Vertebrate Opsins Belonging to Different Classes Vary in Constitutively Active Properties Resulting from Salt-Bridge Mutations†

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ABSTRACT: Vertebrate opsins are classified into one of five classes on the basis of amino acid similarity. These classes are short wavelength sensitive 1 and 2 (SWS1, SWS2), medium/long wavelength sensitive (M/LWS), and rod opsin like 1 and 2 (RH1, RH2). In bovine rod opsin (RH1), two critical amino acids form a salt bridge in the apoprotein that maintains the opsin in an inactive state. These residues are K296, which functions as the chromophore binding site, and E113, which functions as the counterion to the protonated Schiff base. Corresponding residues in each of the other vertebrate opsin classes are believed to play similar roles. Previous reports have demonstrated that mutations in these critical residues result in constitutive activation of transducin by RH1 class opsins in the absence of chromophore. Additionally, recent reports have shown that an E113Q mutation in SWS1 opsin is constitutively active. Here we ask if the other classes of vertebrate opsins maintain activation characteristics similar to that of bovine RH1 opsin. We approach this question by making the corresponding substitutions which disrupt the K296/E113 salt bridge in opsins belonging to the other vertebrate opsin classes. The mutant opsins are tested for their ability to constitutively activate bovine transducin. We demonstrate that mutations disrupting this key salt bridge produce constitutive activation in all classes. However, the mutant opsins differ in their ability to be quenched in the dark state by the addition of chromophore as well as in their level of constitutive activation. The differences in constitutive activation profiles suggest that structural differences exist among the opsin classes that may translate into a difference in activation properties.

Visual pigments are members of the G-protein-coupled receptor (GPCR)[1] family. Like all GPCRs, they contain seven transmembrane helices. Additionally, visual pigments contain a light-sensitive vitamin A-derived chromophore covalently bound to a highly conserved lysine residue in transmembrane helix 7 via a Schiff base linkage. In bovine rod opsin the chromophore attachment site is K296. [For simplicity all amino acid residues referenced in this paper will use the bovine rod opsin numbering system.] Activation of visual pigments begins with the absorption of a photon of light by the chromophore, causing an isomerization of the chromophore from 11-*cis*-retinal to all-*trans*-retinal. This isomerization leads to a series of conformational changes in the protein which result in the formation of the active meta II state (*1*, *2*).

Visual pigments in vertebrates are divided into five classes on the basis of amino acid sequence similarity (*3*, *4*). These classes consist of two rod opsin like classes (RH1 and RH2), two short wavelength sensitive classes (SWS1 and SWS2), and a medium/long wavelength sensitive class (M/LWS) (Figure 1). Each class operates within a given range of maximal absorbance; however, there is some overlap in absorbance between classes. Phylogenetic analysis shows that the ancestral opsin class is the M/LWS class, with the RH1 class arising more recently (*3*−*5*).

All classes of vertebrate visual pigments contain a conserved glutamic acid residue in transmembrane helix 3 (E113). In the RH1 class, it has been shown that the conserved E113 residue serves as the counterion to the Schiff base which exists in a protonated form (*6*−*8*). In SWS1 class opsins there is growing evidence that suggests this residue also serves as a counterion to a protonated Schiff base for photopigments that absorb maximally in the violet range, but for those that have a maximal absorbance in the UV range, the Schiff base is unprotonated in the dark state (*9*, *10*). The protonation state for the other classes of vertebrate opsins has not been experimentally demonstrated; however, their maximal absorbance in the visible light spectrum suggests the Schiff base is most likely protonated.

K296 and E113 play an additional role in the apoprotein state of RH1 pigments. These residues form a salt bridge that holds the protein in an inactive state (*11*, *12*). Disruption of this salt bridge results in a protein that has the ability to constitutively activate its G-protein, transducin (G_t). In addition, reconstitution of this mutant with 11-*cis*-retinal

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[1] Abbreviations: GPCR, G-protein-coupled receptor; RH1, rod opsin like class 1; RH2, rod opsin like class 2; SWS1, short wavelength sensitive class 1; SWS2, short wavelength sensitive class 2; M/LWS, medium/long wavelength sensitive class; DTT, dithiothreitol; MES, 2-(*N*-morpholino)ethanesulfonic acid; DM, *n*-dodecyl *β*-D-maltoside; PMSF, phenylmethanesulfonyl fluoride; G_t, bovine transducin; EthSB, 11-*cis*-retinal covalently bound to ethylamine via a Schiff base.

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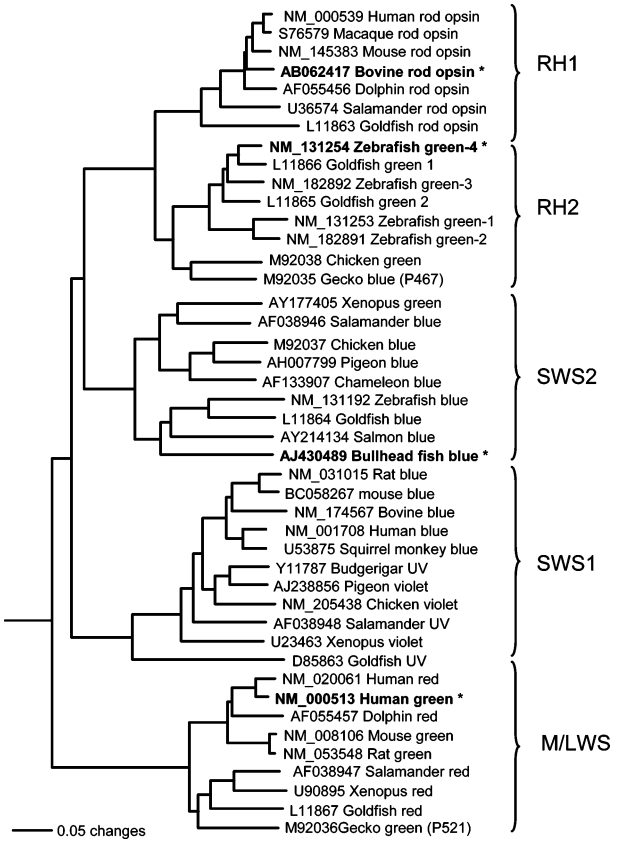

FIGURE 1: Phylogenetic tree depicting the five classes of vertebrate opsins. For each branch the NCBI accession number is listed and followed by the opsin name. Opsins used in this study are shown in bold followed by an asterisk. During tree construction the following sequences were used as outgroups to root the tree: human RGR (BC011349) and chicken peropsin (AY339626).

quenches the activation in the dark state (*11*). Other RH1 mutations, such as G90D (*13*) and A292E (*14*), from transmembrane helices 2 and 7, respectively, have been found to result in constitutive activation of the apoprotein. However, both the G90D and A292E mutations activate the pigment by competing with E113 for the role of the counterion to the positive charge on K296 in the apoprotein state. This effectively causes a break in the salt bridge between E113 and K296. Other mutations such as T94I (*15*), L79A (*16*), W175A (*16*), M257Y (*17*), and E134Q (*18*) have been identified that cause constitutive activation but act through mechanisms not associated with the salt-bridge break.

To emphasize the importance of the K296/E113 salt bridge in other classes of vertebrate opsins, a recent report has shown that an E113Q mutant in the SWS1 class is also constitutively active (*19*). This result suggests that, in the SWS1 class, the E113/K296 salt bridge maintains the critical role of holding the apoprotein in a less active state. The other classes have not been assessed prior to this study.

While at this time the crystal structure of only the dark state of bovine RH1 pigment is known (*20*), research using site-directed spin labeling has established that the activated state of bovine rod opsin requires a movement of helix 6 relative to helix 3 (*21*). Using the same technique it has been demonstrated that opsins with mutations at site E113 have their helices positioned similarly in the dark state to the positions of the helices in WT pigment after photoactivation (*22*). This suggests that conformational changes brought about by disrupting the K296/E113 salt bridge mimic the activated protein.

By making mutations that disrupt the salt bridge in opsins from the other vertebrate opsin classes, we can ask if the other classes have a similar process of activation as the RH1 class. Bovine rod opsin (RH1) (*11*) and salamander UV (SWS1) (*19*) opsin have previously been characterized in regard to their ability to constitutively activate G_t when an E113Q substitution is introduced into the expressed protein. In both cases the constitutive activity was quenched by reconstituting the opsin with 11-*cis*-retinal, producing an inverse agonist affect by the chromophore. If all opsin classes have similar activation processes, then equivalent mutants in all classes of vertebrate opsins should produce constitutively active proteins in the apoprotein state. Additionally, the activity should be quenched when reconstituted with chromophore. Our data show that all classes of vertebrate opsins show constitutive activation when either of the charged residues at sites 113 or 296 are replaced with neutral residues, although the degree of constitutive activation and the dark state response to reconstitution with chromophore varies across the five classes. This study lends support to the idea that structural variation exists among the vertebrate opsin classes.

MATERIALS AND METHODS

Phylogenetic Tree Construction. Nucleotide coding sequences were obtained from NCBI and translated. Amino acid alignments were performed using MUSCLE (*23*). Phylogenetic tree construction was performed in PAUP version 4.0b10 (*24*) using the neighbor-joining method. See Figure 1 for all sequences used to construct the phylogenetic tree.

Mutant Construction and Expression. cDNA from each of the five vertebrate opsin classes were cloned into the expression vector pMT3 (*25*). All cDNA sequences were modified to express the sequence encoding the last eight amino acids of bovine RH1's C-terminal tail (1D4 epitope) fused to the C-terminus of the expressed opsins. The addition of the 1D4 epitope has previously been used with other opsins without modifying their absorbance properties (*26*) yet allowing use of a 1D4 antibody (*27*) in quantitation and purification. The genes used in this study were bovine (*Bos taurus*) rod opsin (RH1), bullhead fish (*Cottus gobio*) SWS2, zebrafish (*Danio rerio*) RH2-4, and human (*Homo sapiens*) green M/LWS. Site-directed mutagenesis using Stratagene's Quikchange procedure was used to create the E113Q and K296A mutants.

COS-1 cell transfections were carried out using a chloroquine/DEAE-dextran-mediated procedure. COS-1 cells were grown to confluency in DMEM enriched with 10% FBS and 2 mM L-glutamine (buffer A). Transfection mix was prepared containing 1 mM chloroquine, 0.25 mg/mL DEAE-dextran, 100 mM Tris-HCl, pH 7.4, and 1 μg of DNA in DMEM enriched with 2 mM L-glutamine. Confluent COS-1 cells in 100 mm $\times$ 20 mm cell culture dishes were washed twice in PBS, pH 7.4, and incubated with 5 mL transfection mix for 6 h. Transfection mix was removed and replaced with buffer A containing 1 mM chloroquine and incubated 12 h. Following incubation the media were replaced with buffer A and incubated an additional 48 h, at which time the cells were harvested.

The COS-1 membranes containing the expressed opsin were isolated according to the previously published protocol (*28*) with the following modification. The final buffer in which the membranes were resuspended contained 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM EDTA, and 1 mM DTT. Concentration of expressed opsin in the membrane preparations was determined using dot-blot analysis against known concentrations of bovine rod opsin. Dot blots were probed using anti-1D4 antibody and detected using an alkaline phosphatase conjugated anti-mouse antibody. Analysis was performed by densometric scanning after visualization on a phosphoimager.

Reconstitution with Chromophore. 11-*cis*-retinal chromophore covalently bound to ethylamine via a Schiff base (EthSB) was constructed by mixing ethylamine and 11-*cis*-retinal in a 10:1 ratio and incubating at 4 °C for 12 h. Formation of the Schiff base was determined by a shift in the absorbance under acidic conditions from 380 to 440 nm. Opsin (20 nM) was reconstituted using 200 μM 11-*cis*-retinal or EthSB and incubated at 4 °C in the dark for 1 h.

Transducin Activation Assay. Transducin was extracted from bovine rod outer segments and purified according to previously published protocols (*11*). All transducin activation assays were performed under either regular room illumination or dim red light to maintain the dark state. Transducin activation assays were carried out in 100 μL reactions containing 10 mM Tris-HCl, pH 7.4 (for the SWS2 opsin, 10 mM MES, pH 6.5, was used), 100 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, 3 μM GTPγ^{35}S (specific activity $\sim$1.6 $\times$ 10^4 cpm/pmol), 3−5 μM transducin, and 20 nM opsin. At indicated time points, 10 μL aliquots were collected on a nitrocellulose filter connected to a vacuum manifold. Nitrocellulose filters were washed with 15 mL of buffer containing 10 mM Tris-HCl, pH 7.4, 100 mM NaCl, 5 mM $MgCl_2$, and 1 mM DTT. Radiation was measured after the addition of 5 mL of scintillation fluid. Moles of bound GTPγS corresponded to moles of activated transducin in a 1:1 ratio. Assays were performed with $n = 3$; error bars represent standard error.

Purification and Spectral Analysis. COS-1 cells expressing opsin, collected from 20 100 mm $\times$ 20 mm plates, were resuspended in 5 mL of 10 mM MES, pH 6.5, and 150 mM NaCl and incubated with 40 μM 11-*cis*-retinal for 1 h. Membranes were solubilized in 10 mM MES, pH 6.5, 150 mM NaCl, 1% *n*-dodecyl β-D-maltoside (DM), and 0.2 mg/mL PMSF for 1 h. Samples were centrifuged in a Beckman 60Ti rotor at 100000*g* for 20 min. Supernatant was passed over a column matrix with α-1D4 antibody bound. The column was washed eight times with 10 mM MES, pH 6.5, 150 mM NaCl, and 0.1% DM and eluted in 400 μL of the same buffer containing 50 mM of a peptide corresponding to the last 18 amino acids of bovine rod opsin's C-terminal tail (sequence: DEASTTVSKTETSQVAPA). Spectral analysis was performed using a Hitachi U-3300 dual-beam spectrophotometer.

RESULTS

Transducin Activation Assays. We first asked if constructing mutants that remove the charge on either E113 or K296, thus breaking the salt bridge, would result in constitutive activation of all classes of vertebrate opsins. To study the activation of the other vertebrate opsin classes arising from disruptions in the E113/K296 salt bridge, we generated appropriate mutations in members of the SWS2, RH2, and M/LWS opsin classes (see Figure 1).

All opsins were expressed in COS-1 cells and maintained in their membrane throughout the experiments. In an assay with COS-1 membranes isolated from cells that did not express any heterologous opsins, we report a low background level of 0.41 $\pm$ 0.03 pmol of G_t activated min^{-1}. The background noise is likely due to spontaneous binding of the GTPγS to the transducin in the system or to other proteins endogenous to the COS-1 membranes. The activity resulting from this low level background noise was minimal and did not interfere with results from other assays.

For all opsins tested, we report constitutive activation occurred when the E113 counterion was replaced with a glutamine residue (Figure 2). Because the salamander SWS1 with an E113Q mutation has previously been shown to be constitutively active (*19*), we can conclude this class behaves in a manner similar to mutations disrupting the salt bridge. Additionally, in all classes tested except the SWS2 class, the constitutive activity was significantly quenched by addition of 11-*cis*-retinal to create the photopigment. This reduced the activity in the dark to a level approaching that of the wild-type opsin. Surprisingly, the SWS2 opsin did not return to an inactive pigment upon addition of chromophore, maintaining activation levels similar to those of the E113Q mutant.

Activation Profiles Exhibited by Each Opsin Class. The differences in the ability of constitutively active opsins to be quenched in the dark state by chromophore addition raised the question as to whether the levels of constitutive activation in the various classes of opsins are similar. Because different opsins will activate bovine G_t with different affinities, we normalized each plot to the activity of the light-activated WT photopigment. Transducin activation assays were performed with (photopigment) and without (opsin) added retinal as follows: WT opsin, WT photopigment, E113Q opsin, E113Q photopigment, and K296A opsin. G_t activation rates were plotted (Figure 3) to obtain the activation profile of each class of opsin.

The assays performed using bovine RH1 opsin produced results that are in agreement with previously published results (*11*). The WT opsin had negligible activity, whereas the photopigment is fully active when exposed to light. The E113Q opsin had a constitutive activity level of 40.0 $\pm$ 4.6% that of WT photopigment, but the activity could be raised to almost full activity levels by addition of chromophore and assaying it in the light. Finally, when the E113Q photopigment was maintained in the dark, complete quenching of the constitutive activity was observed. The K296A opsin had a constitutive activity similar in magnitude to that of the WT pigment.

The data produced by the zebrafish RH2-4 opsin maintains an activation profile similar to that of bovine RH1 opsin. In this class we see a slightly higher activation rate (59.7 $\pm$ 6.1%) in the E113Q apoprotein than observed in bovine RH1 opsin. In contrast to bovine RH1 opsin, measurements of the RH2 E113Q photopigment in the dark state did not show complete quenching of the constitutive activity. However, the quenching observed showed a reduction in the activity

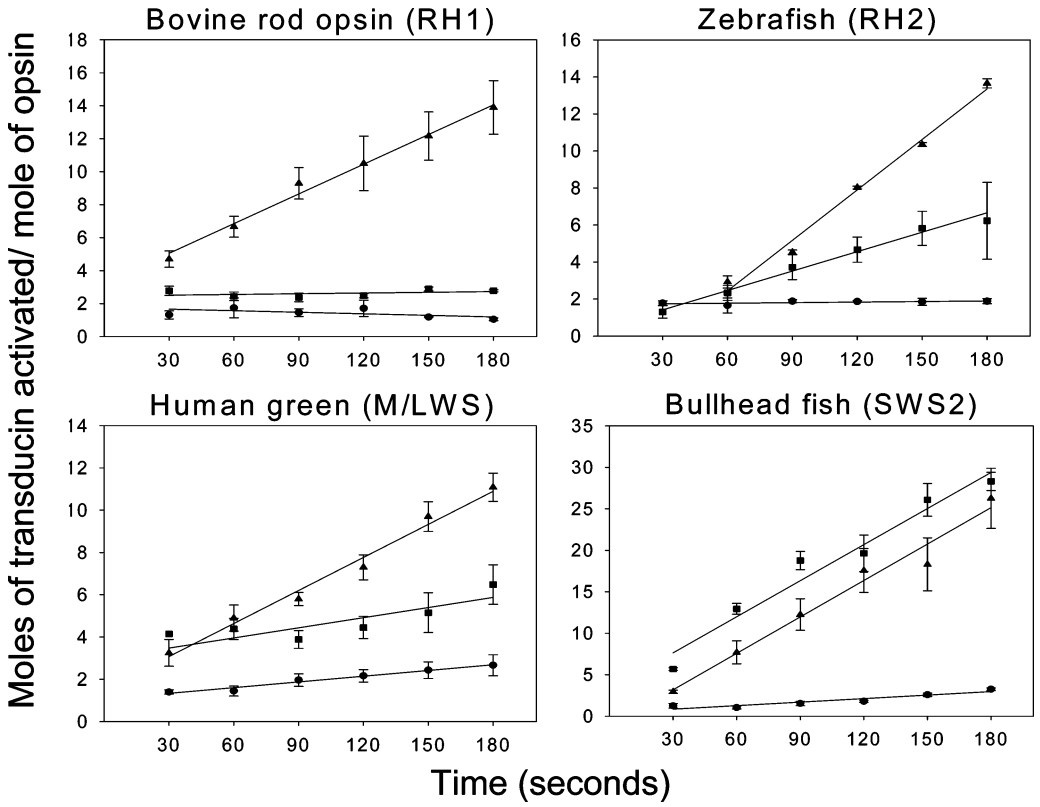

FIGURE 2: Transducin activation assay results showing constitutive activity, resulting from E113Q mutation, for the RH1, RH2, M/LWS, and SWS2 class opsins. For all plots the triangles (▲) represent the E113Q opsin, squares (■) show the E113Q photopigment assayed under dim red illumination to preserve the dark state, and circles (●) represent the wild-type opsin.

level to 45% of its constitutive E113Q opsin state. Additionally, the K296A mutant (data not shown) did not show significant constitutive activity. Reconstitution of the K296A RH-2 opsin with 11-*cis*-retinal covalently bound to ethylamine through a Schiff base (EthSB), previously shown to activate a K296A bovine RH1 mutant (*29*), failed to produce additional activity (data not shown). We conclude that the K296A substitution results in a misfolded protein. Therefore, except for the K296A mutant, these results parallel those observed for the RH1 opsin, which may reflect the close primary sequence homology of the two opsin classes.

The human green opsin, representing the M/LWS opsin class, shows a somewhat different pattern. In this class we observed a much reduced level of activation (20.3 ± 0.9%) in the E113Q opsin, compared to the other classes of opsins. This low level of activity cannot be attributed to a misfolded opsin because the E113Q photopigment shows levels of activation similar to those of the light activated WT photopigment ($p = 0.066$). We see similar levels of activation for the K296A opsin. This suggests that helical movements that arise from the break of the E113/K296 salt bridge are insufficient to produce a fully active opsin. Additional constraints must exist in this class of opsins which prohibit the opsin from reaching a fully activated state until triggered by the isomerization of the chromophore.

The SWS2 opsin, from the bullhead fish, produced yet another pattern of results. The E113Q opsin showed a constitutive activity of 66.7 ± 11.1% of the WT pigment, similar to the RH1 and RH2 classes. However, the E113Q photopigment, when assayed in the light or maintained in the dark, failed to significantly change its level of activation ($p = 0.845$). Results obtained from assaying the K296A mutant revealed a similar level of constitutive activity to the E113Q mutant.

Spectral Analysis of the SWS2 Pigment. The failure of the bullhead fish SWS2 E113Q and K296A mutants to change the levels of activity when reconstituted with chromophore and assayed in the light or dark state led us to ask if the pigments had been properly generated. To answer this question, we looked at the spectral absorption of the SWS2 E113Q opsins reconstituted with 11-*cis*-retinal. With the reconstitution taking place prior to purification of the opsins for spectral analysis, any unincorporated 11-*cis*-retinal would be washed away during purification; therefore, any absorption peak would be attributed to chromophore that has bound inside the binding pocket of the opsin. When the spectra were run at pH 6.5, the SWS2 E113Q photopigments produced an absorption spectra with λ_{max} ~380 nm (Figure 4). To confirm that the chromophore is bound inside the binding pocket and has formed a Schiff base with K296, we reduced the pH of the solution to ~2.5 to force the protonation of the Schiff base. This acidification resulted in a red shift in λ_{max} to ~420 nm, indicating the presence of a protonated Schiff base. This confirmed the SWS2 opsin had reconstituted with the chromophore to form a photopigment. Therefore, the inability of the SWS2 constitutive opsin to be quenched in the dark state must be a result of differences around the binding pocket that are not present in the other classes of opsins.

DISCUSSION

Our results suggest that mutations that disrupt the K296/E113 salt bridge result in the constitutive activation of all

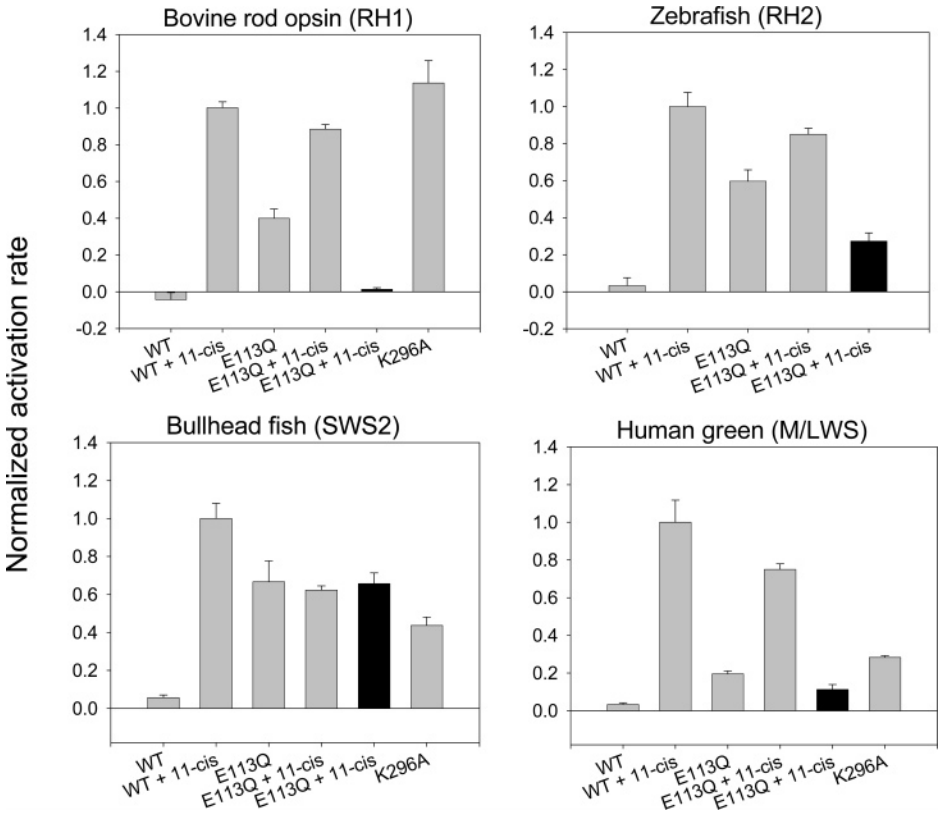

FIGURE 3: Transducin activation rates for the RH1, RH2, M/LWS, and SWS2 pigments. All assays were performed at regular room illumination levels except black bars which represent assays performed under dim red illumination to preserve the dark state. Panels show transducin activation levels for wild-type opsin (WT), wild-type photopigment (WT + 11-*cis*), E113Q opsin (E113Q), E113Q photopigment (E113Q + 11-*cis*), and K296A opsin. Error bars represent standard error.

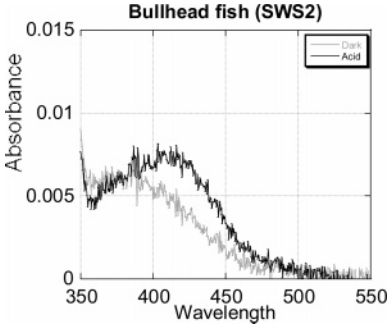

FIGURE 4: Spectral analysis of bullhead fish SWS2 E113Q pigment for verification of chromophore binding. Comparison of dark spectra at pH 6.5 (gray trace) and acid-trapped spectra at pH 2.5 (black trace). Heterologously expressed opsins in COS-1 cells were reconstituted with 11-*cis*-retinal prior to purification and spectral analysis.

classes of vertebrate opsins. Previously, this has only been demonstrated in SWS1 (*19*) and RH1 (*11*) classes. This suggests the role of the salt bridge is highly conserved among all classes of vertebrate opsins. However, our results also suggest opsins belonging to different classes show distinct differences in their constitutive activity profiles. The class that most resembles the RH1 profile (*11*) is the RH2 class. This is not surprising given the close sequence similarities of the RH1 and RH2 opsin classes, arising from their close evolutionary relationship. The SWS2 opsin displayed a very different activation profile in regard to the absence of quenched constitutive activity in the presence of chromophore. The inability of a constitutively active SWS1 opsin to be quenched by reconstitution with its chromophore in the dark state was previously shown for the salamander SWS1 pigment with a triple mutation F86L/T93P/S118T (*30*). However, in the same paper, Kono et al. showed that the use of a 9-demethyl-11-*cis*-retinal chromophore could quench the constitutive activity of their triple mutant. They presented the hypothesis that there was a steric block that occurred within the active pigment which prevented it from re-forming the inactive state upon chromophore addition. Our results with the SWS2 opsin suggest a similar situation may be occurring in this class. The failure of the reconstituted pigment to change its transducin activation levels is in agreement with this hypothesis and suggests that activation may lead to a movement of residues in the binding pocket that sterically block a return to an inactive state while the chromophore is present. Finally, the M/LWS class shows that, by disrupting the E113/K296 salt bridge, a constitutively active protein is formed; however, the resulting activity is the lowest of all the vertebrate opsin classes, resulting in only ~20% of the fully activated photopigment. This indicates that this class may have a more extensive network of hydrogen bonding between its helices which requires additional energy, provided during chromophore isomerization, to be necessary to form a fully active photopigment.

Taken together, our data suggest that structural differences exist among the different opsin classes which affect activation. Past observations have suggested that structural variation among rod and cone photopigments exists. In the literature, there are examples of opsins belonging to the M/LWS (*31*), SWS1 (*26*, *32*, *33*), SWS2 (*32*, *34*), and RH2 (*32*, *35*) classes being sensitive to hydroxylamine bleaching, whereas RH1 photopigments are resistant (*32*, *35*). In addition, the formation of the covalent bond between opsin and 11-*cis*-retinal

is reversible for cone opsins in the dark but essentially irreversible for rod opsins (*36*). It is also known that cone photopigments have an increased rate of chromophore thermal isomerization when compared to rod photopigments (*37*, *38*). These observations suggest the binding pocket of the cone photopigments, which are primarily comprised of the M/LWS, SWS1, SWS2, and RH2 classes, has a conformation which is more open to the external environment than the RH1 class. Differences are also apparent between the SWS and M/LWS classes in that there is a higher spontaneous thermal isomerization rate in the M/LWS class (*38*). Other studies showing that alternative chromophores such as 9-demethyl-11-*cis*-retinal reduce the rate of salamander rod but not salamander cone activation (*39*) also indicate that structural differences may exist around the binding pocket of different opsin classes.

The exact mechanism leading to these differences remains unknown. The SWS2 opsin class may have residues that interact with the chromophore, preventing it from resuming an inactive state once moved into an active conformation. This model would require the E113/K296 salt bridge to reform in the WT opsin once the chromophore is removed from the binding pocket, thus allowing the opsin to return to an inactive state before it can regenerate with another chromophore. The M/LWS class was able to resume an inactive state; the difference in this class was its low constitutive activity level in comparison to the activated WT photopigment. This may indicate a second salt bridge may occur around the binding pocket or a second charged residue may compete with E113 to maintain the protein in an inactive conformation.

It is of interest to note that, in all cone classes measured (SWS2, M/LWS, and RH2), the level of activation for the dark state of the E113Q mutant reconstituted with chromophore was never quenched 100% compared to our WT opsin measurements. With it known that opsin maintains a low level of constitutive activity in its bleached state (*40*), Kefalov et al. (*36*) proposed that the ability of cone opsin to dissociate retinal from opsin may contribute to the increased dark noise in cones relative to rods. With the E113Q mutants shown to be constitutively active, the increased level of activation in our dark state E113Q photopigments over WT may be a reflection of the dissociation of opsin from chromophore.

In summary, this is the first study to demonstrate that mutations in the conserved salt bridge cause constitutive activation in all classes of vertebrate opsins. However, the activation levels and responses to chromophore addition in the dark state vary across the vertebrate opsin classes. While currently the only crystal structure that exists for a G-protein-coupled receptor is the dark state of bovine RH1 opsin (*20*), additional research still needs to be performed to determine structures for other vertebrate opsin classes. Although opsins of different classes have an overall similar structure, our results show that differences do exist, which may result in changes in activation properties as well as active conformations.

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