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Spectral Tuning of Rhodopsin and Metarhodopsin In Vivo

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Summary

Color vision is dependent upon the expression of spectrally distinct forms of rhodopsin in different photoreceptor cells. To identify the structural features of rhodopsin that regulate spectral sensitivity and absorption in vivo, we have constructed a series of chimeric Drosophila rhodopsin molecules, derived from a blue- and a violet-sensitive rhodopsin, and used P element-mediated germline transformation to generate transgenic flies that express the modified pigments in the R1-R6 photoreceptor cells of the compound eye. Our analysis of these animals indicates that multiple regions of the opsin protein are involved in regulating rhodopsin spectral sensitivity and that the native and photoactivated forms of rhodopsin can be tuned independently of each other. These results demonstrate the feasibility of designing receptor molecules with specifically modified activated states.

Introduction

G protein-coupled signal transduction systems are an evolutionarily conserved signaling mechanism that mediates responses to a wide range of extracellular stimuli, ranging from light and odorants to neurotransmitters (Dohlman et al., 1991; Khorana, 1992; Nathans, 1992; Oprian, 1992). G protein-coupled receptors all share several structural features, including seven membrane-spanning segments, an intracellular C-terminal tail rich in serine and threonine residues, and an extracellular N-terminal domain. Stimulation of these receptors leads to the activation of the target G proteins. This is dependent upon the ability of the receptor to respond to and discriminate between different stimuli and to undergo a conformational change that produces a catalytically active receptor molecule. Recently, biochemical and in vitro mutagenesis studies have helped to identify several of the receptor regions involved in stimulus discrimination and G protein interaction (for reviews see Nathans, 1992; Oprian, 1992). However, very little is known about the mechanism by which the ligand initiates the activation process or the sequence of events that occur during receptor activation.

Rhodopsin is the light-sensitive pigment of the eye and differs from other G protein-coupled receptors in that the ligand, the 11-cis retinal chromophore (or 3-hydroxy-11-cis retinal in flies), is covalently attached to the receptor. Upon absorption of light, the 11-12 double bond of retinal isomerizes from the cis to the trans conformation. The light-induced isomerization of the retinal ligand induces a conformational change in the opsin protein, which leads to the conversion of rhodopsin to metarhodopsin. Metarhodopsin is the activated form of the receptor. It directly couples to and activates transducin, thereby initiating the biochemical steps of the signaling cascade (Stryer, 1986). Just as the other members of the G protein-coupled receptor family are activated by different ligands, different forms of rhodopsin are activated by different wavelengths of light. An important advantage of using rhodopsin as a model system for studying G protein-coupled receptors is that the retinal chromophore can serve as a reporter group, allowing the activation process to be studied spectroscopically (Birge, 1981; Lewis and Kliger, 1992).

The spectral sensitivity of human rhodopsin and the cone opsins appears to be regulated by interactions between the 11-cis retinal chromophore and charged or polar amino acids within the opsin apoprotein (Sakmar et al., 1989, 1991; Zhukovsky and Oprian, 1989; Nathans, 1990a, 1990b; Neitz et al., 1991; Chan et al., 1992; Merbs and Nathans, 1992a, 1992b). Although recent work has begun to identify regions involved in the tuning of the native form of rhodopsin, much of the available data have been obtained in vitro, through the characterization of mutagenized opsin constructs expressed in heterologous tissue culture cells (Sakmar et al., 1989, 1991; Zhukovsky and Oprian, 1989; Nathans, 1990a, 1990b; Oprian et al., 1991; Chan et al., 1992; Merbs and Nathans, 1992a, 1992b). With the exception of studies on photoactivated intermediates using low temperature or time resolved spectral techniques, very little is known about the tuning of metarhodopsin absorption (Birge, 1981).

The Drosophila visual system is an excellent experimental system in which to study rhodopsin activation in vivo, in its normal cellular environment (Smith et al., 1991). In both Drosophila and humans, spectrally distinct visual pigments are expressed in different photoreceptor cells (Jacobs, 1981; Hardie, 1985; Nathans et al., 1986a, 1986b). This difference in opsin expression is the basis for color vision in humans and provides a unique experimental tool in Drosophila. The adult fly visual system is made up of at least six different types of photoreceptors, which differ in anatomical location and spectral sensitivity. The genes encoding four Drosophila opsins have been isolated

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and characterized. The Rh1 opsin gene encodes a blue-sensitive pigment that is the major rhodopsin of the compound eye. It is expressed in the R1-R6 class of photoreceptor cells and is genetically defined by the *ninaE* locus (O'Tousa et al., 1985; Zuker et al., 1985, 1988; Feiler et al., 1988). The Rh2 opsin gene encodes a violet-sensitive pigment that is expressed in the ocelli, simple eyes located on the vertex of the head (Cowman et al., 1986; Feiler et al., 1988; Pollack and Benzer, 1988). The Rh3 and Rh4 opsin genes encode ultraviolet (UV)-sensitive pigments that are expressed in nonoverlapping sets of R7 photoreceptor cells (Fryxell and Meyerowitz, 1987; Montell et al., 1987; Zuker et al., 1987; Feiler et al., 1992). The availability of the cloned genes and regulatory promoter elements of four Drosophila opsins has made it possible to target the expression of minor opsins (Rh2, Rh3, or Rh4) to the major class of photoreceptor cells. Expression of the minor opsins in *ninaE* mutant flies, which lack the opsin normally expressed in the R1-R6 cells (Rh1), has allowed for the detailed spectral and photochemical characterization of these minor opsins and demonstrated the utility of using the R1-R6 photoreceptors as an in vivo expression system for studying rhodopsin function (Feiler et al., 1988, 1992; Zuker et al., 1988).

The goal of the present study was to identify regions of the opsin protein that regulate rhodopsin spectral sensitivity and metarhodopsin absorption in vivo. We constructed a series of chimeric opsins in which regions of the blue Rh1 opsin were replaced with the corresponding regions of the violet Rh2 opsin. Transgenic animals expressing these genetically engineered visual pigments were characterized by microspectrophotometry and spectral sensitivity analysis. Here, we show that specific regions of the opsin apoprotein are involved in tuning rhodopsin and metarhodopsin absorption in vivo and that the native and activated forms of the receptor can be tuned independently of each other. These results are discussed in relation to receptor activation and structure.

Results

Generation of Chimeric Rhodopsin Molecules

The study of chimeric proteins has been an extremely useful strategy for identifying regions of related molecules that are responsible for differences in function (Kobilka et al., 1988; Osawa et al., 1990). We utilized this approach to identify regions of the opsin protein that regulate differences in spectral sensitivity and metarhodopsin absorption. Amino acid sequence comparison between the four known Drosophila opsins showed that these pigments comprise 2 related groups: the blue- and violet-sensitive pigments (Rh1 and Rh2) and the UV-sensitive opsins (Rh3 and Rh4). We selected the blue and violet opsins for study, since they are closely related (67% amino acid identity) and display the largest difference in absorption and sensitivity properties (Zuker et al., 1987; Feiler et al., 1988, 1992). Rh1 is maximally sensitive near 480 nm (rhodopsin or R form) and photoconverts to a metarhodopsin (M) form absorbing maximally near 570 nm. Rh2 is maximally sensitive near 420 nm and photoconverts to an M form absorbing near 500 nm. (These wavelengths are slightly shorter than previously reported [Feiler et al., 1988], reflecting a higher degree of precision in the current work.) Using a cassette mutagenesis approach, we constructed a series of chimeric genes in which single or multiple transmembrane segments of the blue Rh1 opsin were replaced with the corresponding region from the violet Rh2 opsin (Figure 1). We chose individual membrane-spanning segments as the experimental unit, since the regulation of rhodopsin absorption is thought to result from interactions between the retinal chromophore and amino acids within the transmembrane segments (Nakayama and Khorana, 1990, 1991).

The chimeric opsins were placed under the regulatory control of the Rh1 opsin promoter and introduced into *ninaE* mutant hosts by P element–mediated germline transformation. These hosts carry an internal deletion in the endogenous Rh1 opsin gene, and thus the only opsin expressed in the R1-R6 photoreceptor cells of these animals is the one encoded by the transgene (O'Tousa et al., 1985; Feiler et al., 1988, 1992; Zuker et al., 1988). The R1-R6 photoreceptor cells of Drosophila dominate the physiological and photochemical properties of the compound eye and mediate most behavioral responses that are dependent on visual input (Heisenberg and Wolf, 1984).

We generated 13 different groups of transgenic flies, each expressing a different blue/violet opsin chimera. Seven of the 13 groups expressed transgenes containing single transmembrane replacements (Rh1 TMD 1 to Rh1 TMD 7). (Nomenclature for the chimeric constructs is as follows. Rh1 TMD1 refers to rhodopsin Rh1, in which the first transmembrane domain [TMD] has been replaced with the corresponding region from Rh2. Rh2 TMD 2 refers to a chimeric Rh2 rhodopsin, in which the second transmembrane domain has been replaced with the corresponding region from Rh1. Multiple transmembrane domain replacements are designated as TMD 1–7, TMD 2–7, etc.) The remaining 6 groups expressed chimeras containing multiple transmembrane replacements (Rh1 TMD 6–7, Rh1 TMD 5–7, Rh1 TMD 4–7, Rh1 TMD 3–7, Rh1 TMD 2–7, and Rh1 TMD 1–7).

Rhodopsin Chimeras Are Functional in Transgenic Animals

To determine whether the chimeric genes were expressed in the proper tissue and at the proper level, we studied their expression by RNA (data not shown) and Western blots and by immunofluorescence staining of tissue sections of the adult eye. Figure 2 shows that the transformed animals express the transgenes in the R1-R6 photoreceptor cells. Western blot analysis of the proteins produced from the different chimeric opsin genes demonstrated a wide range of expression levels, ranging from wild-type levels to a

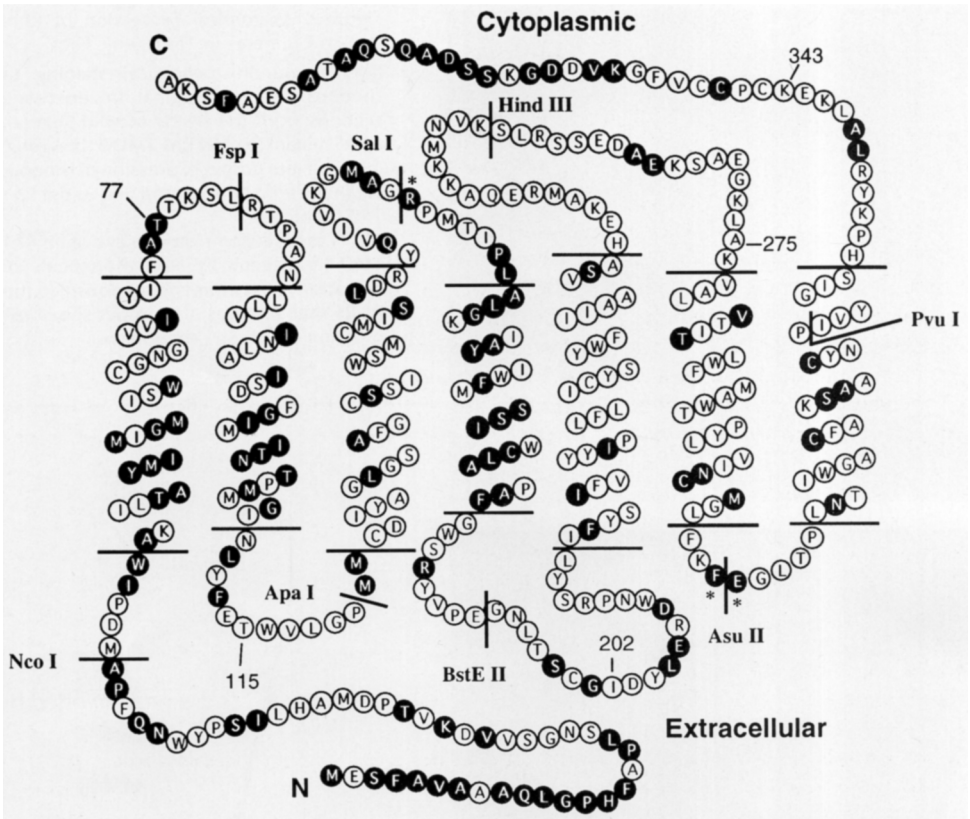

Figure 1. Proposed Structure of the Drosophila Rh1 Opsin

The diagram is based on the models of Hargrave et al. (1983) and Ovchinnikov (1982), using the sequence alignment previously reported (Zuker et al., 1987). The position of the protein with respect to the membrane has been adjusted to account for the proper placement of the counterion (Sakmar et al., 1989; Zhukovsky and Oprian, 1989; Nathans, 1990a). Solid black circles indicate amino acid differences between the Rh1 and Rh2 proteins. The diagram shows the position of the endonuclease restriction sites used for the generation of the chimeras. Asterisks flanking the SalI and AsuII restriction sites indicate amino acid residues that were not changed in some chimeras (affecting only Rh1 TMD 4 and Rh1 TMD 4–7 for the SalI site, but all chimeras containing replacements of TMD 6 or 7 for the AsuII site).

greater than 100-fold reduction (Figure 3). The reduced quantity of protein produced in mutants that express abundant levels of chimeric mRNA (for example, Rh1 TMD 5; data not shown) is most likely due to partial defects in intracellular targeting or reduced folding efficiency. This is not unexpected, given the dramatic alteration in the primary structure of some of these molecules. For instance, the Rh1 TMD 4 chimera differs at 17 amino acid positions from wild-type Rh1.

Figure 4 shows electrophysiological recordings of light-evoked responses from the photoreceptors of control wild-type flies, homozygous *ninaE* mutant hosts, and transgenic flies expressing the chimeric molecules. The on-transients of the electroretinogram have been shown to be of laminar origin and are induced only by activation of the R1-R6 photoreceptor cells (Heisenberg, 1971; Heisenberg and Wolf, 1984). The *ninaE* host strain (Figure 4, top panel) has no rhodopsin in the R1-R6 photoreceptor cells and therefore does not display on-transients in response to light. These flies also display very small signal amplitudes, which are derived from the R7 and R8 cells (Johnson and Pak, 1986). All transgenic animals that express the chimeric opsins display a normal response to light, in which the on- and off-transients of the electroretinogram (ERG) were restored. The differences in the amplitudes of the light response (compare for example Rh1 TMD 1 and Rh1 TMD 3) roughly parallel the amount of rhodopsin present in the different transformed lines. Remarkably, even though several of the chimeras were produced at extremely low levels and may have reduced folding and assembly efficiency, all of the transgenes produced biologically active visual pigment molecules.

Spectral Sensitivity Recordings

The absorption maximum of rhodopsin corresponds to the wavelength of light that is most effective in inducing the isomerization of the 11-cis retinal chromophore to the all-trans form (Styer, 1986; Nathans, 1992). Because the activation of rhodopsin is coupled to a highly amplifying biochemical cascade (Johnson and Pak, 1986; Lagnado and Baylor, 1992), the most sensitive technique for determining rhodopsin absorption in vivo is to measure spectral sensitivity electrophysiologically. In Drosophila, light activation of the phototransduction cascade results in a depolarizing receptor potential. To determine the spectral sen-

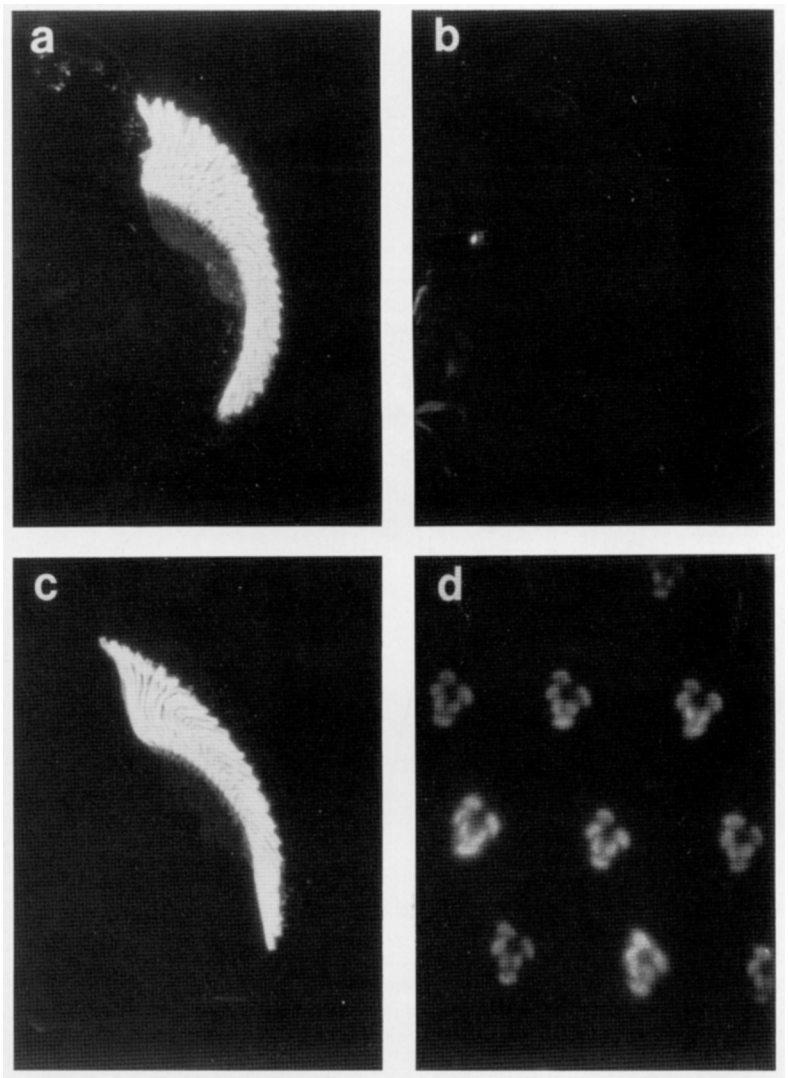

Figure 2. Anatomical Expression of Rhodopsin Chimeras in Transgenic Flies

(a–c) Immunohistochemical staining of rhodopsin in longitudinal frozen tissue sections from the heads of wild-type (a), *ninaE* mutant (b), and Rh1 TMD 2 transgenic (c) flies. Note proper expression of rhodopsin in the wild-type control and in the Rh1 TMD2 chimera.

(d) A cross section from the eye of an Rh1 TMD 1 transgenic fly. Note the specific localization of the chimeric rhodopsin to the R1-R6 rhabdomeres, the proper site of expression of the Rh1 rhodopsin.

sitivity of animals expressing the blue/violet opsin chimeras, we carried out electrophysiological recordings of their light-evoked responses using the light-clamp technique of Franceschini (1979, Invest. Ophthalmol. Suppl., abstract). In essence, the amplitude of the electroretinogram is clamped to a reference value by adjusting light intensity, and therefore the sensitivity of the photoreceptors at any given wavelength is inversely related to light flux. Figure 5B shows the spectral sensitivity of control wild-type flies (bottom panel) and of flies that express the Rh2 opsin in the R1-R6 photoreceptor cells (upper panel). These visual pigments display the known sensitivity profiles with maxima near 480 nm and 420 nm, respectively. In addition, both of these visual pigments show the characteristic peak of sensitivity in the UV region of the spectrum, owing to the presence of a sensitizing pigment in the R1-R6 photoreceptor cells (Kirschfeld et al., 1977, 1978, 1988). Figure 5A shows sensitivity recordings of animals expressing the single transmembrane segment

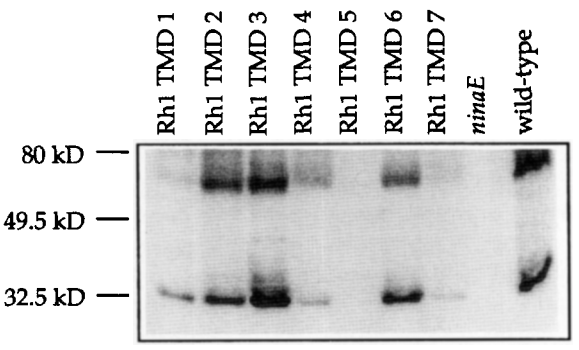

Figure 3. Expression Levels of Chimeric Rhodopsins in Transgenic Flies

A Western blot of protein extracts from the heads of flies expressing the seven single transmembrane domain chimeras (Rh1 TMD 1 through Rh1 TMD 7) is shown. Drosophila rhodopsin appears in monomeric and dimeric forms; numbers to the left of the panel indicate molecular weight markers. Expression levels ranged from nearly wild-type (Rh1 TMD 3) to barely detectable (Rh1 TMD 5).

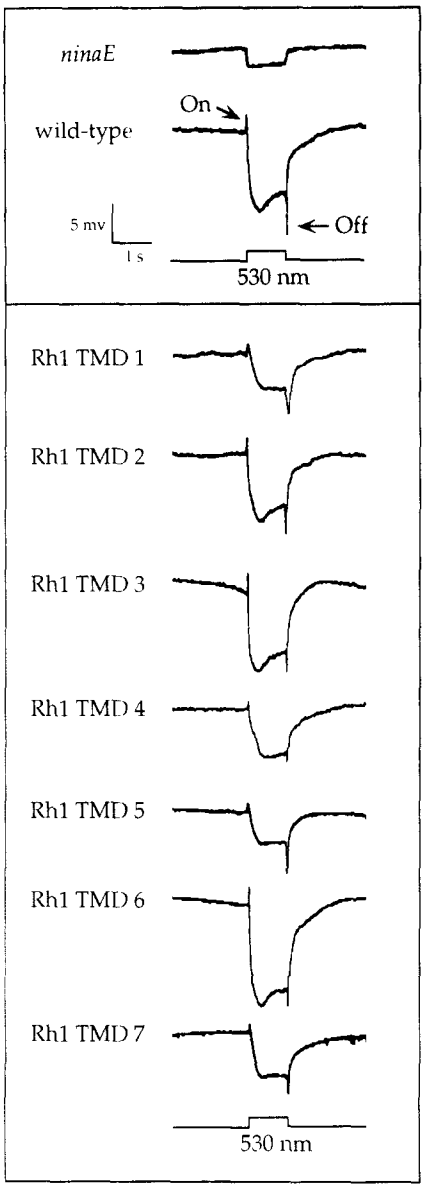

Figure 4. Rh1/Rh2 Rhodopsin Chimeras Rescue the Visual Response of *ninaE* Mutants

ERG recordings from wild-type controls, *ninaE* mutant flies (upper panel), and *ninaE* mutants transformed with the single transmembrane chimeric opsins expressed under the control of the Rh1 promoter (Rh1 TMD 1 through Rh1 TMD 7) (lower panel) are shown. *ninaE* mutants do not express rhodopsin in the R1-R6 photoreceptor cells. These mutants do not display on-transients and show a dramatically reduced response to light (derived primarily from the R7 and R8 photoreceptor cells). In contrast, transgenic animals expressing the chimeric opsins show proper responses following visible light stimulation.

chimeras. The results demonstrate that there were only minor changes in the sensitivity of the blue Rh1 opsin when any one single transmembrane segment was replaced with the corresponding region from the violet Rh2 opsin. Each of the 7 groups of transgenic flies shows a broad peak of sensitivity near 480 nm, which is similar to that of the blue Rh1 opsin. As expected, all of the chimeras show the characteristic peak of sensitivity in the UV region of the spectrum, which does not differ between Rh1 and Rh2.

Figure 5B shows the spectral sensitivity of chimeric opsins in which multiple membrane-spanning segments of Rh1 were replaced with the corresponding regions of Rh2. As predicted, replacement of all seven transmembrane segments of the blue opsin with those from the violet opsin (Rh1 TMD 1-7) generates a molecule which now has spectral sensitivity that is virtually identical to the violet Rh2 rhodopsin. These results indicate that replacement of the N- and C-terminal segments are not required to produce a violet-sensitive rhodopsin. Chimeras that have smaller replacements (Rh1 TMD 3-7, Rh1 TMD 4-7, Rh1 TMD 5-7, and Rh1 TMD 6-7) show either broadened sensitivity in the blue region or sensitivity profiles similar to the blue Rh1 opsin. Thus, replacement of as many as five out of the seven transmembrane segments (as in Rh1 TMD 3-7) is not enough to shift the sensitivity of the Rh1 opsin significantly. Interestingly, replacement of one additional region, the second transmembrane segment, converts the Rh1-like Rh1 TMD 3-7 chimera into an Rh2-like molecule (Rh1 TMD 2-7). These results suggest that the second transmembrane domain is involved in regulating the spectral sensitivity differences between the Rh1 and Rh2 rhodopsins, but is not sufficient by itself to confer this change (as evidenced by the Rh1-like sensitivity of the Rh1 TMD 2 single transmembrane replacement chimera).

Metarhodopsin Is Tuned Independently of Rhodopsin

In invertebrates, the absorption profile of metarhodopsin can be conveniently determined by in vivo microspectrophotometry (MSP) because the photopigment does not bleach following light activation. Thus, by using different wavelengths of adapting light, it is possible to photoconvert the visual pigment molecule from the native R form into a thermally stable photoactivated M form, and vice versa (Minke, 1986). Difference spectra analysis may then be used to determine the absorption profiles of the R and M forms. MSP of wild-type flies shows that the Rh1 rhodopsin absorbs maximally near 480 nm and is photoconverted to a metarhodopsin with an absorption maximum near 570 nm (Figure 6A). The Rh2 photopigment has R and M form absorptions that are blue shifted from those of the Rh1 pigment, with absorption maxima near 420 nm and 500 nm, respectively (Figure 6B).

Figure 6C shows the difference spectra of the single transmembrane segment chimeras. Replacement of transmembrane regions 1, 3, 4, 6, or 7 of Rh1 with those of Rh2 had little effect on the absorption maxima of the M form of these pigments. The difference spectrum of Rh1 TMD 5 was not detectable by MSP, most likely owing to the very reduced level of opsin produced in transgenic animals expressing this chi-

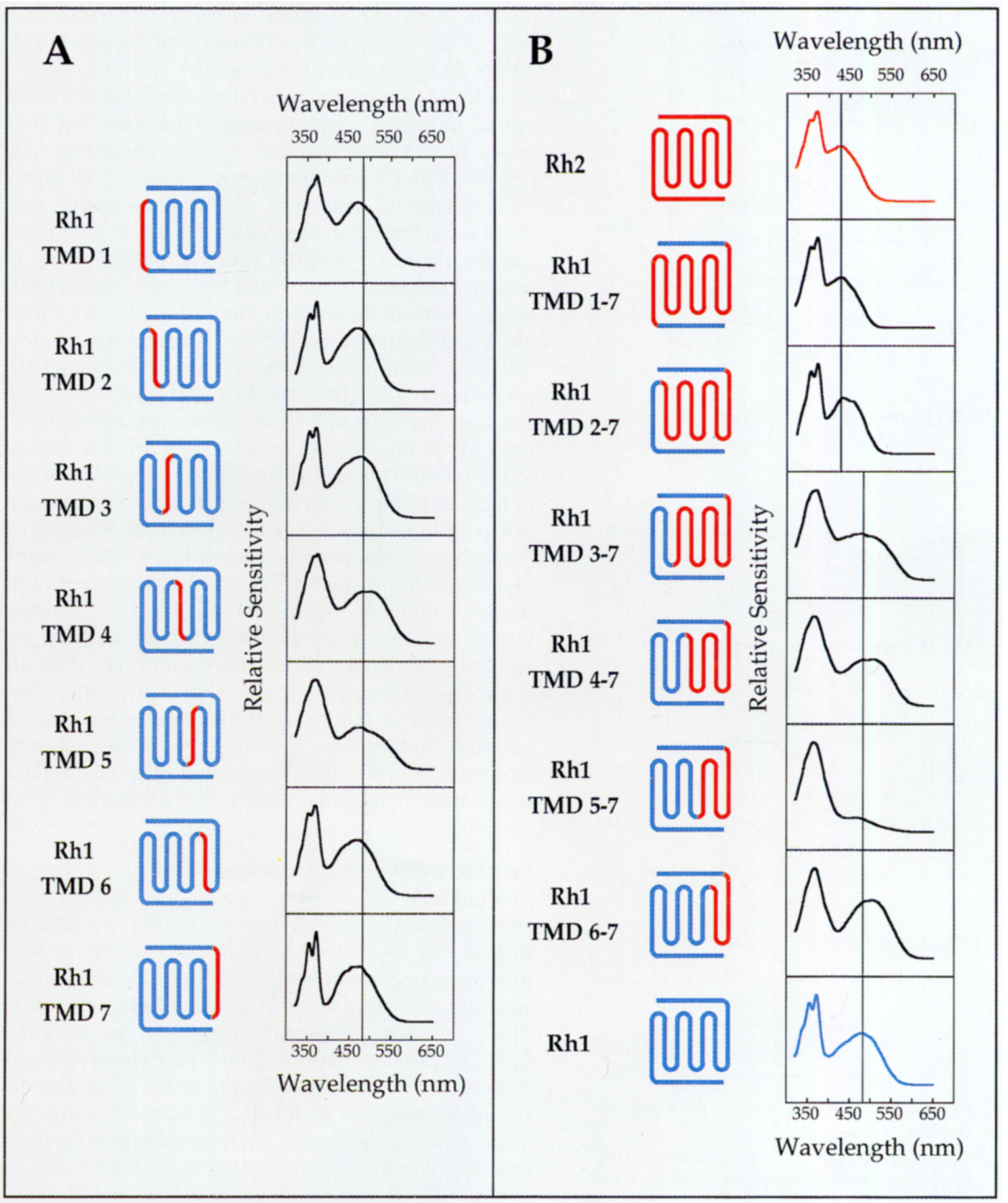

Figure 5. Spectral Sensitivities of the Chimeric Drosophila Opsins

(A) The ERG sensitivity recordings of the single transmembrane chimeras. The diagrams show the schematic structure of the resulting chimeric molecules. Light blue represents Rh1 sequences and dark red represents segments derived from the violet Rh2 rhodopsin. No single transmembrane chimera significantly changed the sensitivity profile of Rh1.

(B) The ERG sensitivity recordings of the multiple membrane-spanning chimeras. The diagram shows the structures of the resulting chimeric molecules. Four of the multiple membrane-spanning chimeras (Rh1 TMD 3–7, Rh1 TMD 4–7, Rh1 TMD 5–7, and Rh1 TMD 6–7) have sensitivity profiles in the visible region that are similar to Rh1 (see vertical cross line at 480 nm). Interestingly, two of the multiple transmembrane domain chimeras (Rh1 TMD 1–7 and Rh1 TMD 2–7) have sensitivity profiles that are now very similar to the Rh2 rhodopsin (see vertical cross line at 425 nm). The sensitivity in the UV region of several chimeras (Rh1 TMD 1 and Rh1 TMD 4, for example) lacks the normal vibrational fine structure shown in the traces for Rh2 and Rh1. The absence of this doublet indicates that the ionone ring and polyene chain of the 3-OH-retinol sensitizing pigment are not coplanar in the affected chimeras, but are mobile and free to rotate (Kirschfeld and Vogt, 1986). The reduced sensitivity of the Rh1 TMD 5–7 chimera to light in the blue region, relative to that in the UV, may be due to an altered chromophore (Fischer et al., 1981). Sensitivity maxima in the visible region for each chimera were as follows: Rh1 TMD 1 (469 nm), Rh1 TMD 2 (468 nm), Rh1 TMD 3 (475 nm), Rh1 TMD 4 (490 nm), Rh1 TMD 5 (470 nm), Rh1 TMD 6 (467 nm), Rh1 TMD 7 (465 nm), Rh2 (427 nm), Rh1 TMD 1–7 (428 nm), Rh1 TMD 2–7 (436 nm), Rh1 TMD 3–7 (472 nm), Rh1 TMD 4–7 (498 nm), Rh1 TMD 5–7 (464 nm), Rh1 TMD 6–7 (501 nm), Rh1 (479 nm).

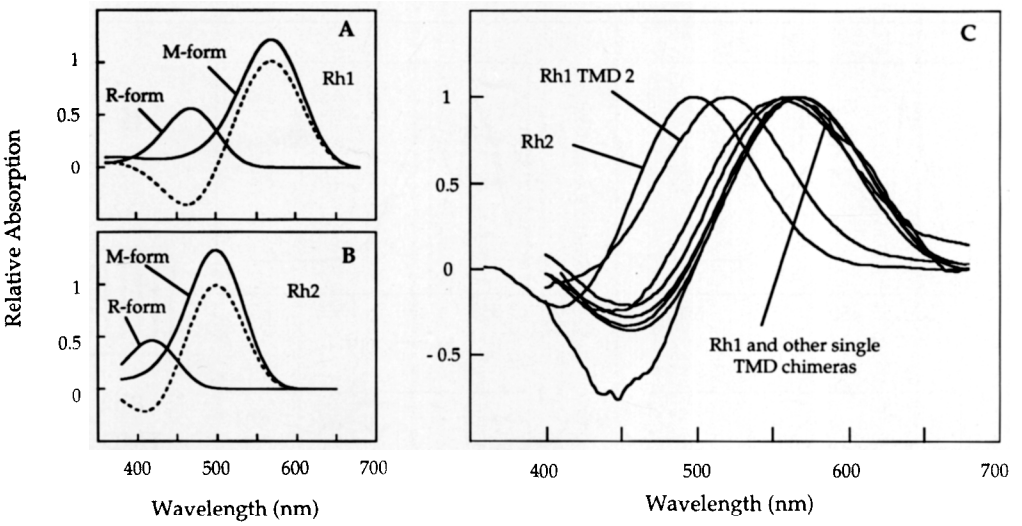

Figure 6. MSP of Transgenic Animals

(A) and (B) The difference spectra recordings (broken lines) from control wild-type (Rh1) and *ninaE* mutants transformed with the Rh2 opsin gene expressed under the control of the Rh1 opsin promoter (Rh2). Also indicated are the modeled curves for the absorption properties of the R and M forms of these pigments (solid lines). The R and M maxima for Rh1 are near 480 nm and 570 nm, respectively. The R and M maxima for Rh2 are near 420 nm and 500 nm, respectively.

(C) The difference spectra recordings from the single membrane-spanning chimeras. Note that the difference spectra for Rh1 TMD 1, Rh1 TMD 3, Rh1 TMD 4, Rh1 TMD 6, Rh1 TMD 7, and Rh1 overlap with an absorption maximum near 470 nm. In contrast, the absorbance of the Rh1 TMD 2 chimera has been significantly shifted toward that of the Rh2 opsin. Absorption maxima for the difference spectrum of each chimera were as follows: Rh1 TMD 1 (557 nm), Rh1 TMD 2 (520 nm), Rh1 TMD 3 (567 nm), Rh1 TMD 4 (566 nm), Rh1 TMD 6 (559 nm), Rh1 TMD 7 (563 nm), Rh2 (499 nm), Rh1 (568 nm).

meric gene (see Figure 3). Surprisingly, the difference spectrum of transgenic flies expressing the Rh1 TMD 2 chimera was significantly blue shifted and had an absorption maximum at 520 nm. These results suggest that amino acids within the second transmembrane segment which differ between the blue and violet opsins (see Figure 1) are largely responsible for the difference in absorption of the Rh1 and Rh2 metarhodopsins. Indeed, it appears that the second transmembrane segment is sufficient to retune the absorbance of the Rh1 M form to that of the Rh2 opsin without an associated change in rhodopsin sensitivity (Figure 7A). We modeled the absorption spectra of the R and M forms of the Rh1 TMD 2 chimera and determined that the R form of the pigment has a maximal absorption near 470 nm, very much like Rh1, and the M form has a maximal absorption near 510 nm, very much like Rh2 (models not shown; see Figure 6 for examples of the method).

To demonstrate that the second transmembrane domain independently regulates the absorption of metarhodopsin, we constructed the reciprocal transgene of the Rh1 TMD 2 chimeric opsin. In this new chimera, Rh2 TMD 2, the second transmembrane segment of the violet Rh2 opsin was replaced with the corresponding region from the blue Rh1 opsin. If the second membrane-spanning region can independently regulate metarhodopsin absorption, then we would predict that this chimeric opsin would have an R form sensitivity very similar to the parental Rh2 opsin, but a metarhodopsin absorption spectrum that is red shifted toward that of the Rh1 donor molecule. Figure 7B shows that this is precisely what was observed. The Rh2 TMD 2 chimeric opsin has an R form sensitivity with a maximum near 430 nm and an M form absorption maximum that has been red shifted to nearly 550 nm. Taken together, these results demonstrate that the second transmembrane domain is involved in regulating metarhodopsin absorption and that the tuning of metarhodopsin, the activated form of the visual pigment molecule, can occur independently of changes in rhodopsin absorption and sensitivity.

The absorption shifts of the TMD 2 chimeras most likely reflect the retuning of metarhodopsin absorption and not the formation of a novel photointermediate. Had the equilibrium between rhodopsin and metarhodopsin been altered by the addition of a novel species, adapting the sample with different wavelengths of light would be expected to shift the equilibrium and the λmax of the difference spectrum. We performed these experiments using many different adapting light combinations, and no shift in the difference spectrum was observed (data not shown). Rather, the amplitude of the difference spectrum decreased, as expected, for a simple two component equilibrium in which illumination at wavelengths other than the λmax of the R and M forms yields only partial photoconversion (Schwemer, 1989). In addition, MSP measurements of the difference spectrum of the Rh1 TMD 3–7 chimera show that this pigment

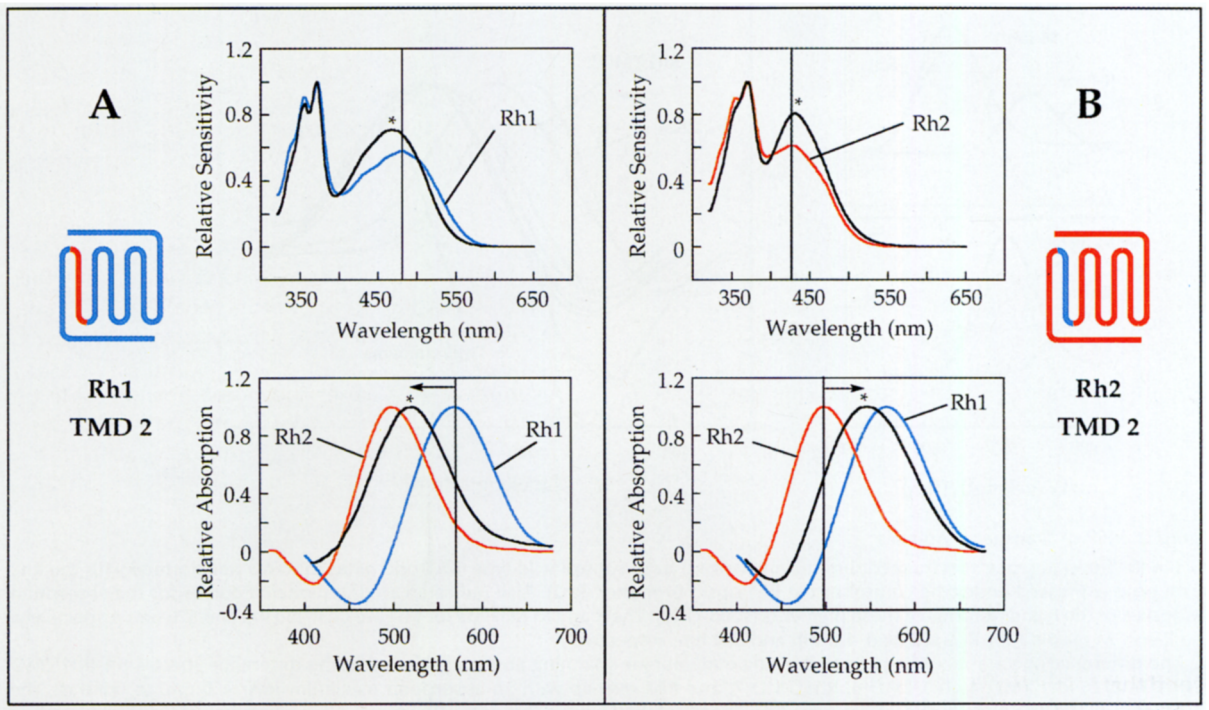

Figure 7. Metarhodopsin Is Tuned Independently of Rhodopsin

The upper panel of (A) shows sensitivity recordings of the Rh1 TMD 2 chimera (black trace, asterisk). The spectrum of wild-type controls is in light blue (labeled). Note the overlap in the sensitivity spectra. The lower panel shows MSP recordings from animals expressing the same chimera (asterisk). The absorption spectra of control Rh1 (light blue) and Rh2 (dark red) rhodopsins (labeled) are also indicated. The Rh1 TMD 2 chimera displays a 48 nm shift in the difference spectrum of its M form toward the Rh2 maximum. The upper panel of (B) shows sensitivity recordings of the Rh2 TMD 2 chimera (λmax = 431 nm; black trace, asterisk). Represented in dark red (labeled) is the spectrum of Rh2 controls. Note the overlap in the sensitivity spectra. The lower panel shows MSP recordings from animals expressing the same chimera (asterisk). The absorption spectra of control Rh1 (light blue) and Rh2 (dark red) rhodopsins (labeled) are also indicated. The Rh2 TMD 2 chimera (λmax = 546 nm) displays a 47 nm shift in the difference spectrum of its M form toward the Rh1 maximum.

has an absorption maximum at 550 nm, near that of Rh1. Addition of the second transmembrane segment to this chimera produces Rh1 TMD 2–7 and blue shifts the difference spectrum to an absorption maximum at 518 nm, near that of Rh2. These results, in combination with the reciprocal absorbance shifts observed for the M forms of the Rh1 TMD 2 and Rh2 TMD 2 chimeras, provide strong evidence that the second transmembrane segment does indeed play an important role in regulating metarhodopsin absorption.

Discussion

Rhodopsin is one of the best characterized members of the G protein–coupled receptor family. Studies into the mechanisms of rhodopsin function have provided fundamental insight into the biology of G protein–coupled receptors in general. Although much is known about the biochemical steps that constitute the phototransduction pathway, our knowledge of the mechanisms underlying color discrimination and the dynamics of rhodopsin activation is limited. Indeed, much of the work on the biology of tuning the visual pigments has been restricted to studies of the red and green cone pigments and to the identification of the counterion in rhodopsin thought to be responsible for its visible spectral properties (Sakmar et al., 1989; Zhukovsky and Oprian, 1989; Nathans, 1990a, 1990b; Neitz et al., 1991; Sakmar et al., 1991; Chan et al., 1992; Merbs and Nathans, 1992a, 1992b).

In this paper, we have utilized Drosophila as a model system to characterize the regulation of rhodopsin and metarhodopsin tuning in vivo. We have shown that the replacement of any single transmembrane segment of the blue Rh1 opsin by the corresponding region from the violet Rh2 opsin was not sufficient to change rhodopsin sensitivity. Additional experiments showed that the second transmembrane domain, in combination with another region(s) in TMDs 3–7, was capable of conferring Rh2-like violet sensitivity to the Rh1 chimera. Other experiments suggested that the first transmembrane segment or perhaps the N- and C-termini, may also play an interactive role in regulating spectral sensitivity (compare Rh2 TMD 2 and Rh1 TMD 3–7 sensitivities in Figure 5B and Figure 7B). These results indicate that spectral tuning of Drosophila rhodopsin occurs by a coordinated process involving more than one region of the protein.

This result differs significantly from the mechanism proposed for the regulation of the human red and green cone pigment absorptions. Comparative studies suggest that perhaps as few as 3 amino acids in the fourth and sixth transmembrane segments may each contribute small incremental but additive shifts to the absorption of the red and green cone pigments (Neitz et al., 1991; Chan et al., 1992; Merbs and Nathans, 1992a, 1992b).

The dramatic difference in the regulation of spectral tuning between the human red and green cone pigments and the Drosophila opsins is most likely attributable to the extreme similarity of the human cone pigments. The human red and green cone opsins are 96% identical at the amino acid level (differing at only 15 amino acid positions) and differ in maximal sensitivity by only 35 nm. By contrast, the Drosophila Rh1 and Rh2 opsins differ from each other at 117 amino acid positions (only 67% identity) and have over a 60 nm difference in spectral sensitivity. This suggests that there may be two mechanisms which regulate rhodopsin sensitivity, one that has large scale spectral effects and occurs in a combinatorial manner, and a second that is involved in the incremental fine tuning of rhodopsin. We expect that both of these mechanisms may operate simultaneously, perhaps with residues in the second transmembrane contributing to a coarse adjustment of spectral sensitivity, and with amino acids in the fourth and sixth transmembrane segments producing small additive and incremental shifts in rhodopsin sensitivity.

An additional interesting result of this study is the finding that chimeric molecules containing multiple novel membrane-spanning segments (for example the blue-sensitive chimeras Rh1 TMD 5–7, Rh1 TMD 4–7, and Rh1 TMD 3–7 have three, four, and five transmembrane segments replaced, representing 21, 38, and 49 amino acid changes, respectively) are still capable of folding and assembling into biologically active rhodopsin molecules. These results highlight the robust nature of the seven transmembrane segment structural motif and provide a dramatic example of the conservation of receptor tertiary structure in the face of significant amino acid sequence divergence.

Finally, we have presented a detailed analysis of the regulation of spectral tuning of the activated state of the visual pigment molecule. Our results demonstrate that metarhodopsin tuning is regulated by amino acid residues in the second membrane-spanning segment. In transgenic flies expressing the Rh1 TMD 2 chimera, the sensitivity of rhodopsin was unchanged, but the absorbance of metarhodopsin was retuned toward that of the Rh2 metarhodopsin. In this chimera, 12 amino acid residues differ from Rh1. Among these, Thr^{101} to Ser, Asn^{102} to Gln, and Thr^{103} to Ser are particularly interesting because these substitutions involve a change in the length of polar side chains. Further evidence that this effect reflected the retuning of metarhodopsin absorption was obtained by studying the sensitivity and absorption of a reciprocal chimera, in which the second transmembrane region of the violet Rh2 opsin was replaced with the same region from the blue Rh1 opsin (Rh2 TMD 2). In this chimera, rhodopsin sensitivity remained close to that of the violet Rh2 opsin, but metarhodopsin absorption was shifted toward that of Rh1. Thus, we have generated visual pigment molecules in which the rhodopsin and metarhodopsin forms are either 40 nm apart (Rh1 TMD 2), 90 nm apart (Rh1 wild-type), or 120 nm apart (Rh2 TMD 2), effectively narrowing or broadening the R to M shift that serves to photoregenerate the native form of rhodopsin in the wild-type (red eyed) fly (Stavenga, 1989).

In summary, this paper shows that it is possible to introduce localized structural changes within the opsin protein that retune metarhodopsin absorption without an associated change in rhodopsin sensitivity. This demonstrates that different regions of the protein interact with the chromophore in the native and activated states and opens up the possibility of custom tailoring light receptor molecules with specifically tuned photointermediate states. Such mutants will provide a means to dissect temporally the pathway of chromophore movement within the opsin protein during photoactivation.

Experimental Procedures

Mutagenesis and Drosophila Transformations

Novel restriction endonuclease sites were introduced into the Drosophila Rh1 and Rh2 opsin genes by in vitro mutagenesis (Saiki et al., 1988; Yon and Fried, 1989). The chimeric genes were assembled by restriction fragment replacement and placed under the regulatory control of the Drosophila Rh1 promoter, as described previously (Feiler et al., 1988). The DNAs were subcloned into a Drosophila P element transformation vector and injected into *ninaE*^{17} mutant embryos (Lindsley and Zimm, 1992). The P element-mediated germline transformations were performed using standard techniques (Karess and Rubin, 1984).

Immunochemistry

Immunohistochemistry and Western blot analysis were performed as previously described (Colley et al., 1991; Feiler et al., 1992).

Electroretinograms

All recordings were carried out on white eyed flies. Glass electrodes were filled with normal saline. Light stimulation was by means of a xenon arc lamp (450 W Osram, Oriel Corp., Stratford, CT). The light beam was passed through a high intensity grating monochromator (Oriel model 77264). A 1 s pulse of dim 530 nm light was used as the stimulus. The ERG signals were amplified and processed as previously described (Feiler et al., 1988).

Spectral Sensitivity Analysis

The spectral sensitivity measurements were calculated from the ERGs of white eyed flies, using the light-clamp technique of Franceschini (1979, Invest. Ophthalmol. Suppl., abstract) (Feiler et al., 1988; Kirschfeld et al., 1988). Briefly, a quartz neutral density wedge (density 0–4) was rotated in the path of the stimulating light in such a way that the amplitude of the ERG remained constant during the scan through the spectrum. Under these conditions, the spectral sensitivity of the eye is inversely related to light flux at any given wavelength. The resolution of the monochromator (Zeiss MM12) was 2 nm at 350 nm. The signal to noise ratio was improved by chopping the light stimulus (5–20 Hz) and averaging over the area of the alternating current signal. With the

exception of Rh1 TMD 1–7, at least two independent transformed lines were analyzed for each chimeric opsin. Each sensitivity graph represents the average of four or more scans.

Microspectrophotometry

The MSP data were obtained from white eyed animals. A Leitz MPV2 single beam microspectrophotometer equipped with Zeiss Ultrafluar optics and a Products for Research C31034A02 photomultiplier was used for the absorption measurements. The recording paradigm and data analysis were conducted as previously described (Kirschfeld et al., 1978; Feiler et al., 1988).

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