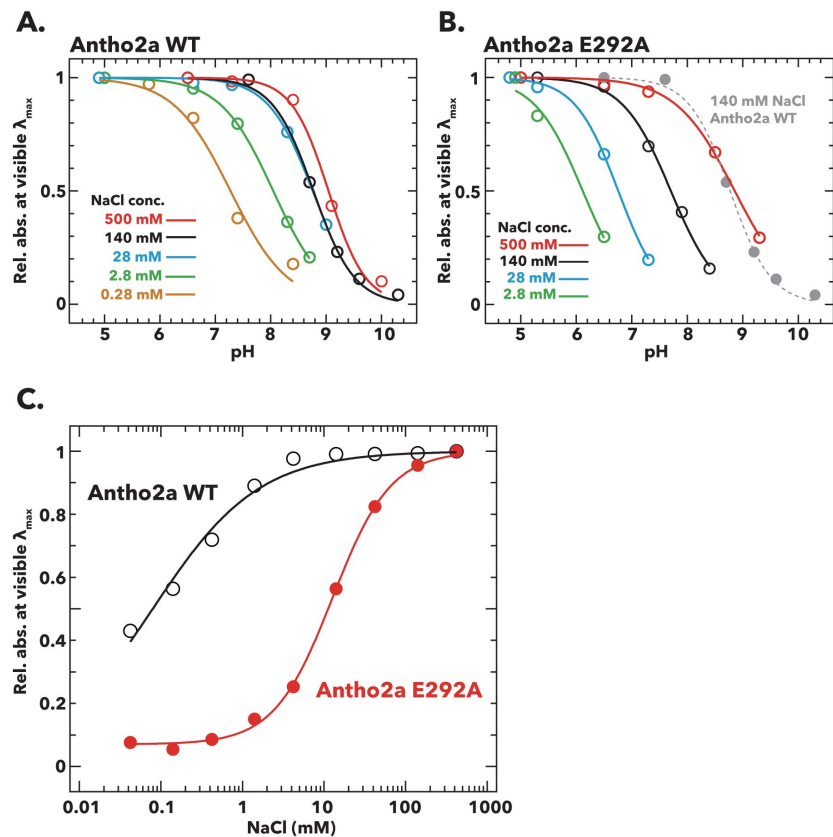


Fig. 3.

Effects of pH and Cl^- concentration on the absorption spectra of the dark states of wild type Antho2a and the Antho2a E292A mutant.

(A, B) Changes in the absorbance at λ_{max} as a function of pH for (A) wild type Antho2a and (B) the E292A mutant at different Cl^- concentrations. The absorbance values at “visible λ_{max} ” (mean absorbance at 503 ± 5 nm for the wild type and 505 ± 5 nm for the E292A mutant, respectively) were normalised for each Cl^- concentration to those at the lowest pH, in which the Schiff base is assumed to be fully protonated (“Rel. abs. at visible λ_{max} ” in the y-axes). Solid and dashed lines represent sigmoid fits to the experimental data for each Cl^- concentration (indicated by different colours). The pH-dependent change of wild type Antho2a at 140mM NaCl is also shown in B (dotted grey line). The full absorption spectra used to generate these plots are provided in Fig. S7 (for wild type Antho2a) and Fig. S9 (for the E292A mutant). (C) Changes in the absorbance at λ_{max} for wild type Antho2a (black open circles) and the E292A mutant (red solid circles) as a function of Cl^- concentration. The absorbance values at visible λ_{max} were normalised to those at 500 mM NaCl for both the wild type and the E292A mutant. The lines in the graph were generated by fitting the Hill equation to the experimental data.

geometry optimisation positioned the Cl^- ion close to the Schiff base ($\sim 3\text{\AA}$) and near Glu292 ($\sim 4.7\text{\AA}$), with Glu292 itself located in proximity to the Schiff base ($\sim 3.3\text{\AA}$) (**Fig. 4A, B**). To validate these models, we calculated the QM/MM vertical excitation energies of the ground state geometries (**Table S1**).

For wild type Antho2a with a protonated neutral Glu292, the calculated λ_{max} using the CAM-B3LYP/cc-pVTZ level of theory was 503 nm (**Fig. 4A**), in good agreement with the experimentally observed value (503 nm; **Fig. 2A**). In contrast, the λ_{max} calculated with a deprotonated negatively charged Glu292 was blue-shifted to 415 nm (**Fig. 4B**), deviating significantly from the experimental value. Finally, the calculated λ_{max} for the E292A mutant was 499 nm (**Fig. 4C**), also in agreement with the experimental value (505 nm). To further substantiate these findings, we recalculated the excitation energies using the RI-ADC(2)/cc-pVTZ method. Although these λ_{max} values are blue-shifted compared to those calculated with the CAM-B3LYP method, they followed a similar trend. Both these computational methods have previously been employed to accurately calculate the excitation energies of rhodopsins (Church et al., 2021). These results strongly suggest that in the dark state of Antho2a, Glu292 is protonated and neutral at pH 6.5, and therefore, it does not function as the counterion.

Effect of the Glu292 mutation on the function of the photoproduct

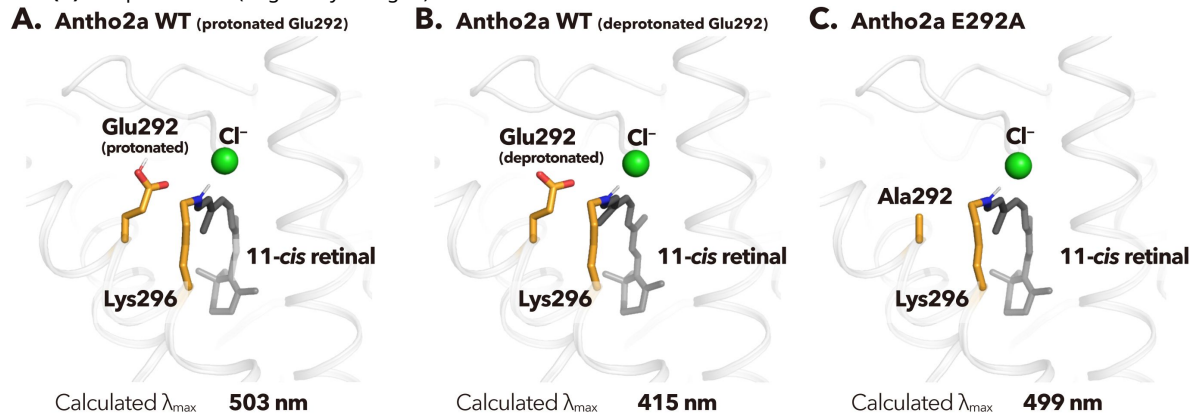
The spectroscopy data indicate that Glu292 is involved in stabilising the protonated Schiff base by facilitating Cl^- -binding in the dark state and also serves as a counterion in the photoproduct. This suggests that Glu292 significantly contributes to the visible light absorption of Antho2a. To explore additional roles of Glu292 in Antho2a, we measured the light-induced Ca^{2+} response in cultured cells expressing wild type Antho2a or the E292A mutant. Notably, cells expressing wild type Antho2a showed a ~ 30 -fold increase in Ca^{2+} levels upon light irradiation (**Fig. 5**, solid black circles), whereas cells expressing the Antho2a E292A mutant showed a smaller Ca^{2+} elevation (< 5 -fold increase) (**Fig. 5**, red open circles). This indicates that the peak Ca^{2+} response in cells expressing wild type Antho2a was approximately nine times greater than in cells expressing the E292A mutant. This result, along with the crucial role of Glu292 in Cl^- binding in the dark state and as a counterion in the photoproduct, suggests that Glu292 plays also a role in G protein activation.

Cl^- -dependent changes in the absorption spectra of the dark states of Antho2c and Antho2e

We tested whether Cl^- concentration affects the pK_a of the Schiff base in another visible light-sensitive opsin, Antho2c ($\lambda_{\text{max}} = 450\text{ nm}$, **Fig. 1B**). The pH-dependent equilibrium between UV- and visible-light absorbing forms was clearly observed at 0 mM or 0.093 mM NaCl, but not at 9.3 mM NaCl, where Antho2c stably absorbed visible light across the measured pH range (pH 4.8 to 7.2, **Fig. 6A–C**). Also, the ratio of UV to visible-light absorption increased with higher Cl^- concentrations at pH 6.5 (**Fig. 6D**). These results demonstrate that Cl^- serves as a counterion in the dark state of Antho2c, as it does in Antho2a. In contrast, wild type Antho2e continues to absorb UV light even at 1 M NaCl (**Fig. 6E**). Notably, Antho2e has an arginine at position 113, which corresponds to the counterion position in vertebrate visual opsins (**Fig. S2**). When this arginine is mutated to alanine (R113A), the mutant becomes sensitive to visible light ($\lambda_{\text{max}} = \sim 420\text{ nm}$) in the presence of Cl^- (**Fig. 6F**), suggesting that Cl^- can serve as the counterion in the R113A mutant.

Fig. 4.

Structural models of the retinal binding pocket in the dark state of **(A, B)** wild type Antho2a and **(C)** the E292A mutant obtained using QM/MM calculations. For wild type Antho2a, two models are shown: **(A)** with a protonated (neutral) Glu292 and **(B)** a deprotonated (negatively charged) Glu292.

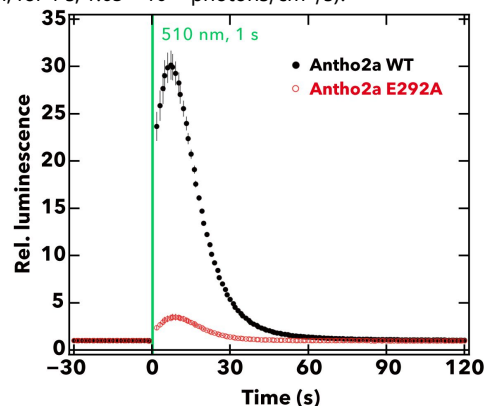


Experimentally obtained λ_{max} of Antho2a WT = **503 nm**

Fig. 5.

Comparison of the light-evoked intracellular Ca^{2+} levels between wild type Antho2a and the E292A mutant.

The graph shows the mean $\pm$ S.E.M ($n = 4$) of the measured relative luminescence values (luminescence values normalised to the baseline) for wild type Antho2a (black) and the E292A mutant (red). The green vertical line indicates the time of cell illumination with green light (510 nm, for 1 s, 1.65×10^{15} photons/cm²/s).



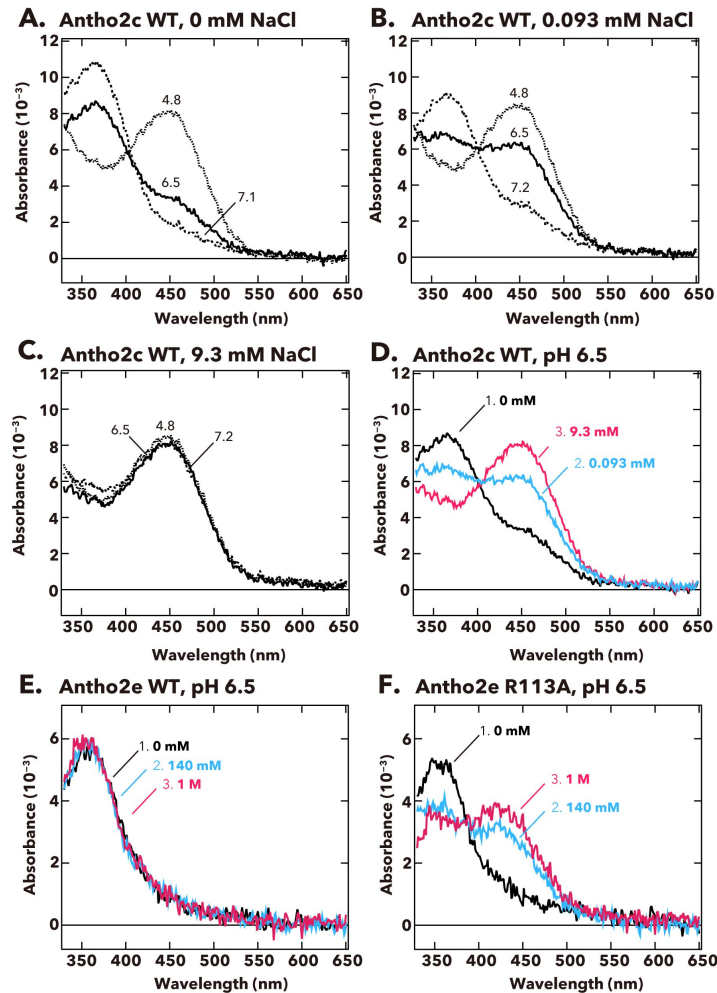


Fig. 6.

pH-dependent changes in the absorption spectra of Antho2c and Antho2e at different Cl^- concentrations at 0°C.

(A–C) Absorption spectra of purified wild type Antho2c pigment at (A) 0 mM, (B) 0.093 mM, and (C) 9.3 mM NaCl concentrations. The corresponding pH values are indicated on each curve in the graphs. (D) Summary of the spectral changes for wild type Antho2c across different Cl^- concentrations at neutral pH (pH 6.5). (E, F) Absorption spectra of (E) wild type Antho2e (Antho2e WT) and (F) its R113A mutant (Antho2e R113A) at different Cl^- concentrations at pH 6.5 at 0°C. Each colour indicates a different Cl^- concentration.

Discussion

In this study, we reveal for the first time the spectral properties of opsins in the ASO-II group from the coral *Acropora tenuis*, showing that their sensitivity spans from UV to visible light. Opsins in this group have a highly conserved Glu292 residue near the Schiff base, which can potentially stabilise the proton on the Schiff base. Indeed, our results show that the pK_a of the protonated Schiff base in the photoproduct ($\lambda_{\max} = 476$ nm) of Antho2a is altered when Glu292 is substituted with alanine (**Fig. 2A, B**; **Fig. S4**), suggesting that Glu292 serves as the counterion of the photoproduct. Conversely, the dark state of Antho2a ($\lambda_{\max} = 503$ nm) exhibits robust visible light absorption only in the presence of Cl^- at physiological pH, and the pK_a of the protonated Schiff base changes with Cl^- concentration in both wild type Antho2a and the E292A mutant. Furthermore, the pK_a for wild type and the E292A mutant are comparable in the presence of sufficient Cl^- (500 mM NaCl, **Fig. 3A, B**), supporting the conclusion that Cl^- , and not Glu292, acts as the counterion of the dark state of *A. tenuis* Antho2a. We found that the type of halide anions in the solution affected the $\lambda_{\max}$ values of the dark state of Antho2a, which is consistent with the observed effect on the $\lambda_{\max}$ of the bovine rhodopsin counterion-less mutant, in which halide ion serves as a surrogate counterion (Nathans, 1990; Sakmar et al., 1991), further substantiating our hypothesis that a solute anion acts as the counterion in the dark state of Antho2a. Moreover, QM/MM calculations for the dark state of Antho2a suggest that Glu292 is protonated and neutral, further supporting the hypothesis that Glu292 does not serve as the counterion in the dark state. Along with the result showing Cl^- has little to no effect on the visible light absorption of the photoproduct (**Fig. S5**), unlike the wild type, we conclude that Cl^- and Glu292, respectively, act as counterions for the protonated Schiff base of the dark state and photoproduct of Antho2a. This represents a unique example of counterion switching from exogenous anion to a specific amino acid residue upon light irradiation.

Our spectroscopic data also showed that the other visual light sensitive opsin, Antho2c, exhibited the Cl^- dependency on the Schiff base pK_a of the dark state, which suggesting that opsins in the ASO-II group may share a spectral tuning mechanism based on the Cl^- counterion. Interestingly, Antho2e had an arginine at position 113 and when it was mutated to alanine (R113A), the mutant showed sensitivity to visible light in the presence of Cl^- (**Fig. 6F**). We hypothesise that the positive charge of Arg113 disturbs interaction between the Cl^- and the protonated Schiff base or that it completely inhibits Cl^- binding, rendering Antho2e UV-sensitive.

It is noteworthy that although Cl^- has been reported to serve as a surrogate counterion in “counterion-less” mutants of animal opsins (such as E113Q in bovine rhodopsin and E181Q in retinochrome) (Nathans, 1990; Sakmar et al., 1991; Terakita et al., 2000), Antho2a is, to our knowledge, the first example in a wild type animal opsin that employs Cl^- as a counterion. Interestingly, within the microbial rhodopsin family, heliorhodopsin (TaHeR) incorporates a Cl^- into the Schiff base region under high Cl^- concentrations at pH 4.5. This Cl^- stabilises the protonated retinal Schiff base when its primary counterion, E108, is neutralised (Besaw et al., 2022). The observed 18 nm red shift at low pH is consistent with E108 protonation. The TaHeR E108A mutant shows the same $\lambda_{\max}$ under high Cl^- concentrations, further supporting the role of Cl^- as a counterion. At pH 8, the Schiff base proton is stabilised by the negatively charged E108 (Shihoya et al., 2019).

The E292A mutation in Antho2a drastically decreases the Cl^- binding affinity in the dark state at pH 6.5 (K_d : Antho2a WT = 0.079 mM, Antho2a E292A = 12.7 mM). Based on these results, supported by our QM/MM calculations of the dark state, we hypothesise that protonated Glu292 not only serves as a counterion of the photoproduct but also constitutes part of the Cl^- binding site in the dark state of Antho2a. In Cl^- -pumping microbial rhodopsins, where Cl^- serves as a counterion, the Cl^- binding site near the Schiff base is typically formed by hydrogen-bonding between Cl^- , the

protonated Schiff base, and key amino acids such as serine, threonine and aspartic acid, and water molecules (Besaw et al., 2020 [↗](#); Hosaka et al., 2016 [↗](#); Kolbe et al., 2000 [↗](#)). Additionally, the bovine rhodopsin double mutant E113Q/A292E exhibits a higher sensitivity to hydroxylamine than the wild type, indicating instability of the Schiff base and increased solvent accessibility (Tsutsui and Shichida, 2010 [↗](#)). These observations suggest that Glu292 in Antho2a may also facilitate the accessibility of Cl⁻ ions to the Schiff base environment. To determine the precise nature of the Cl⁻ binding site and clarify the roles of Glu292 and other residues in the Cl⁻-dependent counterion system of Antho2a, detailed spectroscopic and structural experiments will be necessary.

We also found that the cells expressing the Antho2a E292A mutant show a lower Ca²⁺ elevation upon light stimulation compared to the wild type (Fig. 5 [↗](#)). This effect could be due to Glu292 not only serving as a counterion in the photoproduct but also allosterically influencing G protein activation. It has been reported that the introduction of Glu or Asp at position 292 can affect various molecular properties of opsins. For instance, the A292E mutation in human rhodopsin has been shown to result in constitutively active apoproteins, leading to congenital night blindness (Dryja et al., 1993 [↗](#); Jin et al., 2003 [↗](#)). These data suggest that Glu292 has the potential to influence functional properties, such as G protein activation. Several studies have reported that the counterion can affect diverse properties of opsins beyond their absorption spectra. For instance, in bovine rhodopsin, Glu113 is involved not only in visible light absorption in the dark state but also in the efficient activation of G proteins by the photoproduct (Terakita et al., 2004 [↗](#)). It has been suggested that these pleiotropic functions of the counterion have been achieved through evolutionary modifications of the protein structure once the counterion was acquired (Terakita et al., 2004 [↗](#)). This concept may similarly apply to Antho2a.

One of the notable features of the opsins in the ASO-II group that use chloride as a counterion is the relatively low pK_a of the Schiff base (e.g., ~9.0 for Antho2a in the dark state, Fig. 3A [↗](#)) compared to other animal opsins with a “regular” amino acid counterion (e.g., >16 for bovine rhodopsin, with Glu113 as a counterion (Steinberg et al., 1993 [↗](#)) or 11 for jumping spider Rh1, with Glu181 as a counterion (Nagata et al., 2019 [↗](#))). We hypothesise that opsins in the ASO-II group, with lower Schiff base pK_a, may change their spectral sensitivity and G protein activation profile within the physiological pH range of corals. For instance, these opsins could exhibit decreased sensitivity to visible and increased sensitivity to UV light under alkaline pH conditions. The extra- and intracellular pH environments in symbiotic cnidarians such as corals are spatially and temporally variable due to the photosynthesis of symbiotic algae (Barott et al., 2017 [↗](#)). For example, pH values in the cytosol of algae-hosting cells (a group of endodermal cells with algal occupancy) in corals reportedly increase by approximately 0.5 pH units upon light treatment (Venn et al., 2009 [↗](#)). The pH values in the gastrovascular cavity (coelenteron), which is in contact with endodermal cells, range from 6.6 to 8.5 (Agostini et al., 2012 [↗](#); Al-Horani et al., 2003 [↗](#); Cai et al., 2016 [↗](#)) and increase in the presence of light, presumably due to the consumption of CO₂ by photosynthesis (Barott et al., 2017 [↗](#)). Additionally, alkalinisation in extracellular region of calcifying cells reportedly increases ~1.0 pH units from dark to light conditions reaching levels above pH 9.0 (Al-Horani et al., 2003 [↗](#)). These pH changes, generally an increase due to photosynthetic activity, may result in variable light sensitivity of the “low pK_a” opsins in the ASO-II group. Recent studies have reported that members of the ASO-II group may be associated with the symbiotic relationship between host anthozoans and symbiotic algae. For example, using the symbiotic sea anemone *Exaiptasia diaphana*, Gornik et al. (2021) showed that the mRNA levels of several opsins in the ASO-II group were higher in symbiotic adults than in apo-symbiotic ones (Gornik et al., 2020 [↗](#)). In sea anemone, it has also been reported that behavioural responses to light differ between symbiotic and apo-symbiotic individuals (Foo et al., 2020 [↗](#); Kishimoto et al., 2023 [↗](#)), with these responses potentially being driven by the activation of ASO-II opsins that are up-regulated in the presence of symbiotic algae. Moreover, single-cell RNA-seq analysis in the reef-building coral *Stylophora pistillata* has revealed that an opsin belonging to the ASO-II group is specifically expressed in algae-hosting cells (a group of endodermal cells with 50% algal occupancy) (Levy et al., 2021 [↗](#)). Although further studies are needed, we suggest that the unique

use of Cl^- as the counterion in opsins of ASO-II group, rather than a negatively charged amino acid, may be associated to their pH-sensitive light response and, ultimately, to their role in photosynthesis-related functions in symbiotic cnidarians.

It is widely accepted that opsins have evolved from a non-opsin GPCR (Feuda et al., 2012). Our finding of the native chloride counterion in opsins shows a possibility that in the first stage of the evolutionary process, the “primitive” opsins having Lys296 but no counterion residue could absorb not only UV light but also a wide range of wavelengths that extends into the visible region by embracing chloride ions. Namely, the chloride counterion system might have been a preliminary step in the evolution of amino acid counterions in animal opsins. Further empirical and bioinformatic studies are required for disentangling the evolutionary trajectories of the Schiff base – counterion system, including the chloride counterion.

Materials and Methods

Experimental design

We first identified and cloned opsins from a reef-building coral, *Acropora tenuis*, and then expressed opsins belong to the ASO-II group in mammalian cultured cells. We performed spectroscopic measurements of purified pigments of the opsins in different pH and Cl^- conditions to identify their effects on the acid dissociation constant of the protonated Schiff base of the opsins, leading to the determination of the counterion. Computational modelling and QM/MM calculations were also conducted to elucidate the retinylidene Schiff base environment in the dark state of Antho2a. Light-evoked Ca^{2+} responses were assessed by aequorin-based bioluminescent reporter assay to evaluate the G protein activation of Antho2a.

Identification of *Acropora tenuis* opsins and phylogenetic tree inference

Acropora tenuis (Dana, 1846) is a common reef-building coral distributed throughout the Indo-Pacific Ocean. Candidate sequences of *A. tenuis* opsins were identified by homology search against public genome and transcriptome datasets (Shinzato et al., 2020; Voolstra et al., 2015) and their phylogenetic relationships to known opsins were inferred by subsequent phylogenetic tree reconstruction. We first conducted BLASTP and TBLASTN searches with an E-value cut-off of 10^{-10} using *Acropora palmata* Acropsin 1–3 (JQ966100 – JQ966102), two *Nematostella vectensis* opsins (BR000676 – BR000677), human rhodopsin (NM_000539), and squid rhodopsin (X70498) as queries. We aligned the collected opsin homologs and excluded sequences which did not contain a retinal-binding lysine residue (Lys296) in the seventh transmembrane helix. We modified the fragmented sequences by reference to the genome sequence of *A. tenuis* and opsin sequences of other *Acropora* species (*A. palmata* or *A. millepora*). The candidate sequences of *A. tenuis* opsins were combined with the representative opsin sequences. The final sequence set was aligned using MAFFT (Katoh and Standley, 2013) and trimmed by TrimAl (Capella-Gutiérrez et al., 2009) with the “gappyout” function. The ML tree was reconstructed using RAXML-NG v1.1.0 (Kozlov et al., 2019) assuming the LG + G4 model of protein evolution, which was selected by ModelTest-NG v0.2.0 (Darriba et al., 2020). The ML branch supports were estimated with 1000 bootstrap replicates.

Sample collection, total RNA extraction, and cDNA synthesis

Colonies of *A. tenuis* were collected from < 3 m depth on the fringing reef on Sesoko Island, Okinawa (N26°37.58', E127°52.01') and were maintained in flow-through aquaria at Sesoko Station (Tropical Biosphere Research Center, University of Ryukyus, Okinawa, Japan). Four days after spawning, motile larvae and small branches of adult colonies were preserved in RNAlater® Stabilization Solution (Thermo Fisher Scientific, MA, USA). Total RNAs were extracted from the

larval and adult samples using TRIzol reagent (Thermo Fisher Scientific) or Sepasol-RNA I Super G (nacalai tesque, Kyoto, Japan) and purified using Qiagen RNeasy mini kits (Qiagen, Hilden, Germany) following the manufacturer's protocol. cDNAs were synthesised from the total RNA by reverse transcription using High-Capacity cDNA Reverse Transcription kits (Thermo Fisher Scientific).

Expression and purification of *Acropora tenuis* opsins

The coding regions of *A. tenuis* opsins were amplified by PCR with gene-specific primers and were tagged with the epitope sequence of the anti-bovine rhodopsin antibody Rho1D4 (ETSQVAPA) at their C-termini. Site-directed mutants were produced by overlap extension PCR using PrimeSTAR Max DNA Polymerase (TAKARA, Shiga, Japan) with site-specific primers and were also tagged with the 1D4 epitope sequence. The tagged cDNAs were inserted into the pUSRa vector (Kayada et al., 1995 [\[4\]](#)) digested with HindIII and EcoRI or the pMT vector (Ridge and Abdulaev, 2000 [\[5\]](#)) digested with EcoRI and NotI using In-Fusion HD cloning kit (TAKARA). The plasmids (15 µg per 100 mm culture dish) were transfected into COS-1 cells using the polyethyleneimine (PEI) transfection method. The transfected cells were maintained for 24 h after transfection at 37°C under 5% CO₂ and then 11-*cis* retinal was added to the medium (1 µL of 4 mM 11-*cis* retinal to 100 mm culture dish) following 25°C or 30°C incubation for another 24 h in the dark before collecting the cells. The reconstituted pigments were extracted from the cell membranes with 1% dodecyl β-D-maltoside (DDM, Dojindo, Kumamoto, Japan), 50 mM HEPES, and 140 mM NaCl (pH 6.5). The solubilised samples were mixed with 1D4-conjugated agarose beads overnight, and the mixture was transferred into Bio-Spin columns (Bio-Rad, Hercules, CA, USA) and washed in the buffer containing 0.02% DDM, 50 mM HEPES, and 140 mM NaCl (pH 6.5, buffer A). The purified pigments were eluted with buffer A containing 0.5 ~ 1 mg/mL 1D4 peptide (custom peptide synthesis by GenScript Japan Inc., Tokyo, Japan). To obtain pigments in solutions of various anions (SO₄²⁻, Br⁻, and I⁻) other than Cl⁻, samples were prepared as described above and in the final step the mixture of solubilised samples and 1D4-agarose beads was washed with buffer A followed by the additional wash with buffers including different sodium salts of anions (0.02% DDM, each of 70 mM Na₂SO₄, 140 mM NaBr, or 140 mM NaI, and 50 mM HEPES). Then the pigments were eluted with the buffer including the appropriate sodium salt of anion containing 0.5 ~ 1 mg/mL 1D4 peptide. Alternatively, for some pigments that were unstable under the absence of Cl⁻, we quickly removed Cl⁻ by gel-filtration chromatography on PD MiniTrap desalting columns with Sephadex G-25 resin (Cytiva, Marlborough, MA, USA). The columns were first equilibrated with the buffer including 0.02% DDM, 70 mM Na₂SO₄, and 50 mM HEPES, 500 µL of samples were loaded onto the columns and eluted with the buffer. We collected 800 µL fractions and used them for subsequent spectroscopic analyses.

UV-visible spectroscopy

Spectroscopic measurements were performed at 0°C using a V-750 UV-visible spectrophotometer (JASCO Corporation, Tokyo, Japan). The pH of the samples was adjusted with 100 mM CAPS including NaOH for alkaline conditions and 500 mM NaH₂PO₄ for acidic conditions. pH values were measured using a pH meter (B-211; HORIBA, Kyoto, Japan) immediately after each spectroscopic measurement. The concentration of Cl⁻ in the samples was adjusted by addition of different concentrations of NaCl solutions which were prepared in 70 mM Na₂SO₄ buffer (see above). A 100-W halogen lamp was equipped on the spectrophotometer and used to illuminate samples with a set of optical interference filters (420 nm, Toshiba) and cut-off filters (O-55 or O-56, AGC Techno Glass Co., Shizuoka, Japan). Absorption spectra of some UV-absorbing pigments were recorded using the V-750 UV-visible spectrophotometer, equipped with a 300-W xenon lamp (MAX-350; Asahi Spectra Co., Tokyo, Japan) that was used for illumination of samples in combination with a UV-transmitting filter (UTVAF-50S-36U, SIGMA KOKI, Tokyo, Japan).

HPLC analysis

An HPLC analysis was carried out to analyse the conformations of retinal present in the purified pigments. 100 μ L of purified pigments were mixed with 210 μ L of cold 90% methanol which was stored in -20°C and 30 μ L of 1 M hydroxylamine to convert retinal chromophore in a sample into retinal oxime. The retinal oxime was extracted with 700 μ L of *n*-hexane. 200 μ L of the extract were injected into a YMC-Pack SIL column (particle size 3 μ m, 150 $\times$ 6.0 mm) and eluted with *n*-hexane containing 15% (v/v) ethyl acetate and 0.15% (v/v) ethanol at a flow rate of 1 mL/min.

Bioluminescent reporter assays for

Ca^{2+} measurements in cultured cells

Ca^{2+} levels in opsin-expressing cultured cells were assessed by an aequorin-based luminescent assay as described previously (Koyanagi et al., 2022 [DOI](#)). Briefly, the plasmid containing open reading frames of each opsin was transfected into HEK293S cells in 35 mm dishes by the PEI method with the aequorin plasmid obtained by introducing a reverse mutation A119D into the plasmid [pcDNA3.1+/mit-2mutAEQ] (Addgene no. 45539) (de la Fuente et al., 2012 [DOI](#)). The transfected HEK293S cells were incubated for ~ 24 hours at 37°C under 5% CO_2 with the addition of 0.2 μM /dish of 11-*cis* retinal 4 $\sim$ 5 hours after the transfection. Before the luminescence measurements, the culture medium was replaced with a medium containing Coelenterazine *h*, and the cells were incubated to equilibrate with the media at 25°C for at least 2 hours. Dishes of cells were then stimulated with light and luminescence values were recorded using GloMax 20/20n Luminometer (Promega). A green (495 nm) LED light (colour: “Cyan”, Ex-DHC; BioTools Inc., Gunma, Japan) and arrayed LEDs on a board with spectral emission peaks at 390 nm and 510 nm (SPL-25-CC; REVOX Inc., Kanagawa, Japan) were used as light sources.

Computational modelling and QM/MM calculations

The three-dimensional structure of Antho2a was predicted from the primary amino acid sequence using AlphaFold2 (Jumper et al., 2021 [DOI](#)). The 11-*cis* retinal chromophore linked to the protonated Schiff base was incorporated into the AlphaFold model using as a template the high-resolution structure of bovine rhodopsin solved by time-resolved serial femtosecond X-ray crystallography (Gruhl et al., 2023 [DOI](#)). A Cl^- anion was initially placed in close proximity to the retinal protonated Schiff base, as observed in the microbial chloride-pump halorhodopsin (Mous et al., 2022 [DOI](#)). Water molecules were added using HomolWat (Mayol et al., 2020 [DOI](#)). We determined the pK_a values of the titratable amino acid residues at pH 6.5 using the PROPKA program (Olsson et al., 2011 [DOI](#); Søndergaard et al., 2011 [DOI](#)) and subsequently, the protein was protonated using the tleap program in the AMBER software package (Case et al., 2016 [DOI](#)). The geometry of this initial model was first relaxed by molecular mechanics energy minimisation with the Amber ff14SB force field (Maier et al., 2015 [DOI](#)) using steepest descent for 10,000 steps before switching to a conjugate gradient minimiser for an additional 10,000 steps. During energy minimisation, a positional restraint of 10 $\text{kcal mol}^{-1}\text{\AA}^{-2}$ was applied to all atoms, including hydrogens. The SHAKE algorithm (Ryckaert et al., 1977 [DOI](#)) was used to constrain the motion of bonds involving hydrogen. Finally, the geometry of the system was optimised using hybrid quantum mechanics/molecular mechanics (QM/MM) calculations without considering any external environment and with the backbone of the protein frozen (Mrogiński et al., 2021 [DOI](#); Senn and Thiel, 2009 [DOI](#)). The QM part consists of the retinal chromophore linked to the lysine sidechain cut between the C δ and C ϵ atoms forming the protonated Schiff base, along with Cl^- , Glu292 and Ser186. The hydrogen link atom (HLA) scheme was used at the QM/MM boundary. The QM part was treated using the BP86-D3 (BJ) functional (Becke, 1988 [DOI](#); Grimme et al., 2011 [DOI](#)) in conjunction with the cc-pVDZ basis set (Dunning, 1989 [DOI](#)) and the def2/J auxiliary basis set for the resolution of identity (RI) (Weigend, 2008 [DOI](#)). The Chain of Spheres exchange (COSX) algorithm was utilised in combination with the resolution of identity for the Coulomb term (RI-J). The rest of the protein was treated with the Amber ff14SB force field. Water molecules were treated with the TIP3P model (Jorgensen et al., 1983 [DOI](#)). The

QM/MM calculations were performed using the quantum chemistry program Orca 5.0.2 (Neese, 2022 [↗](#)) interfaced with the DL_POLY module of the ChemShell 3.7.1 software package (Metz et al., 2014 [↗](#); Sherwood et al., 2003 [↗](#)). The optimised ground state geometries and partial charges were used to calculate the vertical excitation energies using the simplified time-dependent density functional theory (STD-DFT) (Bannwarth and Grimme, 2014 [↗](#); Runge and Gross, 1984 [↗](#)) at the CAM-B3LYP/cc-pVTZ level of theory (Dunning, 1989 [↗](#); Yanai et al., 2004 [↗](#)) using the Orca program. The excitation energies were also calculated using the RI-ADC(2) method (Hättig, 2005 [↗](#)) with frozen core orbitals and the cc-pVTZ basis set in association with the corresponding auxiliary basis by utilising the Turbomole 7.5.1 program package (Furche et al., 2014 [↗](#)). The three-dimensional models were visualised using the molecular graphics program PyMOL 2.5.5.

Supplementary table and figures

QM Region	sTD-DFT CAM-B3LYP		ADC(2)	
	ΔE_{calc} nm (eV)	f	ΔE_{calc} nm (eV)	f
RET+Lyr296+Cl+Ser186+ Glu292 (deprotonated)	415 (2.99)	1.54	374 (3.32)	1.64
RET+Lyr296+Cl+Ser186+ Glu292 (protonated)	503 (2.47)	1.19	416 (2.98)	1.43
RET+Lyr296+Cl+Ser186+ Ala292	499 (2.49)	1.19	426 (2.91)	1.36

Table S1.

Vertical excitation energies (ΔE_{calc}) and oscillator strengths (f) computed by quantum mechanics/molecular mechanics (QM/MM) calculations using different QM methods with the cc-pVTZ basis set.

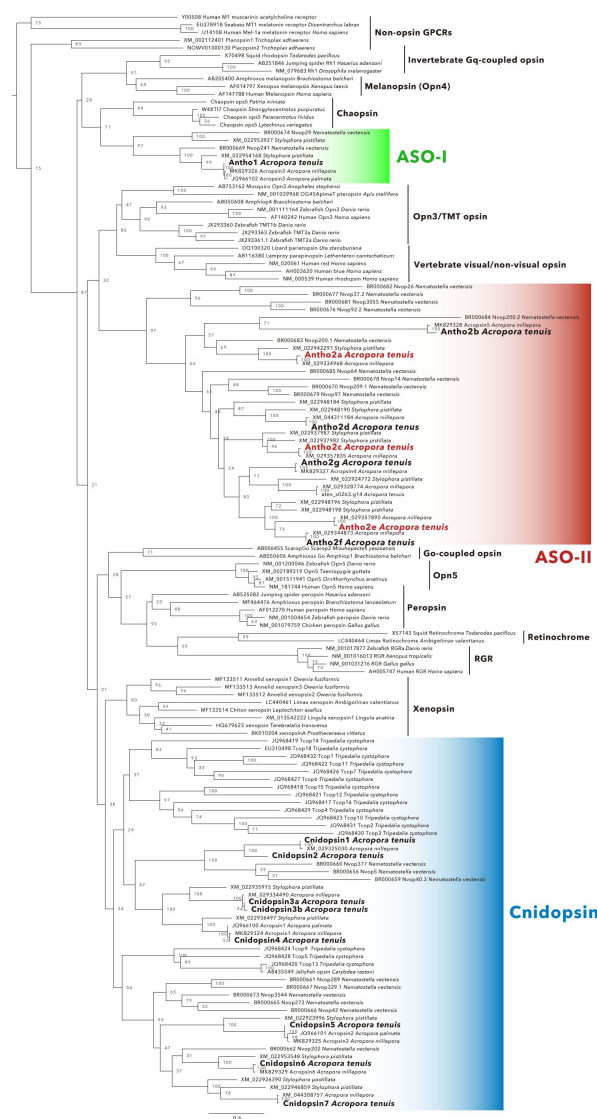


Fig. S1.

Maximum-likelihood (ML) tree of animal opsins, with non-opsin GPCRs included as an outgroup (a simplified version of the ML tree is shown in **Fig. 1A**). The tree includes opsins belonging to the eight main groups (see main text) as well as of opsins from the recently identified subgroups xenopsins and chaopsins⁶. The sixteen *A. tenuis* opsins (eight opsins in the cnidopsin group, one opsin in the ASO-I group, and seven opsins in the ASO-II group) that were identified in this study are shown in bold. The three opsins in the ASO-II group for which absorption spectra were successfully obtained are highlighted in red. Numbers at nodes represent support values for the ML branch estimated by 1000 bootstrap samplings ($\geq 70\%$ are indicated). Scale bar = 0.6 substitutions per site.

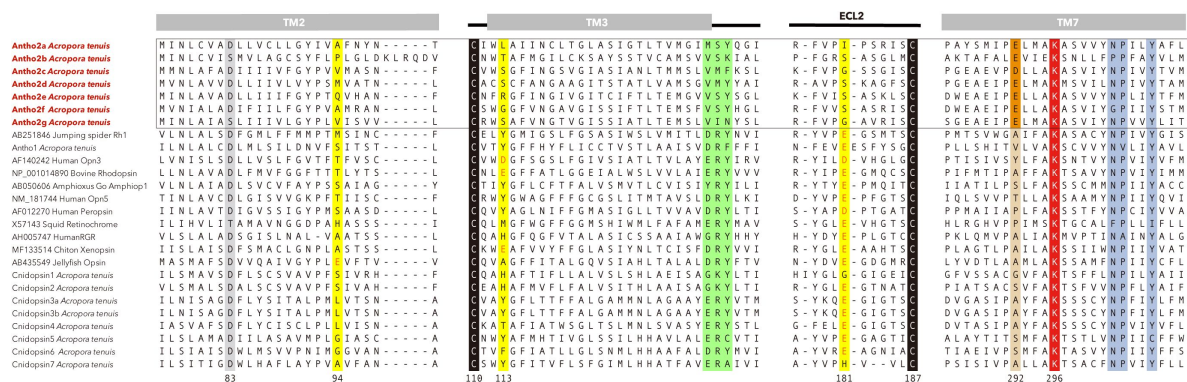


Fig. S2.

Amino acid sequence alignment of opsins in the ASO-II group (in red) with other animal opsins.

Only selected regions of the full alignment are shown, with their secondary structure elements displayed at the top. The alignment reveals residues and motifs in the ASO-II group that are broadly conserved throughout the large family of Class A GPCRs, such as the key functional residue D83 (grey) in TM2, a disulphide bridge between C110 in TM3 and C187 in ECL2 (black), and the NPxxY motif in TM7 (blue). Strikingly, ASO-II opsins lack the highly conserved and functionally key E(D)RY motif on TM3 (green). ASO-II opsins also possess residues specific to animal opsins, such as the retinal binding K296 (red). While they do not have E/D at any of the three established counterion positions (yellow), they feature a highly conserved Glu/Asp at position 292 (orange).

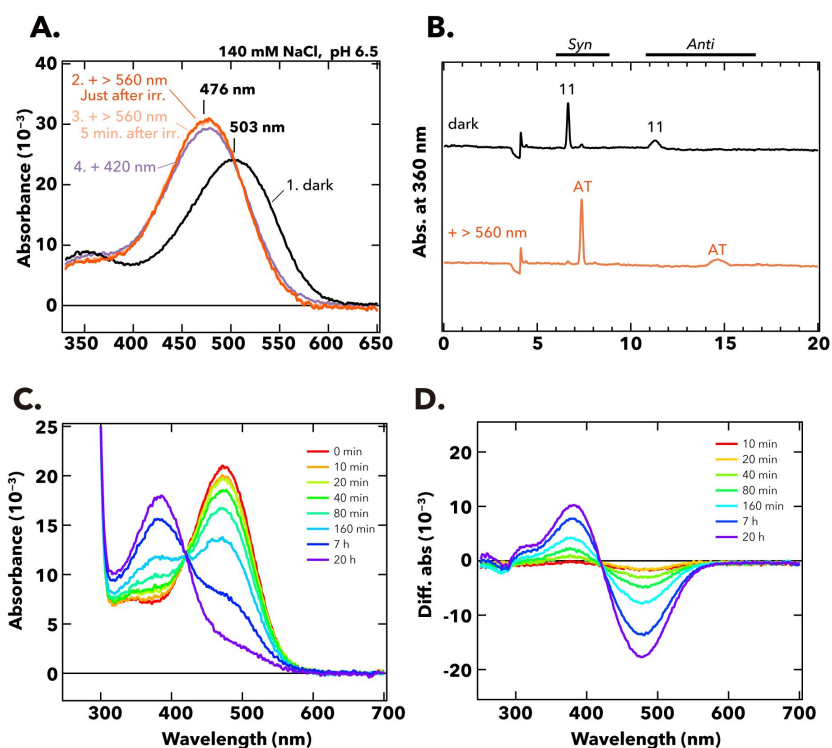


Fig. S3.

Photo- and thermal reactions of wild type Antho2a.

(A, B) Absorption spectra and isomer compositions of the chromophore retinal of Antho2a WT before and after light irradiation. (A) Absorption spectra were measured at 0°C in the dark (curve 1, black), after orange light irradiation (> 560 nm, curve 2 and curve 3, deep and pale orange), and after subsequent violet light irradiation (420 nm, curve 4, purple). Irradiation of wild type Antho2a with orange light shifted the absorption maximum from 503 nm in the dark (curve 1, black) to 476 nm in the photoproduct (curve 2 and curve 3, orange). Upon subsequent irradiation with 420 nm light (while the photoproduct was stable), the λ_{max} of the photoproduct stayed at 476 nm, with only a slight decrease of the peak absorbance (curve 4, purple) possibly resulting from degradation of the photoproducts upon light irradiation. (B) The configuration of retinal in Antho2a WT before (black) and after (orange) irradiation with orange light (> 560 nm) was analysed with HPLC. Retinal was extracted in its oxime form. AT, all-*trans* retinal; 11, 11-*cis* retinal. (C, D) Thermal stability of the photoproducts of wild type Antho2a in 140 mM NaCl at pH 6.5 and 0 °C. (C) Changes in the absorption spectra of Antho2a WT after irradiation with orange (>560 nm) light (with the sample kept in the dark at 0°C). Each coloured curve corresponds to a different time after light irradiation. (D) Difference spectra obtained by subtracting the spectrum of Antho2a WT in the dark from the spectra measured at different time points after irradiation (shown in C).

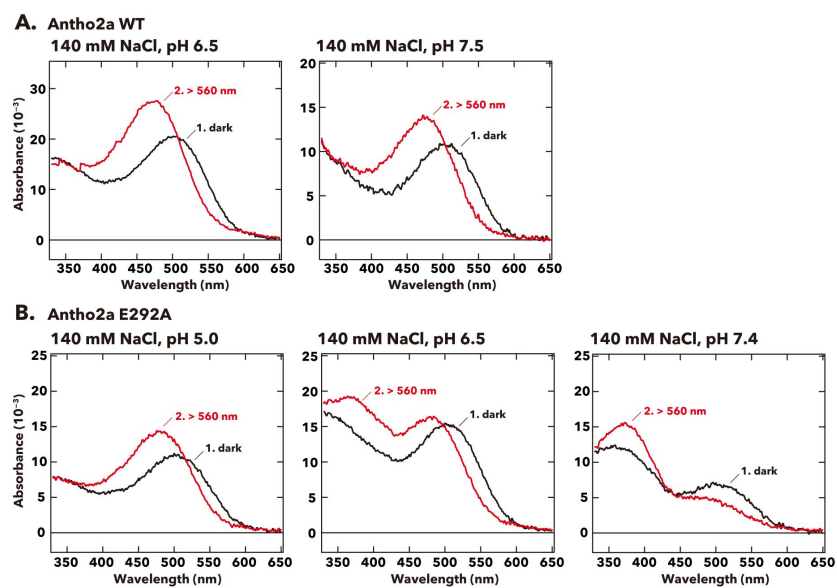


Fig. S4.

(A, B) Effect of pH on the absorption spectra of the dark state and photoproduct of (A) wild type Antho2a and (B) the E292A mutant at 140 mM NaCl.

Each graph shows spectra before (curves 1, black) and after (curves 2, red) irradiation with orange (> 560 nm) light.

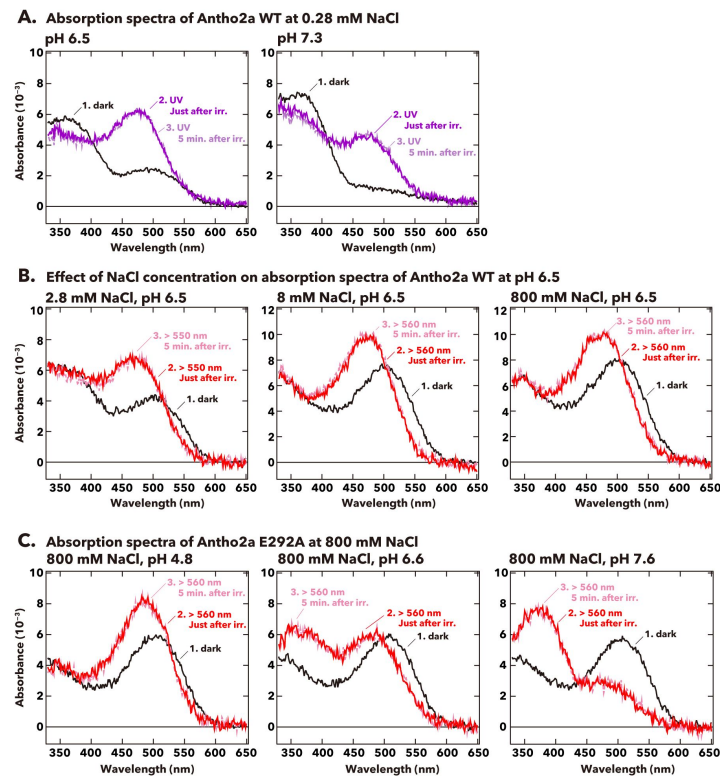


Fig. S5.

Effect of pH and NaCl concentration on absorption spectra of the dark states and photoproducts of Antho2a.

(A) Absorption spectra of wild type Antho2a at 0.28 mM NaCl under different pH conditions (pH 6.5 and pH 7.3). (B) Absorption spectra of wild type Antho2a at pH 6.5 with varying NaCl concentrations (2.8 mM, 8 mM, and 800 mM). (C) Absorption spectra of the E292A mutant of Antho2a at 800 mM NaCl and different pH conditions (pH 4.8, pH 6.6, and pH 7.6). Each graph shows spectra before (curves 1, black) and after (curves 2, deep purple/red, and 3, pale purple/pink) irradiation with UV light (< 410 nm, A) or orange light (> 550 nm or > 560 nm, B and C). Curves 2 and 3 in each graph represent, respectively, the first measurement (immediately after irradiation) and a subsequent measurement (< 5 min. after irradiation). The minimal change in the absorption spectra over this time scale indicates that the spectra remain stable after irradiation.

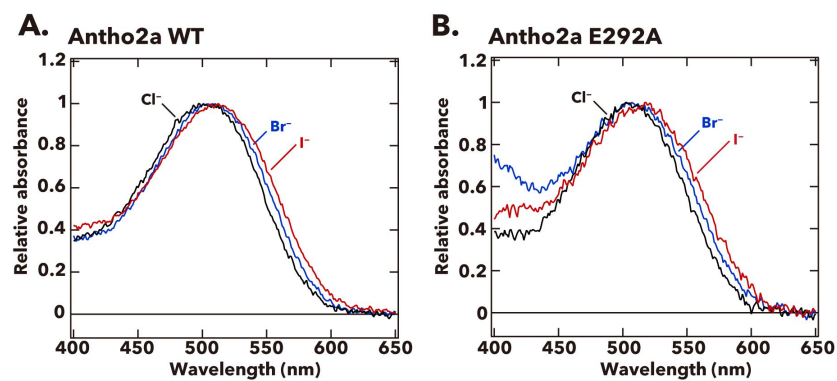


Fig. S6.

Effect of halide anions on the absorption spectra of (A) wild type Antho2a and (B) the Antho2a E292A mutant at pH 6.5 and 0°C.

The graphic shows the normalised absorption spectra of the pigments prepared in 140 mM NaCl (black curves), 140 mM NaBr (blue curves), and 140 mM NaI (red curves).

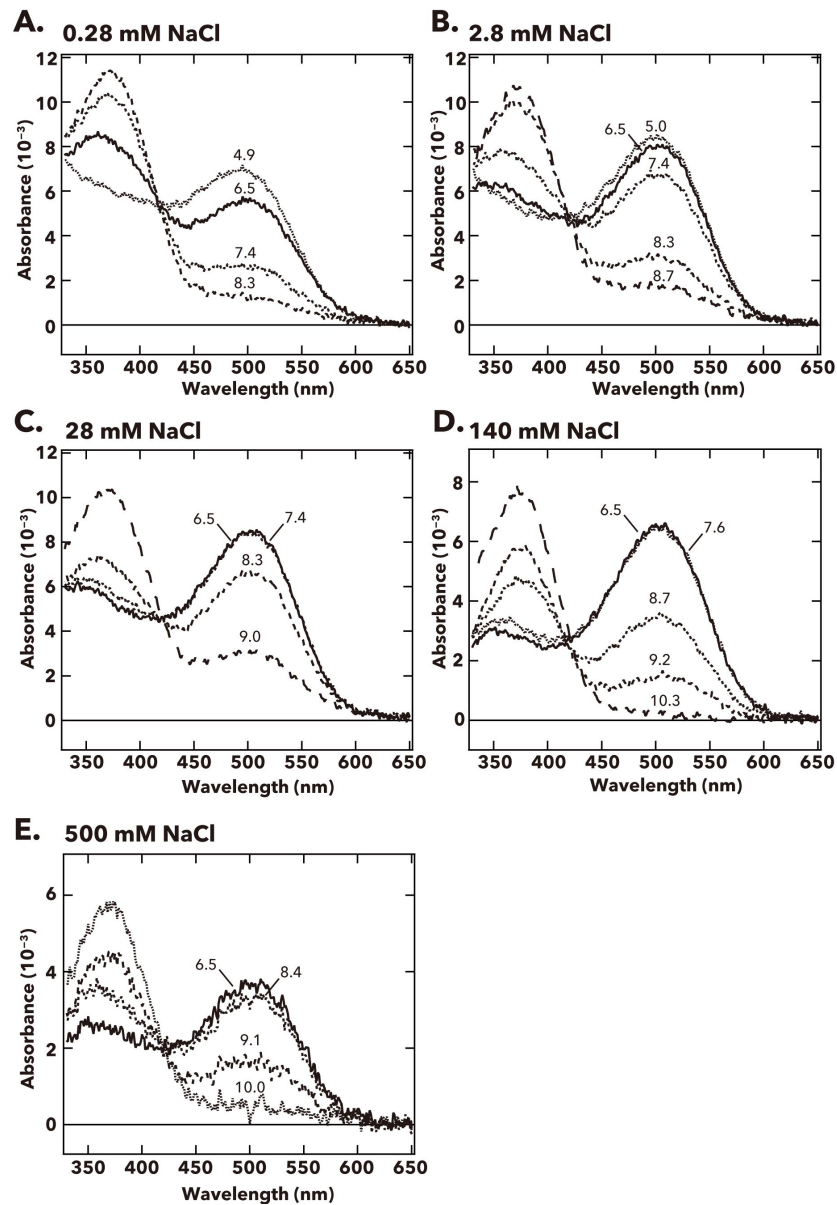


Fig. S7.

pH-dependent changes in the absorption spectra of Antho2a WT at (A) 0.28 mM, (B) 2.8 mM, (C) 28 mM, (D) 140 mM, and (E) 500 mM Cl^- at 0°C.

The pH values of the solution, measured right after each spectroscopic measurement, are indicated on the corresponding curves in each graph.

Fig. S8.

pH-dependent changes in the absorption spectra of Antho2a WT and Antho2a E292A at 0 mM NaCl (containing 70 mM Na₂SO₄) at 0°C.

The pH values at which the absorption spectra were measured are indicated on the corresponding curves.

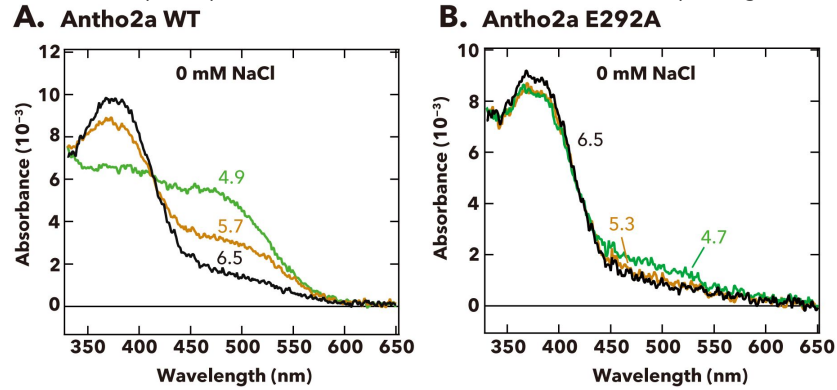
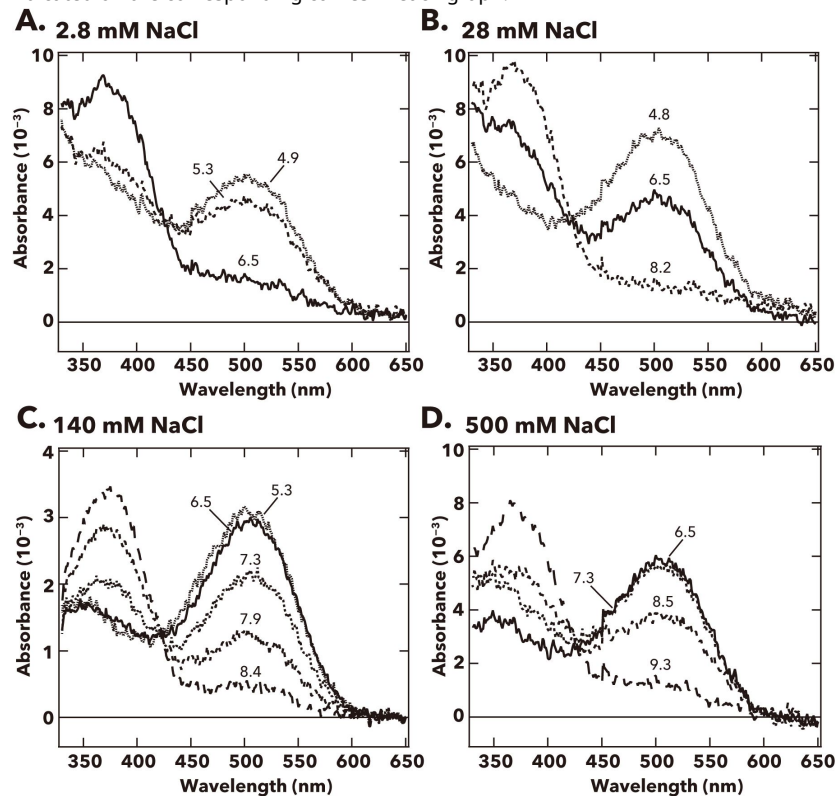


Fig. S9.

pH-dependent changes in the absorption spectra of Antho2a E292A at (A) 2.8 mM, (B) 28 mM, (C) 140 mM, and (D) 500 mM Cl⁻ at 0°C.

The pH values are indicated on the corresponding curves in each graph.



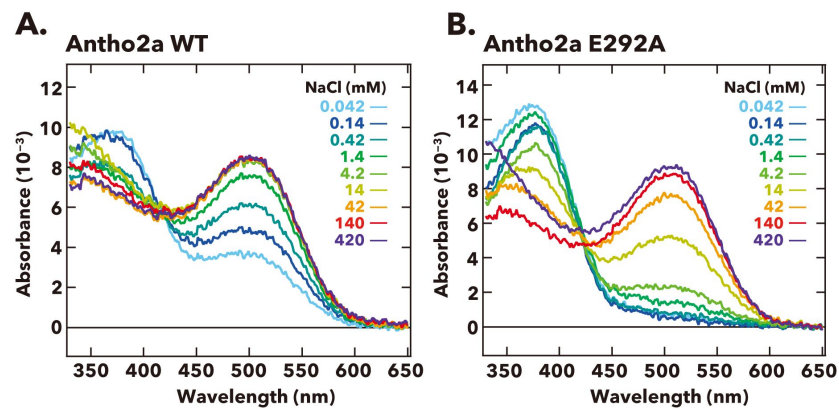


Fig. S10.

Absorption spectra of (A) wild type Antho2a and (B) the Antho2a E292A mutant under different Cl^- concentrations at pH 6.5 and 0°C .

Each colour indicates a different concentration of Cl^- .

Data and materials availability

The cDNA sequences of *Acropora tenuis* opsins in this paper are available in GenBank: eight Cnidopsins (accession no. LC844924–LC844931), seven opsins in the ASO-II group (accession no. LC844932–LC844938), and one opsin in the ASO-I group (accession no. LC844939). All data needed to evaluate the conclusion in this paper are present in the paper and the Supplementary Materials. Additional data related to this paper may be requested from the authors.

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Additional information

Author contributions

YS, MK, and AT conceptualised the study; YS, MK, GFXS, XD, and AT designed the study; YS, TS, YK, and MI performed the experiments and analyses; SS and XD performed the computational modelling; YS and AT wrote the original draft with inputs from SS and XD; All authors reviewed the manuscript and gave final approval for the manuscript.

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Reviewer #1 (Public review):

The chromophore molecule of animal and microbial rhodopsins is retinal which forms a Schiff base linkage with a lysine in the 7-th transmembrane helix. In most cases, the chromophore is positively charged by protonation of the Schiff base, which is stabilized by a negatively charged counterion. In animal opsins, three sites have been experimentally identified, Glu94 in helix 2, Glu113 in helix 3, and Glu181 in extracellular loop 2, where a glutamate acts as the counterion by deprotonation. In this paper, Sakai et al. investigated molecular properties of anthozoan-specific opsin II (ASO-II opsins), as they lack these glutamates. They found an alternative candidate, Glu292 in helix 7, from the sequences. Interestingly, the experimental data suggested that Glu292 is not the direct counterion in ASO-II opsins. Instead, they found that ASO-II opsins employ a chloride ion as the counterion. In the case of microbial rhodopsin, a chloride ion serves as the counterion of light-driven chloride pumps. This paper reports the first observation of a chloride ion as the counterion in animal rhodopsin. Theoretical calculation using a QM/MM method supports their experimental data. The authors also revealed the role of Glu292, which serves as the counterion in the photoproduct, and is involved in G protein activation.

The conclusions of this paper are well supported by data, while the following aspects should be considered for the improvement of the manuscript.

(1) Information on sequence alignment only appears in Figure S2, not in the main figures. Figure S2 is too complicated by so many opsins and residue positions. It will be difficult for general readers to follow the manuscript because of such an organization. I recommend the authors show key residues in Figure 1 by picking up from Figure S2.

(2) Halide size dependence. The authors observed spectral red-shift for larger halides. Their observation is fully coincident with the chromophore molecule in solution (Blatz et al. Biochemistry 1972), though the isomeric states are different (11-cis vs all-trans). This suggests that a halide ion is the hydrogen-bonding acceptor of the Schiff base N-H group in solution and ASO-II opsins. A halide ion is not the hydrogen-bonding acceptor in the structure of

halorhodopsin, whose halide size dependence is not clearly correlated with absorption maxima (Scharf and Engelhard, Biochemistry 1994). These results support their model structure (Figure 4), and help QM/MM calculations.

(3) QM/MM calculations. According to Materials and Methods, the authors added water molecules to the structure and performed their calculations. However, Figure 4 does not include such water molecules, and no information was given in the manuscript. In addition, no information was given for the chloride binding site (contact residues) in Figure 4. More detailed information should be shown with additional figures in Figure SX.

(4) Figure 5 clearly shows much lower activity of E292A than that of WT, whose expression levels are unclear. How did the authors normalize (or not normalize) expression levels in this experiment?

(5) The authors propose the counterion switching from a chloride ion to E292 upon light activation. A schematic drawing on the chromophore, a chloride ion, and E292 (and possible surroundings) in Antho2a and the photoproduct will aid readers' understanding.

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Reviewer #2 (Public review):

Summary:

This work reports the discovery of a new rhodopsin from reef-building corals that is characterized experimentally, spectroscopically, and by simulation. This rhodopsin lacks a carboxylate-based counterion, which is typical for this family of proteins. Instead, the authors find that a chloride ion stabilizes the protonated Schiff base and thus serves as a counterion.

Strengths:

This work focuses on the rhodopsin Antho2a, which absorbs in the visible spectrum with a maximum at 503 nm. Spectroscopic studies under different pH conditions, including the mutant E292A and different chloride concentrations, indicate that chloride acts as a counterion in the dark. In the photoproduct, however, the counterion is identified as E292.

These results lead to a computational model of Antho2a in which the chloride is modeled in addition to the Schiff base. This model is improved using the hybrid QM/MM simulations. As a validation, the absorption maximum is calculated using the QM/MM approach for the protonated and deprotonated E292 residue as well as the E292A mutant. The results are in good agreement with the experiment. However, there is a larger deviation for ADC(2) than for sTD-DFT. Nevertheless, the trend is robust since the wt and E292A mutant models have similar excitation energies. The calculations are performed at a high level of theory that includes a large QM region.

Weaknesses:

I have a couple of questions about this study:

(1) I find it suspicious that the absorption maximum is so close to that of rhodopsin when the counterion is very different. Is it possible that the chloride creates an environment for the deprotonated E292, which is the actual counterion?

(2) The computational protocol states that water molecules have been added to the predicted protein structure. Are there water molecules next to the Schiff base, E292, and Cl⁻? If so, where are they located in the QM region?

(3) If the E292 residue is the counterion in the photoproduct state, I would expect the retinal Schiff base to rotate toward this side chain upon isomerization. Can this be modeled based on the recent XFEL results on rhodopsin?

<https://doi.org/10.7554/eLife.105451.1.sa1>

Reviewer #3 (Public review):

Summary:

The paper by Saito et al. studies the properties of anthozoan-specific opsins (ASO-II) from organisms found in reef-building coral. Their goal was to test if ASO-II opsins can absorb visible light, and if so, what the key factors involved are.

The most exciting aspect of this work is their discovery that ASO-II opsins do not have a counterion residue (Asp or Glu) located at any of the previously known sites found in other animal opsins.

This is very surprising. Opsins are only able to absorb visible (long wavelength light) if the retinal Schiff base is protonated, and the latter requires (as the name implies) a "counter ion". However, the authors clearly show that some ASO-II opsins do absorb visible light.

To address this conundrum, they tested if the counterion could be provided by exogenous chloride ions (Cl⁻). Their results find compelling evidence supporting this idea, and their studies of ASO-II mutant E292A suggest E292 also plays a role in G protein activation and is a counterion for a protonated Schiff base in the light-activated form.

Strengths:

Overall, the methods are well-described and carefully executed, and the results are very compelling.

Their analysis of seven ASO-II opsin sequences undoubtedly shows they all lack a Glu or Asp residue at "normal" (previously established) counter-ion sites in mammalian opsins (typically found at positions 94, 113, or 181). The experimental studies clearly demonstrate the necessity of Cl⁻ for visible light absorbance, as do their studies of the effect of altering the pH.

Importantly, the authors also carried out careful QM/MM computational analysis (and corresponding calculation of the expected absorbance effects), thus providing compelling support for the Cl⁻ acting directly as a counterion to the protonated retinal Schiff base, and thus limiting the possibility that the Cl⁻ is simply altering the absorbance of ASO-II opsins through some indirect effect on the protein.

Altogether, the authors achieved their aims, and the results support their conclusions. The manuscript is carefully written, and refreshingly, the results and conclusions are not overstated.

This study is impactful for several reasons. There is increasing interest in optogenetic tools, especially those that leverage G protein-coupled receptor systems. Thus, the authors' demonstration that ASO-II opsins could be useful for such studies is of interest.

Moreover, the finding that visible light absorbance by an opsin does not absolutely require a negatively charged amino acid to be placed at one of the expected sites (94, 113, or 181) typically found in animal opsins is very intriguing and will help future protein engineering efforts. The argument that the Cl⁻ counterion system they discover here might have been a

preliminary step in the evolution of amino acid based counterions used in animal opsins is also interesting.

Finally, given the ongoing degradation of coral reefs worldwide, the focus on these curious opsins is very timely, as is the authors' proposal that the lower Schiff base pKa they discovered here for ASO-II opsins may cause them to change their spectral sensitivity and G protein activation due to changes in their environmental pH.

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