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Anion sensitivity and spectral tuning of cone visual pigments *in situ*

(wavelength regulation/retinal chromophore/Schiff base counterion/lyotropic anions/microspectrophotometry)

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ABSTRACT **We tested the effect of anions on the absorbance spectrum of native visual pigments as measured by microspectrophotometry in individual cone outer segments of four species of fish and one species of amphibian. In all species tested, the long-wavelength-absorbing cone pigments were anion sensitive, and their λ_{max} could be tuned over a range of 55 nm depending on the identity of the anion present. Cl^- and Br^- were the only anions that produced native pigment spectra by red shifting λ_{max} from its value under anion-free conditions. Lyotropic anions such as NO_3^-, SCN^-, BF_4^-, and ClO_4^- caused substantial and graded blue shifts of λ_{max}. The apparent K_d of binding sites on the pigment for Cl^- and for ClO_4^- was ≈2 mM. Taken together with previous findings on three visual pigments from the reptilian, avian, and amphibian classes, our results support the hypothesis that all long-wavelength-absorbing vertebrate visual pigments are spectrally tuned in part through the binding of a chloride ion. We propose that the site of anion tuning is near the protonated Schiff base of the chromophore, whose counterion may be complex and include Cl^- as an exchangeable anion. This counterion configuration may resemble the one present in the light-driven Cl^- pump halorhodopsin.**

Color vision in vertebrates is based on sets of two, three, or four different photopigments that reside in separate classes of cone photoreceptors. The wavelength of peak absorbance (λ_{max}) of these pigments ranges from the UV (360 nm) to the far red (635 nm). All vertebrate visual pigments are integral membrane proteins and contain 11-*cis*-retinal or 11-*cis*-dehydroretinal as the chromophore that is covalently bound through a Schiff base linkage to a lysine residue on the protein (opsin) moiety of the pigment (1). Although great progress has been made in recent years in unraveling the structure of visual pigments, the molecular basis of spectral tuning is still only incompletely understood.

A variety of mechanisms have been invoked to account for photopigment tuning (for reviews, see refs. 2 and 3). For example, protonation of the retinal Schiff base shifts λ_{max} from 360 to 430 nm (4). Blue-absorbing pigments may contain an unperturbed chromophore behaving much like a protonated retinal Schiff base in a nonpolar solvent (5). Closer interaction of the chromophore with the protein perturbs the electronic structure of the chromophore and results in an additional red shift of λ_{max}. This shift is commonly called the "opsin shift" and very likely comprises multiple components. For example, λ_{max} is extremely sensitive to the charge environment provided by the counterion, which is paired with the protonated Schiff base (3). A decreased interaction between protonated Schiff base and counterion, perhaps caused by an increased distance between the two, may be responsible for the opsin shift in green-absorbing rhodopsins (λ_{max}, ≈500 nm). Amino acid side chains, which form the hydrophobic binding pocket for the chromophore, also can interact with and perturb the chromophore. In fact, much or all of the λ_{max} difference between certain red/green pigment pairs seems to be due to a difference in only three critical amino acid residues close to the chromophore, which are nonpolar in the green-absorbing pigment and more polar in the red-absorbing pigment. This holds for the human green and red cone pigments (6), the green-absorbing pigment P521 of gecko (7), and the red-absorbing cone pigment iodopsin of chicken (8), as well as the green- and red-absorbing cone pigments of the characin fish *Astyanax fasciatus* (9). These six pigments are closely related and display >90% sequence similarity in transmembrane segments (sequence similarity defined as in ref. 24).

None of the current models of wavelength regulation in visual pigments has explicitly considered the possibility that an external anion may be involved in determining λ_{max}. Evidence for this possibility was first provided for the gecko P521 pigment (10–12), then for chicken iodopsin (13, 14), and recently for the red-absorbing cone pigment in frog (15). For example, when the gecko pigment was studied in a Cl^--free environment, its λ_{max} was found to be ≈500 nm; when Cl^- was added, λ_{max} shifted to the native value of 521 nm (10, 11). A similar Cl^--induced shift from 520 to 565 nm was found in chicken iodopsin (13, 14). In this paper, we provide evidence that suggests that Cl^--mediated spectral tuning of long-wavelength-absorbing cone pigments is a widespread phenomenon and may indeed be universal among vertebrates.

MATERIALS AND METHODS

The following species were examined: the cyprinid fish *Danio malabaricus* (giant danio), the characin fish *Metynnis argenteus* (silver dollar) and *Astyanax fasciatus* (Mexican cavefish), the anabantid fish *Helostoma temminckii* (kissing gourami), and the urodele amphibian *Ambystoma tigrinum* (tiger salamander) in its larval form. Eyes were dissected under infrared illumination with the aid of a closed-circuit television system from animals that were dark adapted overnight. Isolated retinae were immersed in ice-cold media for periods ranging from 30 min to 5 hr. Media compositions are listed in the legend to Table 2. After dissection, retinae were preincubated in glucuronate or sucrose medium for a minimum of 30 min before being transferred to the final experimental medium for an additional incubation of 20 min to several hours.

For spectral recordings, retinae were teased apart with forceps into several smaller pieces, which then were mounted in the final experimental medium between coverslips. Absorbance spectra were recorded from outer segments of individual photoreceptors, which were either free or still attached to the retina. Spectral measurements were carried out at room temperature (18°C–23°C) over the range of 375–745 nm by the use of a single-beam wavelength-scanning

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Abbreviations: λ_{max}, wavelength of maximum absorbance; bR, bacteriorhodopsin; hR, halorhodopsin.

microspectrophotometer. The measuring beam was adjusted to be ≈0.6 × 2 μm in the plane of the specimen; over this image, a given outer segment was positioned in transverse orientation. A typical spectrum consisted of the average of 8–24 spectral scans; these were subjected to digital filtering. Details of the technique have been published (16).

RESULTS

Fig. 1*A* shows absorbance spectra recorded from red-absorbing cones in the giant danio in three media: control Cl^- medium, Cl^--free glucuronate medium, and Cl^--free ClO_4^- medium. In general, the spectra had similar shapes in the three conditions, although there was a consistent increase in spectral half-bandwidth and a reduction in maximum absorbance in the glucuronate medium compared to the Cl^- or ClO_4^- medium. A striking change, however, was seen in λ_{max}: in glucuronate and ClO_4^- media, λ_{max} was blue shifted by approximately 25 nm and 50 nm, respectively, compared to the control medium.

Fig. 1*B* shows similar results obtained from red-absorbing cones in the salamander retina. Cl^- replacement in this species resulted in λ_{max} shifts of the same magnitude as those observed in the danio, in spite of the difference in chromophore type (A_2 vs. A_1; see Table 1). Moreover, the red-absorbing cone pigment in the kissing gourami, another A_2-utilizing species that we investigated extensively, displayed very similar spectral changes in response to changes

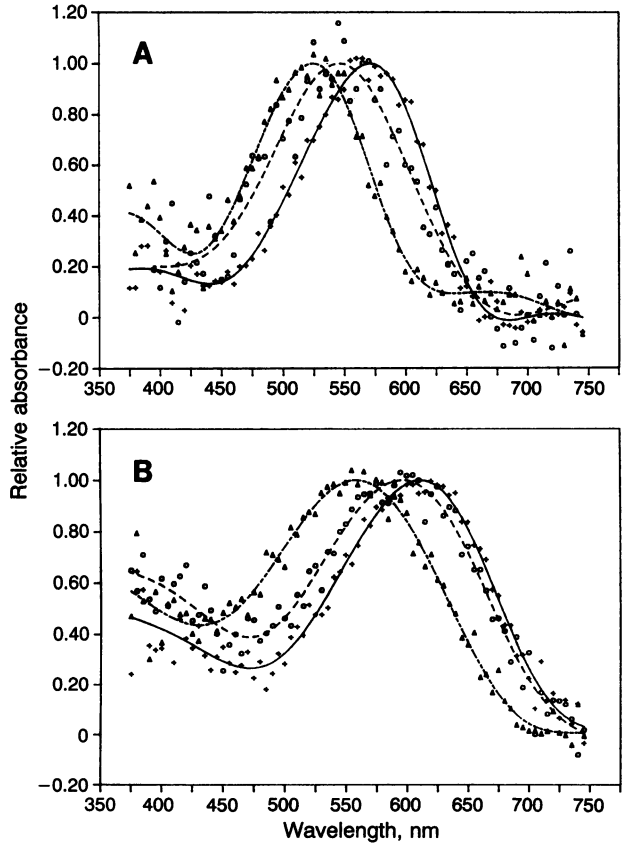

FIG. 1. Normalized absorbance spectra of red-absorbing cone pigments of giant danio (*A*) and larval tiger salamander (*B*) recorded from individual outer segments in Cl^- (+), glucuronate (○), and ClO_4^- (△) media. Each data set was obtained as the average of 8–24 spectral scans, with the measuring beam traversing a given outer segment transversely. The continuous curves are the result of Fourier smoothing. λ_{max} in danio: 572, 546, and 525 nm in Cl^-, glucuronate, and ClO_4^- media, respectively. λ_{max} in salamander: 612, 597, and 559 nm in Cl^-, glucuronate, and ClO_4^- media, respectively.

in anionic conditions. We also performed several experiments in both danio and kissing gourami, in which we incubated multiple retinal tissue samples in ClO_4^- or NO_3^- medium. Through absorbance measurements on a subset of these samples, we verified the presence of characteristic λ_{max} shifts in red-absorbing cones. The remaining subset of samples was then returned from the ClO_4^- or NO_3^- medium to the control Cl^- medium, and absorbance spectra were determined after further incubation. In these samples, λ_{max} of the original pigment was found to be restored with little loss in absorbance. Thus, λ_{max} shifts induced by ClO_4^- or NO_3^- appeared to be fully reversible upon reintroduction of Cl^- ions.

A summary of our results obtained on the anion sensitivity of visual pigments is given in Table 1. In all species examined, we recorded absorbance spectra not only from long-wavelength-absorbing cones but also from other photoreceptor types encountered. We found no systematic shift in λ_{max} for any photopigment other than long-wavelength-absorbing pigments (L pigments; see *Discussion*), regardless of the identity of the anion present. This includes the characin fish *M. argenteus* and *A. fasciatus*, which possess two closely spaced long-wavelength-absorbing cone pigments (L_1 and L_2) residing in unequal members of their double cones. The λ_{max} values of these pigments obtained in our control medium are listed in Table 1. In *Metynnis*, the λ_{max} values of the two pigments were shifted to 576 ± 7 (n = 9) and 548 ± 4 (n = 9) nm, respectively, in ClO_4^- medium. Similarly, in *Astyanax* the two pigments were shifted to 544 ± 8 (n = 5) and 506 ± 2 (n = 6) nm, respectively, in ClO_4^- medium.

Our results on the anion selectivity of spectral shifts of cone pigments in three species are shown in Table 2. Anions are listed from top to bottom in the order of increasing blue shift. Several media containing bulky anions such as glucuronate, sulfate, iodide, as well as sucrose medium (the latter containing zwitterionic Hepes sulfonate as the sole anion) yielded λ_{max} values close to those found for pure sucrose, an isotonic medium that completely lacked ions. We assume that these large anions do not bind to sites involved in spectral tuning and that in these media water molecules rather than anions occupy the sites. Hence, we designated the glucuronate medium our Cl^--free, neutral condition, and assessed spectral shifts with reference to this condition. Table 2 shows that only Cl^- and Br^- were effective in red shifting the λ_{max} of the pigments from the neutral to the native state. A second group of anions produced variable blue shifts whose magnitude at 100 mM anion concentration followed approximately the lyotropic selectivity sequence (17).

The concentration dependence of λ_{max} shifts caused by Cl^- and ClO_4^- in the red-absorbing cone pigment of danio is shown in Fig. 2. A half-maximal shift in λ_{max} was induced by ≈2 mM Cl^- or ClO_4^-. This value can be considered an apparent dissociation constant (K_d) for binding of the anion to sites on the protein. These dissociation constants are surprisingly low for binding of monovalent ions to a protein, and indicate the presence of fairly specific binding sites. For ClO_4^-, the concentration effective in tuning the pigment is remarkably low; this rules out the possibility that its spectral effect is due to a nonspecific lyotropic action such as partial denaturation of the protein (17).

Fig. 2 also includes results from experiments with equimolar mixtures of Cl^- and ClO_4^- added to glucuronate medium. The λ_{max} in the equimolar mixture was always intermediate to the λ_{max} in medium containing only Cl^- or ClO_4^-. Furthermore, when both Cl^- and ClO_4^- were present at saturating but unequal concentrations, the more abundant anion determined the λ_{max}. For example, in danio for the condition of 10 mM Cl^- in 100 mM ClO_4^-, λ_{max} was 527 ± 2 nm (n = 8). These results are consistent with a model in which tuning anions compete for a single binding site on the

Table 1. λ_{max} and anion sensitivity of visual pigments tested

Species	Chromophore	LWC	MWC	SWC	Rod
Danio	A_1	**572 ± 2 (14)**	487 ± 3 (24)	418 ± 2 (9)	505 ± 2 (13)
Gourami	A_2	**630 ± 5 (18)**	531 ± 5 (14)	—	523 ± 2 (8)
Astyanax	$A_1 + A_2$	**596 ± 7 (16)**	**554 ± 5 (15)**	453 ± 3 (12)	520 ± 6 (8)
Metynnis	A_2	**628 ± 4 (11)**	**602 ± 5 (9)**	505 ± 4 (11)	533 ± 3 (5)
Salamander	Mostly A_2	**611 ± 3 (13)**	—	NT	520 ± 2 (15)

Boldface entries, anion-sensitive pigments; lightface entries, anion-insensitive pigments. Mean ± SD of λ_{max} is given in nm; number of determinations is in parentheses. The λ_{max} values for anion-sensitive pigments are based on measurements in Cl^- medium only. The λ_{max} values for anion-insensitive pigments are based on determinations in Cl^--free media as well. Classification of a pigment as anion sensitive or anion insensitive was based not only on the data analyzed for and included in this table but also on numerous spectral recordings for which λ_{max} was estimated by visual inspection. The table is not exhaustive. The range of the instrument (set to scan between 375 and 745 nm) precluded measurement of UV-absorbing pigments in danio and salamander. Also, no data are included for pigments rarely encountered such as the blue-absorbing cone pigment in salamander. Based on spectral criteria of λ_{max} and half-bandwidth, the danio appears to possess purely A_1-based pigments, whereas the kissing gourami and silver dollar appear to contain purely A_2-based pigments. *Astyanax* photoreceptors seem to contain a fairly even mixture of A_1 and A_2 chromophores, while the larval tiger salamander predominantly utilizes the A_2 chromophore. LWC, MWC, and SWC; long-, medium-, and short-wavelength-absorbing cone; NT, not tested.

pigment. When Cl^- or Br^- is bound to the site, the pigment is red shifted to its native λ_{max}; when ClO_4^- or another lyotropic anion is bound to it, the pigment is blue shifted; and when both ions are present in the medium, the resulting spectrum is the occupancy-weighted time average of the pure Cl^- and ClO_4^- spectra.

DISCUSSION

We have shown in five species that all long-wavelength-absorbing cone pigments were anion sensitive, whereas short-wavelength-absorbing cone pigments and rod pigments were anion insensitive. This includes pigments from three families of teleost fish and one order of amphibian. Taken together with previous findings on a reptilian, an avian, and a frog pigment (10–15), our results suggest that the anion sensitivity of long-wavelength-absorbing photopigments is universal among vertebrates.

As in the brief microspectrophotometric study by Novitskii *et al.* (15) on red-absorbing cones in the frog, our measurements demonstrate anion sensitivity of native cone pigments studied *in situ* under physiological conditions (e.g., cation concentrations and pH). Previous work on detergent-extracted pigments provided evidence for anion sensitivity of the green-absorbing pigment P521 in gecko (10–12) and the red-absorbing cone pigment iodopsin in chicken (13, 14). The anion selectivity and affinity of the fish and amphibian pigments studied here are very similar to the corresponding results obtained on gecko and chicken pigments. In all these pigments, Cl^- and Br^- alone cause a red shift, whereas NO_3^-, SCN^-, and other lyotropic anions induce blue shifts in λ_{max}. Anions such as sulfate, phosphate, and glucuronate as well as sulfonate-containing pH buffers such as Hepes appear to be inert. Moreover, the affinity of the gecko and chicken pigments for Cl^- (apparent K_d between 0.8 and 2 mM; see refs. 10, 13, and 14) is comparable to that obtained by us for the danio.

The magnitude of the λ_{max} shift caused by blue-shifting anions roughly follows the lyotropic series (for reviews, see refs. 17–19). The anion most effective in blue shifting λ_{max} was ClO_4^-; this anion has a low charge density, high polarizability, and is poorly hydrated as well as amphiphilic (20). ClO_4^- and similar lyotropic anions such as BF_4^-, SCN^-, and NO_3^- may preferentially bind to sites on proteins that possess both weak dipoles and hydrophobic residues (18, 19), an

Table 2. Ionic dependence of λ_{max} of red-absorbing cone pigments

Medium	Species		
	Danio	Gourami	Salamander
Cl^-	572 ± 2 (14)	630 ± 5 (18)	611 ± 3 (13)
Br^-	569 ± 2 (14)	623 ± 3 (11)	603 ± 5 (6)
Sucrose	550 ± 4 (15)	600 ± 4 (7)	NT
$Gluc^-$	546 ± 3 (10)	614 ± 5 (5)	595 ± 6 (7)
I^-	546 ± 3 (13)	607 ± 5 (5)	594 ± 4 (7)
SO_4^{2-}	543 ± 6 (11)	596 ± 5 (4)	NT
Pure sucrose	542 ± 6 (12)	NT	NT
F^-	540 ± 5 (13)	NT	NT
TCA^-	537 ± 3 (8)	583 ± 5 (4)	590 ± 4 (7)
NO_3^-	533 ± 2 (10)	586 ± 3 (9)	593 ± 4 (8)
SCN^-	531 ± 3 (7)	583 ± 3 (15)	571 ± 4 (6)
BF_4^-	528 ± 4 (7)	581 ± 4 (7)	566 ± 6 (9)
ClO_4^-	521 ± 4 (8)	575 ± 5 (12)	562 ± 6 (9)

Mean ± SD of λ_{max} is given in nm; number of measurements is in parentheses. Compositions of media were as follows: control Cl^- medium, 100 mM NaCl/2.5 mM KCl/1 mM $CaCl_2$/1.6 mM $MgCl_2$/5 mM D-glucose/20 mM Hepes, titrated to pH 7.5 with 1 M NaOH; other media, same composition and pH as control medium except that NaCl was replaced with 100 mM NaBr, NaI, $NaClO_4$, etc., and K, Ca, and Mg were added as hydroxides rather than as Cl^- salts; sucrose medium, control medium minus NaCl, with K, Ca, and Mg hydroxides rather than chlorides, and with 200 mM sucrose; pure sucrose medium, 250 mM sucrose in ultrapure water. $Gluc^-$, glucuronate; TCA^-, trichloroacetate; NT, not tested.

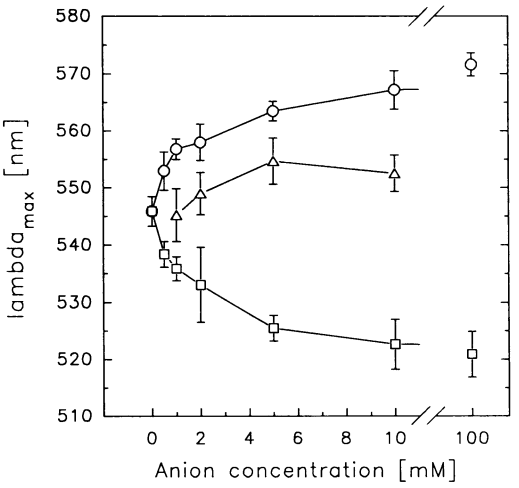

FIG. 2. Dependence of λ_{max} of the red-absorbing cone pigment of giant danio on the concentration of anions, which were added as Na salts to glucuronate medium. Data points show mean ± SD of 5–10 measurements for each condition. ○, Cl^-; □, ClO_4^-; △, equimolar mixtures of Cl^- and ClO_4^- (each anion at 1, 2, 5, and 10 mM).

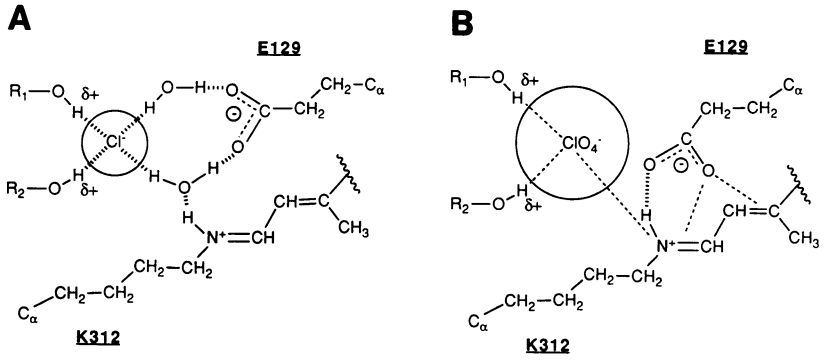

FIG. 3. Schematic of the anion binding site in anion-sensitive pigments, viewed from the side of the A, B, C, and D helices. Extracellular space is up. (*A*) Cl^--bound state. (*B*) ClO_4^--bound state. Amino acids are numbered as for human red and green cone pigments (24). |||, Hydrogen bonding; - - -, electrostatic interactions. The retinylidene moiety is truncated at C-12 by the wavy line. In the Cl^--bound state, the site includes the protonated Schiff base, a glutamate residue in helix C (E129), which is conserved in all vertebrate visual pigments sequenced so far, a Cl^- ion, two hydroxyl groups, and two structural water molecules. By analogy with transmitter-gated Cl^- channels (21, 22), hydroxyl groups provide partial solvation for the Cl^- ion, attracting Cl^- via fractional positive charges as well as hydrogen bonding to it. Candidates for the donors of these OH groups are T103, S107, and T132, which are conserved in Cl^--sensitive cone pigments and are replaced by nonpolar residues in Cl^--insensitive pigments. Water molecules are included as they seem to be a critical component of a "soft" Schiff base counterion complex (36, 37). In addition, two or more water molecules may serve here to counteract the electrostatic repulsion between two adjacent anions, by analogy with the structure of aqueous Cl^-–Cl^- ion pairs where electrostatic repulsion between the two Cl^- ions is overcome through the interposition of three hydrogen-bonded water molecules (38). In the absence of Cl^- (see *B*), the complex counterion collapses and converts to a simple counterion configuration in which the carboxyl group of E129 directly hydrogen bonds to the Schiff base nitrogen, and water molecules take up the former position of the Cl^- ion. The formation of a simpler, "harder" counterion (as probably present in rhodopsins) is usually associated with a blue shift (36)—in this case 20–25 nm. ClO_4^- binds to the Cl^--free site as an unhydrated ion unable to engage in the formation of a water-bridged counterion complex; hence, it will not disturb the direct, hydrogen-bonded ion pairing between Schiff base and glutamate carboxylate. However, the added ClO_4^- will interact electrostatically with the positive charge, which is delocalized over the Schiff base end of the chromophore and will tend to polarize it further to the Schiff base, an action that generally causes a blue shift (2)—in this case, ≈30 nm.

environment that is likely to exist in the vicinity of the chromophore Schiff base. Such an amphiphilic environment may attract not only blue-shifting lyotropic anions but also red-shifting anions such as Cl^- and Br^-. For example, in γ-aminobutyric acid- and glycine-gated Cl^- channels, five α-helical M2 transmembrane segments rich in serine and threonine residues come together to form the pore of the Cl^- channel (21, 22). These channels pass Cl^-, Br^-, I^-, and SCN^- ions (23), and their anion selectivity probably is determined by interaction of permeant ions with hydroxyl groups from the serine and threonine residues.

Pigment Classification. Comparison of the amino acid sequences of visual pigments combined with phylogenetic analysis provides a rational framework for classification of these pigments, which is based on sequence similarity and evolutionary relationships. We have performed such an analysis on 25 vertebrate visual pigments (unpublished observations). Our results lead us to propose that these pigments can be grouped into three major classes which we designate the L, M, and S pigment classes (see also ref. 7). Within each class, members are closely related. L pigments are long-wavelength absorbing and anion sensitive. In any given species, L pigments occur singly or pairwise (L_1 and L_2), and the λ_{max} values of the two members of a pair differ only by 30–40 nm. L pigment pairs probably arose recently through gene duplication (9, 24). Members of the L pigment class display a very high degree of sequence similarity and differ principally in a few critical and conserved tuning residues (see Introduction). Examples of singly occurring L pigments that have been sequenced are chicken iodopsin (8) and the gecko P521 pigment (7); examples of L pigment pairs are the *Astyanax* (9) and *Metynnis* long-wavelength pigments. On the basis of their sequences (24), the red- and green-absorbing cone pigments in humans also should be classified as paired L pigments; hence, we expect them to be anion sensitive. Indeed, in preliminary experiments on monkey cones (*Macaca fascicularis*), we found that the red/green pigments shifted from 566/534 nm in Cl^- medium to 550/525 nm in glucuronate medium and to 520/500 nm in medium containing SCN^- or ClO_4^-. M pigments are medium-wavelength absorbing and anion insensitive. This class includes all rod pigments (rhodopsins) and most green-absorbing cone pigments—i.e., the cone M pigments are close cousins of the rhodopsins. For example, the green-absorbing cone pigment in chicken (λ_{max} = 508 nm) is much more closely related to chicken rhodopsin (λ_{max} = 503 nm) than to chicken iodopsin (25, 26). S pigments are short-wavelength absorbing (blue, violet, UV) and anion insensitive; in fact, there appear to be multiple classes of S pigments. We hypothesize that all vertebrate visual pigments with $\lambda_{max} > \approx 515$ nm for A_1-based pigments or $\lambda_{max} > \approx 550$ nm for A_2-based pigments are L pigments and consequently are anion sensitive.

Spectral Tuning by Anions in Other Retinal-Based Pigments. It is instructive to compare anion tuning of cone pigments and of bacterial retinal-binding opsins such as bacteriorhodopsin (bR) and halorhodopsin (hR). The latter two pigments are structurally similar to vertebrate opsins and function as light-driven proton and chloride pumps, respectively (for reviews, see refs. 27–29). Like vertebrate opsins, they use retinal as a chromophore and link it to the protein as a protonated Schiff base paired with a counteranion. In bR, the Schiff base counterion appears to be a complex made up of the three charged residues D85, D212, and R82 (28, 30). Whereas wild-type bR is completely anion insensitive, mutant bRs, in which any one of these three residues is changed to an uncharged amino acid, become anion sensitive (31). The loss of a fixed negative charge in some of these mutants can apparently be compensated for by an external anion that serves as a surrogate for the lost internal charge and participates in the Schiff base counterion complex, thereby tuning the chromophore directly at the Schiff base.

In hR, the absorbance spectrum is anion sensitive, even in the wild-type protein, and displays the same anion selectivity and polarity of λ_{max} shifts as the cone opsins studied here (29). The Schiff base environment in hR is homologous to that in bR: the residues corresponding to D212 and R82 of bR are conserved (D238 and R108 in hR); however, D85 in bR is replaced by T111 in hR (29). It seems likely, therefore, that the Schiff base counterion in hR consists of D238, R108, and an exchangeable (and transportable) Cl^- ion, which takes the

place of the lost carboxylate and hydrogen bonds to T111. Der *et al.* (32) have offered a similar argument to explain light-driven Cl^- pumping by hR at normal pH and by bR at acid pH. Interestingly, in the bovine rhodopsin mutant E113Q, external Cl^- also appears to be able to substitute for a carboxylate as the Schiff base counterion (33–35).

Molecular Model of the Site of Anion Tuning. We propose that anion-sensitive cone pigments in their native Cl^--liganded state possess a complex Schiff base counterion similar to the one presumed to be present in certain bR mutants and in wild-type hR, and that various anions can tune the chromophore by modifying the structure of the counterion complex. A hypothetical molecular model of the anion binding site, which could explain the observed λ_{max} shifts, is presented in Fig. 3. The basic features of the site are explained in the legend to Fig. 3, both for the native state of the pigment (Fig. 3*A*, with bound Cl^-) and for the blue-shifted state (Fig. 3*B*, with bound ClO_4^-).

Physiological Significance. Several physiological roles for the binding of Cl^- to cone pigments are conceivable. Since the K_d for Cl^- is 1–2 orders of magnitude lower than physiological concentrations of Cl^-, the anion binding site on these pigments will be occupied by Cl^- nearly 100% of the time even though bound Cl^- could exchange rapidly. Clearly, one effect of full occupancy of this site by Cl^- is to red shift λ_{max} by 20–25 nm compared with the unoccupied site. Hence, Cl^- binding constitutes an important component of spectral tuning in these pigments. Moreover, in the gecko P521 pigment (39) and in chicken iodopsin (14), 11-*cis*-retinal continually exchanges in the dark, and Cl^- reduces the rate of this dark exchange. Crescitelli's (40) studies on the effect of raising the pH on the gecko pigment suggest that binding of Cl^- shifts the pK_a of the chromophore Schiff base in the alkaline direction. This would provide a striking parallel to results in hR in which the Schiff base pK_a is shifted from 7.4 in sulfate medium to 8.9 in Cl^- medium (41). We have found consistently (unpublished observations) that outer segments of red-absorbing cones in danio gradually lose absorbance in Cl^--free glucuronate medium at pH 7.5. The rate of loss of absorbance in this medium appears to be retarded at pH 6.5 and greatly accelerated at pH 8.5. By contrast, red cone outer segments exposed to Cl^- medium maintain high absorbance for hours throughout the pH range 6.5–8.5, and a gradual loss of absorbance is apparent only at pH 9.5. These results are consistent with a stabilizing role for Cl^-, probably through a direct influence on Schiff base protonation and hydrolysis—e.g., via a shift of the Schiff base pK_a in the alkaline direction. Cl^- may thus play an important role in regulating pigment bleaching and regeneration in Cl^--sensitive pigments.

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