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Evidence from *Chlamydomonas* on the Photoactivation of Rhodopsins without Isomerization of Their Chromophore

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SUMMARY

Attachment of retinal to opsin forms the chromophore N-retinylidene, which isomerizes during photoactivation of rhodopsins. To test whether isomerization is crucial, custom-tailored chromophores lacking the β-ionone ring and any isomerizable bonds were incorporated in vivo into the opsin of a blind mutant of the eukaryote *Chlamydomonas reinhardtii*. The analogs restored phototaxis with the anticipated action spectra, ruling out the need for isomerization in photoactivation. To further elucidate photoactivation, responses to chromophores formed from naphthalene aldehydes were studied. The resulting action spectral shifts suggest that charge separation within the excited chromophore leads to electric field-induced polarization of nearby amino acid residues and altered hydrogen bonding. This redistribution of charge facilitates the reported multiple bond rotations and protein rearrangements of rhodopsin activation. These results provide insight into the activation of rhodopsins and related GPCRs.

INTRODUCTION

Many biological signaling pathways begin with activation of membrane receptor proteins that sense light or chemicals. Some of these receptors in turn activate G protein signaling cascades that lead to cell response. Because conserved processes of activation are anticipated (Gouldson et al., 2004; Mirzadegan et al., 2003) among GPCRs (G protein-coupled or catalyzing receptors) of which animal rhodopsin is a well-studied prototype, how rhodopsins are activated by light is of significant general interest. Rhodopsins are membrane receptor proteins that use a light-absorbing chromophore to capture the light and as a consequence of light absorption, gain enzymatic activity. Rhodopsins are found in bacteria, archaea, eukaryotes such as *Peranema* (a euglenoid) (Saranak and Foster, 2005), *Chlamydomonas* (a green alga) (Foster et al., 1984), and *Allomyces* (a chytrid-motile fungus) (Saranak and Foster, 1997), and animals. The existence of similar conserved amino acids in all rhodopsins in the region surrounding the chromophore (Saranak and Foster, 2005) indicates the likelihood of common or at least similar mechanisms of rhodopsin activation. The commonality applies whether the similarities are due to ancestral or convergent evolution.

Photons are absorbed by the N-retinylidene chromophore formed by retinal binding to a specific lysine of the photoreceptor molecule, opsin. For 50 years the dominant hypothesis has been that light-triggered isomerization of N-retinylidene in rhodopsin is sufficient by itself to activate vision (Hubbard and Kropf, 1958; Kandori et al., 2001). However, with a *Chlamydomonas* mutant lacking a native chromophore, a series of incorporated retinal analogs where each sequential double bond was individually locked restored rhodopsin-dependent phototaxis (light-oriented swimming) (Foster et al., 1989). This phototaxis system is thought to be the origin of rhodopsin-based vision (Foster, 2009). Because no specific bond isomerization proved to be crucial for the initial step of activation, it seemed likely that the charge redistribution in the chromophore-binding site was primarily responsible for activation (Foster et al., 1989, 1991). However, in every case at least one of the remaining double bonds in the tested chromophores could be isomerized. As a consequence, it could be claimed that isomerization about any double bond is sufficient for activation. Here, we show the new result that isomerization about any of the bonds is not necessary.

The N-retinylidene chromophores of rhodopsins have two main roles. First, they absorb a photon to activate rhodopsin, and second, their occupation of a specific site within the receptor protein inhibits spontaneous activation of rhodopsins in the absence of light, so-called inverse agonists. The β-ionone moiety (ring of **1,** Figure 1) of the chromophore is responsible for the inverse-agonist activity and likely requires isomerization to remove it. However, the β-ionone moiety is not necessary for rhodopsin activation. Chromophores formed from nonlocked analogs lacking β-ionone enable photoactivated proton pumping with bacteriorhodopsin and activate bovine rhodopsin (Rao et al., 1985). Hence, in order to test whether the activation itself requires isomerization, we have used truncated chromophores lacking the β-ionone moiety so that the inverse-agonist activity does not have to be removed by isomerization.

Our goal is to clarify the roles of N-retinylidene isomerization and charge separation along the chromophore (Colonna et al., 2007; Groma et al., 2004) with respect to the initiation of the phototransduction process of eukaryotic rhodopsins. Our experimental model system is the FN68 mutant of the alga *Chlamydomonas* whose carotenoid biosynthesis is defective. Exogenous addition of retinoids is required to restore rhodopsin-dependent

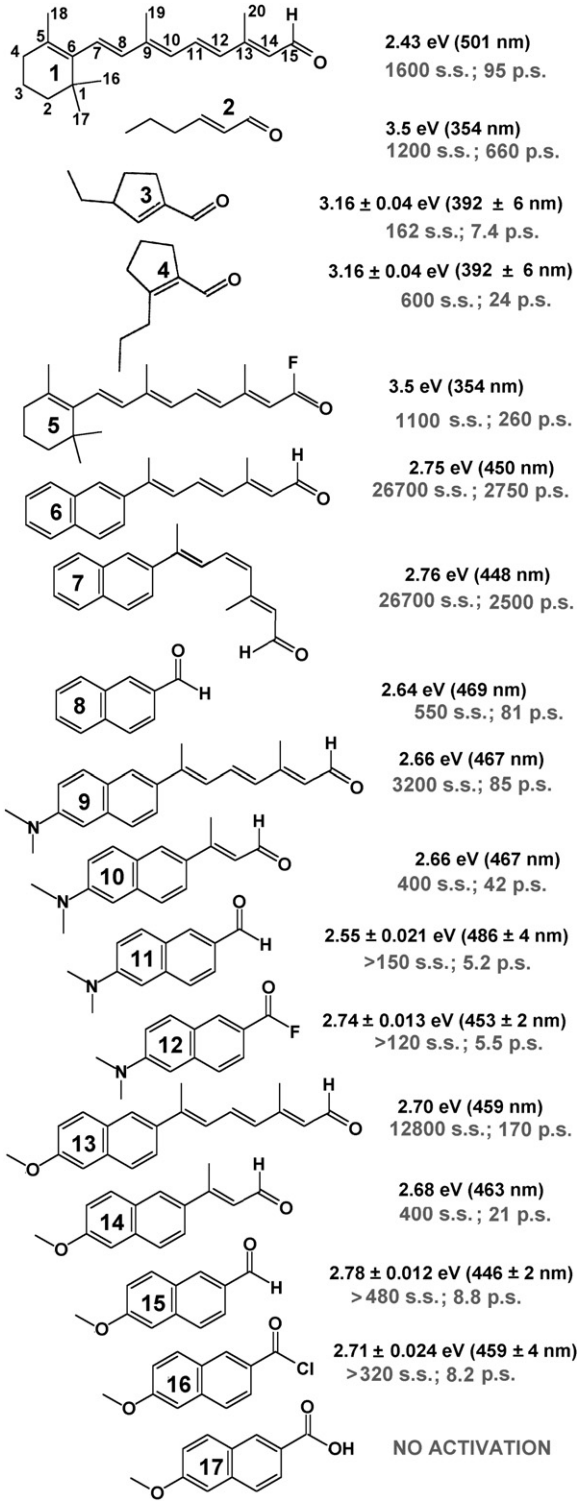

Figure 1. Structures of the Retinal Analogs Incorporated into *Chlamydomonas* Opsin

The analogs with their action spectral peaks ± uncertainty, and sensitivities (s.s., the sensitivity shift or the fold increase in sensitivity upon addition of the analog; p.s., the absolute peak sensitivity in units of nm^2 s/photon) for all analogs that recover phototaxis are shown.

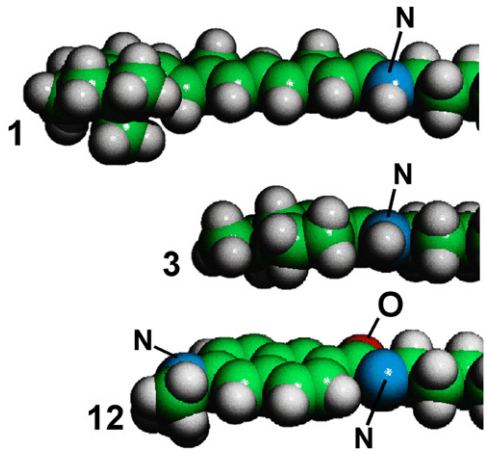

Figure 2. Space-Filling Structures

The structures show the relative sizes and lengths of the native all-*trans* N-retinylidene (**1**) chromophore and two truncated (**3** and **12**) chromophores attached to the N of the lysine residue in opsin.

phototaxis in this mutant. A physiological assay for recovery of phototaxis has been chosen because it is the only method that conclusively shows in vivo activities reflecting physiological function. The phototaxis assay also has the advantage that only photoactivation is recorded. Because cells self-modulate the light by their rotation, there is no background response due to potential chemical activation of rhodopsins. New rigid ligands were designed, synthesized, and incorporated into the opsin, forming chromophores that cannot isomerize about any bond in response to light. The β-ionone pocket of the opsin remains unoccupied in all compounds newly reported here (**3**, **4**, **8**, **10–12**, and **14–16**, Figure 1). Using an in vivo physiological assay with an ~30,000-fold range of measurable sensitivity (Saranak and Foster, 1994), we demonstrate here for *Chlamydomonas* rhodopsin that truncated chromophores completely blocked from isomerization at *all* bonds (**12**, **16**, Figures 1 and 2) fully activate rhodopsin and restore phototaxis. A complete threshold action spectrum for each analog-substituted rhodopsin was measured to determine the relative sensitivity of each pigment (Forster et al., 1988) and whether the shape and peak of the action spectrum are consistent with the binding of each analog to an opsin in the chromophore pocket environment (Foster, 2001).

A question left unanswered in our previous work was how the proposed charge separation along the chromophore might activate rhodopsin. We present evidence here for a strong interaction between the chromophore and the polarizable amino acids located in the chromophore binding sites of all rhodopsin proteins. Our results indicate that charge separation (Birge and Zhang, 1990; Colonna et al., 2007; Groma et al., 2004; Salem and Bruckmann, 1975) is sufficient for receptor activation when the β-ionone binding site is not occupied. Hence, we confirm the primary activation step of rhodopsins is electronic rather than geometric or mechanical. The initial stages of rhodopsin activation with full-length native chromophores may likely involve at least two steps. In step one the primary activation step, *not* requiring isomerization, couples the chromophore activation to the local region of the opsin protein. It is this first step in

the photoactivation mechanism that we wish to establish in this paper. In step two, the secondary step, several hundred nanoseconds later, a change in chromophore shape results from isomerization that removes the inhibitory effect of the β-ionone at the free end of the chromophore.

RESULTS

Short Truncated Chromophores

Previously, we showed that many elements of the normal chromophore including the β-ionone group are not required for rhodopsin activation. *Trans*-2-hexenal (**2**), a short acyclic aldehyde, restored rhodopsin-dependent phototaxis in a green alga *Chlamydomonas* (Foster et al., 1989; Nakanishi et al., 1989), in the chytridiomycete *Allomyces reticulatus* (Saranak and Foster, 1997), and the curling behavior in a euglenoid *Peranema trichophorum* (Saranak and Foster, 2005). Shape changes in the chromophore due to isomerization may not be crucial to receptor photoactivation in these three organisms in different eukaryotic kingdoms. To clarify reports of variable activity for full-length retinal analogs that are isomerization locked at their 13,14 position (see **1** in Figure 1 for the numbering), we synthesized and tested two new compounds, *trans*-locked (**3**) and *cis*-locked (**4**) hexenal (Figure 1), for which double-bond isomerization is impossible. These truncated chromophores recovered phototaxis in the *Chlamydomonas* mutant FN68 (Figures 3A and 3B) with sensitivity comparable to the native chromophore, taking into account the geometry and hydrophobicity differences of the chromophores. In this regard we note a short analog like **3**, but with an isopentyl group instead of the ethyl group, showed a peak sensitivity (p.s.) between 60 and 120 nm^2 s/photon compared with the 7.4 nm^2 s/photon reported for **3**, implying that increasing the hydrophobicity greatly increases incorporation and net activity. The activity of these compounds demonstrates that isomerization about the 13-14 bond is not required. Hence, a different event must lead to activation of the chromophore. Apart from the possibility of isomerizing about the syn-anti imine bond connected to the lysine N moiety of opsin, no other isomerization can take place in these short chromophores.

Naphthalene Chromophores

To explore locking all the isomerizable bonds, including the syn-anti imine linkage to the opsin protein, naphthalene aldehyde analogs were prepared and screened. We previously reported that replacing the β-ionone of the native chromophore with the naphthalene moieties **6** and **7** generated high activity (Foster et al., 1989). Here, we have explored the naphthalene moieties further with two series of naphthalenoid compounds. The first series incorporated the 6-(N,N-dimethylamino)-2-naphthyl group and comprised one compound of normal retinal length (**9**), a truncated aldehyde with only one double bond in the chain (**10**), and a simple naphthaldehyde without a conjugated chain (**11**). These 6-(N,N-dimethylamino)-2-naphthaldehydes show similar action spectra as compounds **6** and **7**, in which a naphthalene replaces the cyclohexane moiety. The 6-methoxy-naphthaldehyde series (**13–15**) also gave overlapping spectra (Figure 4B).

It is interesting to note that usually shortened chromophores result in blue-shifted (higher energy absorbing) pigments as solutions in methyl alcohol (Figure 4B). However, the shortest 2-naphthaldehyde chromophores (**8**, **11**, and **15**) formed a pigment with the opsin in the cells with either a slight spectral red shift (lower energy) or no significant shift in comparison to longer chromophores. This minimal shift of the action spectra of the naphthaldehyde analogs with progressively shorter conjugation is in contrast to the large blue shift observed for the action spectra of the equivalent retinal analogs (Figure 4A) (Foster et al., 1991). The naphthalene transition dipole likely interacts with an induced dipole of a nearby polarizable group or a transition dipole such as that of a tryptophan in the protein. This suggestion implies a particularly polarizable environment near the lysine region of the receptor-binding pocket (Okada et al., 2004).

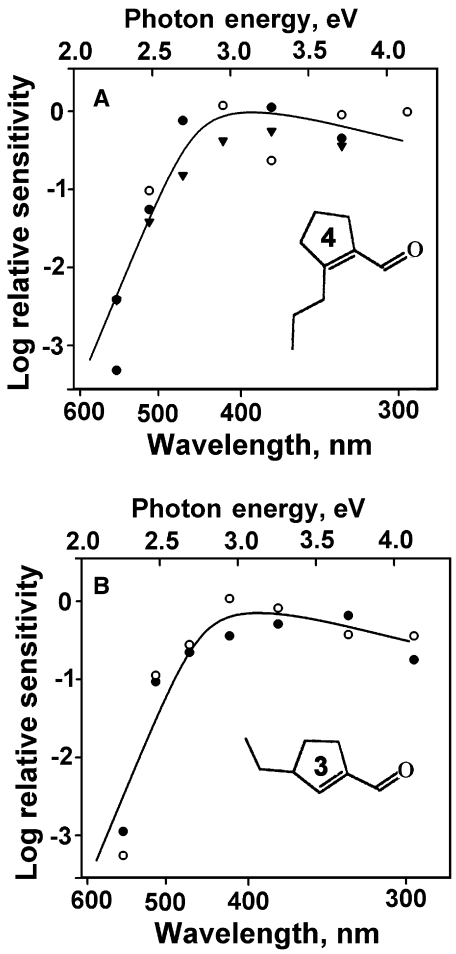

Figure 3. Threshold Action Spectra

Relative log sensitivity was plotted as a function of photon energy. Sensitivity is the reciprocal of the phototactic threshold determined by linear extrapolation to zero of the response versus the log light intensity (see Experimental Procedures; Foster et al. [1989]). The spectra of conformationally locked n-hexenals are shown in (A) for *cis*-locked n-hexenal (**4**) (dissolved in methanol ●, ○; or ethylacetate ▼) and in (B) for *trans*-locked hexenal (**3**) (dissolved in methanol ●, or ethylacetate ○). The solid line is a common fit to both the *trans*- and *cis*-locked spectra. The short conjugated double-bond system results in a broadened action spectrum, as seen previously for similar compounds (Foster et al., 1991). (An analog like **3**, but with a pentyl group instead of the ethyl group, showed the s.s. of 3200.)

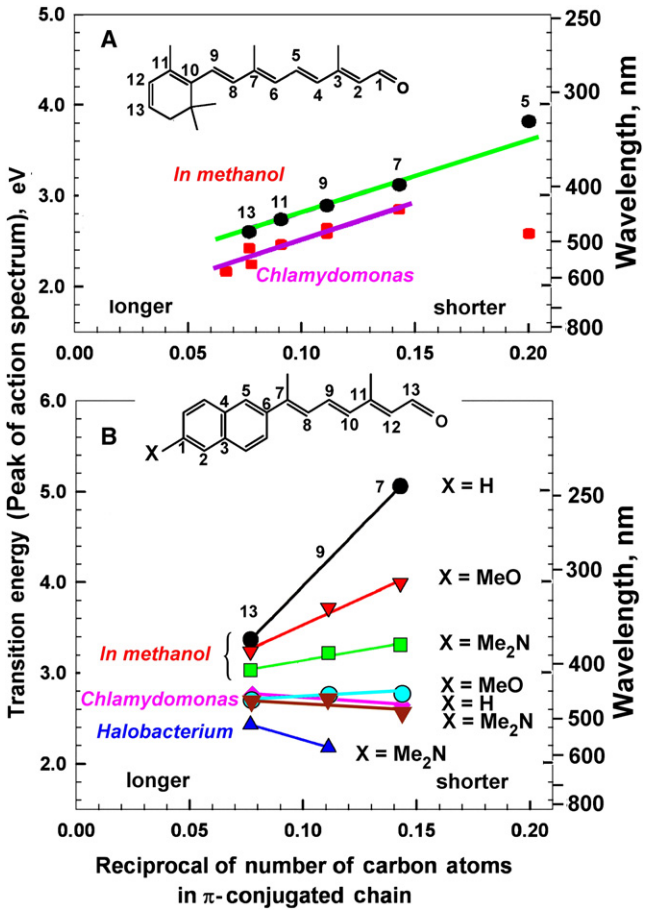

Figure 4. Analysis of Action Spectral Peaks of Two Series of Analogs Incorporated into *Chlamydomonas*

Action or absorption spectral peak (transition energy) was obtained for each analog in the series with different conjugated chain lengths ending at the number labeled in the structure. The peak of the spectrum (transition energy) was plotted as a function of the reciprocal of the number of carbon atoms in the conjugated chain.

(A) Retinal analogs with or without β-ionone in methanol or *Chlamydomonas* rhodopsin (modified from Foster et al., 1989).

(B) Naphthaldehyde analogs incorporated into *Chlamydomonas* or *Halobacterium salinarium* opsin or in methanol. For the retinal and naphthaldehyde series in methanol, the shorter the analogs (to the right in the figure), the more blue shifted (higher energy) was the absorption of the solutions. However, the shortest 2-naphthaldehyde analogs (**8**, **11**, and **15**) formed chromophores with *Chlamydomonas* opsin with either a slight spectral red shift (lower energy) or no significant shift in comparison to longer chromophores in contrast with the large blue shift observed for the action spectra of the equivalent retinal analogs in *Chlamydomonas* (A) (Foster et al., 1989). Measured by absorption spectroscopy, bacteriorhodopsin with more polarizable amino acids in very close proximity with the chromophore shows larger red shifts (lower transition energies) than *Chlamydomonas* rhodopsin (B).

Comparison of *Chlamydomonas* and *Halobacterium salinarium* rhodopsins is instructive because bacteriorhodopsin has more polarizable groups in very close proximity with the chromophore, so there are larger coupled-oscillator interactions with the protein, lowering the transition energy even further relative to that in *Chlamydomonas* as observed (Figure 4B). In *Chlamydomonas* the neighboring polarizability is further reduced by substitution of

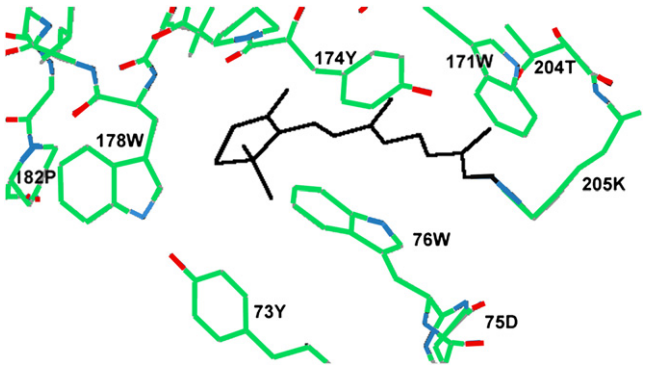

Figure 5. Retinal Binding Site of a Rhodopsin

The retinal binding site in sensory rhodopsin II is based on the WebMol view of the 1H2S structure (BACS2_NATPH).

Phe for Tyr and Trp at homologous locations in the protein. There is an even larger shift to lower energy (red shift) in the absorption spectra of bacteriorhodopsin with the shortest conjugated naphthalene analogs (Figure 4B). The results imply that the maximum interaction with the protein occurs near the attachment site of the chromophore where it binds the lysine residue of opsin. Taking these structures and spectra into account, the results suggest that the polarizability of the amino acids surrounding the chromophore, and properly oriented hydrogen bonds (due to proximity of Glu-162 and Glu-292 and probable water molecules in the retinal binding site of *Chlamydomonas*) (Okada et al., 2004), exists in the opsin to pick up and respond to induced charge motion that accompanies the light absorption of the chromophore. A transient electric field of about 10^7 V/cm has been suggested due to the charge motion (Kikuchi and Suzuki, 1997). Crystal structures of rhodopsins clearly show this arrangement of polarizable amino acids and hydrogen bonds (Figure 5) (Okada et al., 2002; Palczewski et al., 2000). The spectral results with these naphthaldehyde analogs demonstrate strong electronic coupling of the chromophore to opsin.

Completely Nonisomerizable Chromophores

Finally, to make chromophores completely nonisomerizable at all bonds including the syn-anti imine bond to the opsin, we made acid fluoride and chloride (**12** and **16**) analogs of the shortest naphthalenes. As we demonstrated previously with analog **5** (Foster et al., 1989), they formed an amide bond instead of an imine bond at the lysine-binding site. This modification prevents *syn-anti* isomerization about this bond and leaves the lysine N unprotonated rather than protonated, as in the case of the imine bond.

The question of whether light might isomerize the amide bond of acyl halides, **5**, **12**, and **16** has been definitively studied by Wang et al. (1991). They observed, by UV-resonance Raman spectroscopy, that the amount of *cis* formed with UV photoexcitation of the more common *trans* form decreases, from Gly-Gly to Gly-Lys (minuscule) to Ala-Ala (absent), by the sterical size of the two bond-forming moieties. Hence, in the cases of **5**, **12**, and **16**, the potential for cisoid forms from the preferred *trans* forms with Lys can be ignored because both of the moieties that are bound together are at least as sterically hindered as Ala-Ala.

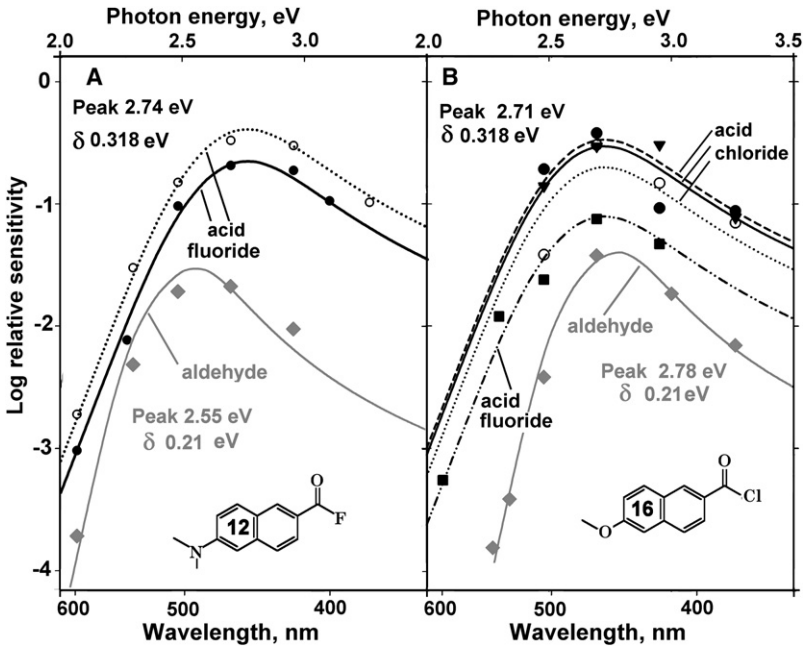

Figure 6. Action Spectra of Phototaxis Recovered by Completely Isomerization-Locked Chromophores

(A) The chromophore formed on incorporation of the acid fluoride of 6-(N,N-dimethylamino)-2-naphthaldehyde (**12**) relative to the addition of the aldehyde (**11** in Figure 1; plotted one log unit downward for clarity).

(B) The chromophore formed on addition of the acid chloride (**16**) or fluoride of 6-methoxy-2-naphthaldehyde relative to the incorporation of the aldehyde (**15** in Figure 1; plotted one log unit downward for clarity). The aldehydes were fit to the standard *Chlamydomonas* rhodopsin curve only shifted with respect to peak and sensitivity. Because the chromophores formed following addition of either the acid fluorides or chlorides will be the same, the curves were fit to a common broadened curve (half-width at half maximum, δ = 0.318 eV, and the slope of the low-energy cutoff, α = 7.5 eV^{-1}).

The action spectrum (Figures 6A and 6B) of each of these amide-linked, truncated chromophores was shifted to shorter wavelengths (higher energies) relative to the comparable analogs forming imine bonds and as anticipated broadened (half-width at half maximum increased from 0.21 to 0.318 eV) due to the carbonyl O on the chromophore. As shown in Figures 1 and 6, there was no difference between the sensitivities (see the Experimental Procedures section for the criteria of the sensitivity analysis) of the acid fluorides or acid chlorides and the corresponding aldehydes (note that the aldehydes are plotted one log unit downward in Figures 6A and 6B for clarity because they would otherwise overlap in their sensitivity).

The fact that these isomerization-blocked chromophores are just as normally active implies that: (1) activation energy from isomerization of the chromophore is not needed to trigger the protein; (2) a long-lived protein-excited state is not needed to couple the excitation of the chromophore to the protein; (3) an isomerization reaction within the chromophore is not needed to get irreversible activation; and (4) stabilization of the active protein state by an altered chromophore is not necessary to get full activation of rhodopsin. Furthermore, the activity of these amide-bound analogs shows that release of the chromophore from the binding site is not required because only imine-bond hydrolysis proceeds at a significant rate following light activation. Additionally, protonation of the lysine N is not necessary because full activity is observed in both the amide- and imine-bound chromophores. On the other hand, probable analogs in carboxylic acid (**17**) and ketone (Nakanishi et al., 1989) structures have no detectable efficacy because no covalent linkage to the protein is formed to keep the chromophore in the site.

Importance of Chemical Substituents to Interpretation of Sensitivity Measurements

Previously, we found that the sensitivity of β-ionone containing analogs varied over a 100-fold range depending on the precise geometry of the chromophore (Foster et al., 1989). This study with a new series of analogs provides further examples, but without the β-ionone ring. It is imperative to compare activity of analogs only within a common substituent and geometry. Chromophores with three or four conjugated double bonds with β-ionone rings show 0.68 of the p.s. of the normal six double-bond chromophores (Foster et al., 1991). Chromophores with dimethylamino groups show average relative sensitivity shift (s.s.) of 0.2 and p.s. of 0.48 (comparison of **11** to **8** and **9** to **6**); and with methoxy groups average relative s.s. of 0.60 and p.s. of 0.085 (comparison of **15** to **8** and **13** to **6**). Once these detailed geometric and substituent effects are taken into account, then we can see that analogs **12** and **16** show p.s. in the same range as **1**, the native chromophore.

DISCUSSION

Phototaxis is a dynamic response with specific kinetics peaking at 2–6 Hz and has a particular sensitivity depending on the concentration and efficacy of the added chromophore as well as the ability of the chromophore to reach the binding site. Some chromophores that are bulky or do not partition readily into a membrane (too hydrophilic) are not easily incorporated. For example, in prior work (Foster et al., 1991), we had to make the shortest conjugated chain chromophores hydrophobic enough to incorporate by attaching a saturated chain at the free ends. All of our results are strongly positive, as evidenced by the phototaxis sensitivities measured for construction of all action spectra always showing greater than 100-fold recovery due to the incorporation of the analogs. The shape and peak of each action spectrum are consistent with the theoretical expectation for the spectrum to be obtained as a result of each analog in the binding pocket of a rhodopsin. The spectra are not that of the free analogs in solution or of a native rhodopsin. The efficiency and dynamics of the activation of the locked analogs are the same as the comparable unlocked analogs. All these results indicate that the locked analogs act by forming rhodopsins.

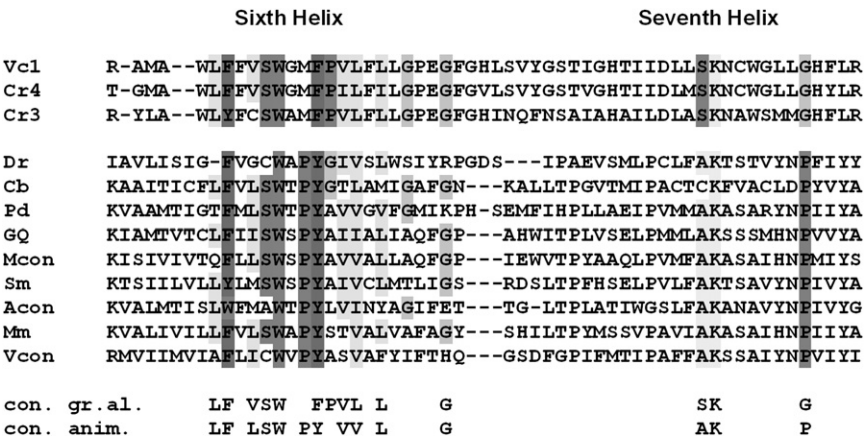

Figure 7. Sequence Similarity of Amino Acids Near the Retinal Binding Site of Rhodopsins

The amino acid sequences of helices VI and VII of selected algae (Vc1, *Volvox carteri*; Cr4, *Chlamydomonas reinhardtii* cop4; Cr3, *Chlamydomonas reinhardtii* cop3) and animals (Dr, *Danio rerio;* Cb, *Cataglyphis bombycinus* UV sensor; Pd, *Platynereis dumerilii* rhabdomeric opsin; GQ, GQ-coupled opsin; Mcon, Mollusc consensus opsin; Sm, *Schistosoma mansoni* rhodopsin; Acon, Arthropod consensus opsin; Mm, *Mus* melano opsin; Vcon, vertebrate consensus opsin) are aligned and colored (Shapely Colors) to show their similarity (ClustalW) (http://www.GPCR.org). Although the overall (second through seventh helix) amino acid sequence of *Chlamydomonas* rhodopsin studied here is closest to the archaea sensory rhodopsins ($\sim 10^{-6}$ probability of this degree of similarity by chance), helices fifth through seventh show significant continuous similarity to the same helices in a variety of animal rhodopsins (K.W.F. unpublished data) and conserve the most important functional groups (con. gr. al., consensus green algae; con. anim., consensus animal).

Although *Chlamydomonas* has at least two rhodopsins serving as phototaxis photoreceptors, in the presence of rho_{497} (rhodopsin A or Channel rhodopsin 1) (Sineshchekov et al., 2002), the other rhodopsins, which are active at different wavelengths, do not play a significant role as indicated by their lack of influence on the spectrum as a result of their low concentrations (J.S., unpublished data). In a threshold assay as used here, the action spectra reflect only the major pigment.

Rhodopsins can be sorted by their spectral width into three classes (Saranak and Foster, 2005): class 1, those with narrow spectral width (0.16 eV, width at half maximum) like bacteriorhodopsins; class 2, those with intermediate spectral width (0.21 eV), as in eukaryotes such as *Peranema* (an euglenoid) and *Chlamydomonas* (a green alga); and class 3, those with the widest spectral width (0.31eV), as found only in motile fungi (chytrids) like *Allomyces* and animals. Given the amino acid sequence similarity of the first, second, and fifth through seventh helices (Figure 7) of the rhodopsins from animals and chytrids (class 3) and the eukaryote rhodopsins of class 2 and others of class 1 as noted for many years (Rothschild et al., 1989) and the proposed common mechanisms of rhodopsin activation, it seems probable to us that rhodopsins have a common origin (K.W.F., unpublished data). Whether by independent convergent evolution (Larusso et al., 2008) or common evolutionary origin, the similarities of amino acid sequence imply that these particular amino acids and their relative location play an essential role for rhodopsin function. Analysis of the protein residues of the conserved amino acid sequences among the different rhodopsins from fungi, *Chlamydomonas*, and various animals (class 2 and 3 rhodopsins) (Figure 7) combined with our results with the naphthalene chromophores, suggests that the initial rhodopsin photoactivation step studied here in the absence of isomerization is likely to be electronic and not geometric or mechanical.

Study of the Charge Redistribution Steps of Photoactivation

Previously, we have observed that leaving the β-ionone ring in its pocket (by eliminating isomerization) variably decreases but does not eliminate response to light. Together with the fact that the phototaxis sensitivity did not vary with truncated chromophores lacking the β-ionone moiety suggests that the role of isomerization is to move the chromophore out of the β-ionone site. By eliminating the β-ionone moiety that is not required for activity (compare the space-filled models of chromophores **1**, **3**, and **12** in Figure 2), we have been able to study the initial and crucial step, charge redistribution, that occurs independently of any later chromophore-shape change as a result of isomerization.

Implication of the Effective Rhodopsin Activation by Short Chromophores

As already discussed for *Chlamydomonas* and *Allomyces* rhodopsins, short chromophores formed from hexenal function well. We have confirmed that result with additional locked analogs with only two conjugated double bonds. They also activate rhodopsin normally, suggesting that the most important protein-chromophore interaction is near the attachment site (Rothschild et al., 1989), Lys-296 of rho_{497} or rhodopsin A of *Chlamydomonas* (Figure 5), and that this interaction is simply sufficient for activation.

In all known examples of rhodopsins of all classes, the chromophore sits in a cage of highly polarizable amino acids and oriented hydrogen bonds. The cage is triggered by the photoactivation of the chromophore "firing" an electron "cannon ball" down its length causing an 11 Debye dipole moment change on the chromophore (equivalent to a whole charge displaced half the length of the native chromophore) in about 10 fs (Colonna et al., 2007). This well-known interaction of the chromophore with the protein (reviewed by Singh and Hota, 2007) has been detected by circular dichroism spectra (Pescitelli et al., 2008) and by analysis of the available absorption spectra (Houjou et al., 1998). It can switch the protein to a transiently stable state by swinging several single-group rotations (Kosower, 1991) like falling dominos. We think this is a reasonable interpretation of the observed motions of the chromophore and adjacent Trp in the activated transition going to metarhodopsin II (meta II) as seen in bovine rhodopsin (Ahuja et al., 2009). As a result of the

number of residues in the conformation change to this state, rhodopsin cannot directly or immediately revert to the unactivated state. Hence, principal consequences are thought to be: (1) single-group rotation of the universally conserved (or convergent) Trp of the sixth helix in the transition to meta I (Schertler, 2005) coming to interact with conserved Phe and/or Tyr of fifth helix in the neighborhood of the Pro or Gly; (2) the change of the sixth helix Pro kink (Shi et al., 2002); (3) the bending, rotation, and sliding of the sixth helix relative to the third helix (opening apart on the cytoplasmic side); (4) the motion of a proton near the chromophore pocket; and (5) numerous changes in hydrogen bonding (Nygaard et al., 2010; Patel, 2005). Note that both the Trp and Pro are conserved in many GPCRs as well as rhodopsins and also specifically interact with ligands (Cherezov et al., 2007). In *Chlamydomonas* and other class 2 rhodopsins, there is a negative charge next to the chromophore that becomes protonated in the active state (Foster et al., 1991). In bovine rhodopsin (class 3), it is believed that a similar group is deprotonated in a charge switch (Peters et al., 1977; Yan, 2003). Conserved amino acids between *Chlamydomonas* and bovine rhodopsins (Figure 7) in the sixth helix include a Phe or Tyr followed by a Ser or Cys (three away or about one helical rotation), the aforementioned Trp (four away or one helical rotation and directed toward the chromophore), and again Phe or Tyr (seven away or two turns from the beginning of this conserved sequence). In addition to these amino acids, in class 3 there are highly conserved Pro in both the fifth, sixth, and seventh helices and multiple Phe and Tyr in the fifth helix in proximity to the Pro. The multiple Phe and Tyr are also present in class 2 rhodopsins, whereas the Pro are alternatively Gly, which are presumably serving the same function as the Pro.

In Other Systems

One example of how polarizable groups are arranged in a rhodopsin is shown for sensory rhodopsin II (class 1) (Figure 5), where Trp-171, Tyr-174, and Trp-178 of the sixth helix are all near the chromophore. Note that similar amino acids are differently arranged around the chromophore in the different rhodopsins, but there are always polarizable amino acids near the chromophore. This arrangement suggests that irreversible capture of the energy of the excitation photon could be single-group rotations of adjacent amino acids near the chromophore put in motion by the light-induced electron density movement down the chromophore. If enough motion occurs it would be hard to revert back because there is no mechanism for immediate reversal. Similarly, the pattern of hydrogen bonding would be significantly altered, and this would not be immediately reversed either. All this rearrangement leads to the sixth helix bending out a little (meta II in bovine rhodopsin) at this conserved site because the relatively loosely bound interaction of helices five and six is free to move, rotate, and slide (Liu et al., 2004). As suggested by Nakamichi and Okada (2006), these motions likely break most of the electrostatic restraints on relative motion of the helices. Eventually, the rhodopsin does revert to the *unactivated* state. After 11-*cis* retinal rebinds with vertebrate opsins, rhodopsin refolds in a process that may take 15–60 s under the most ideal conditions in the presence of excess 11-*cis* retinal (Sakamoto and Khorana, 1995).

A similar activation scenario may be envisaged for some class A GPCRs that have amino acid sequences similar to class 3 rhodopsins (Cherezov et al., 2007; Shi et al., 2002). The binding of a diffusible ligand causes charge motion and, consequently, single-group rotations in the neighborhood of the bound ligand. Particularly noteworthy is that the analogous Trp in β_2-adrenergic receptor is similarly restrained, like the Trp discussed above in *Chlamydomonas* and animal rhodopsins (classes 2 and 3), suggesting that "the signal propagation mechanisms are largely conserved in members of the GPCR family" (Ranganathan, 2007), including the conformational changes associated with activation (Rosenbaum et al., 2007). We propose an electronic mechanism that initiates the "global toggle switch" of Trp motion in these receptors (Cherezov et al., 2007; Schwartz et al., 2006) and the vertical motion of fifth through seventh helices. In both cases, either the chromophore's excitation or the sliding of a ligand into its binding site initiates a relatively large number of motions, which cannot easily or quickly be reversed. This Trp motion is further supported by the recent NMR studies of the meta II conformation (Ahuja et al., 2009).

In addition to the rearrangement, the interior volume of rhodopsin is increased about 60 $Å^3$ for bovine rhodopsin (Strassburger et al., 1997). These motions of a number of components and the temporary irreversibility and increased volume of a normally well-packed protein, particularly on the cytoplasmic side, suggest that an increase in entropy is playing a role in the success of the signal capture. A transient photon or ligand-binding event is extended by this entropic signal capture to a certain length of time to interact with the next stage in the signaling cascade. This process is an extraordinary amplification because it promotes the probability of successful coupling of activation to the next stage.

This new view of rhodopsin activation is supported by work in other systems. Our early efforts in vivo with *Chlamydomonas* (Foster et al., 1989, 1991) showing that isomerization of any specific bond along the chromophore was not essential to activate rhodopsin, was demonstrated to be broadly true for animal rhodopsins as well (Fan et al., 2002; Kuksa et al., 2002). Groma et al. (2004) showed that polarization or charge separation is the cause of the isomerization of a rhodopsin in archaea. Furthermore, the proposed charge separation state was observed by Bryl (2003), who found a fast initial absorbance in structurally and, hence, electrically, asymmetric chromophores attributed to a charge separation state (30 fs) resulting in charge delocalization prior to the isomerization state (500–700 fs) (Amsden et al., 2007). In response to the charge separation, at about 100 fs, there was a large dielectric response of the protein matrix involving a large number of small amplitude motions of charges and dipoles presumably due to the unusually large charge transfer (Song and El-Sayed, 1998), just as we propose. This order of events is also supported by the observation that there is initially a light-induced bond stretch rather than a bond-angle twist of bacteriorhodopsin's excited state chromophore (class 1) (Song and El-Sayed, 1998). As anticipated, this charge separation disrupts the hydrogen-bonding network (Kennis et al., 2002).

The vectorial nature of our proposed charge separation is supported by the fact that although electrically symmetric chromophores absorb light, they do not produce responses, at least in bacteriorhodopsin (Zadok et al., 2002). This mere dumping of

heat into the site when a photon is absorbed must be insufficient to activate. An electrically asymmetric chromophore seems necessary, suggesting a directed charge separation in the activation process. Finally, the proposed protein changes are seen spectroscopically in the case of bacteriorhodopsin even with nonisomerizable analogs (Aharoni et al., 2001; Losi et al., 2000). The anticipated protein motion could, with improvements in technology, be observed with near-IR spectroscopy (Amsden et al., 2007) and cryogenic X-ray diffraction of appropriate crystals (Nakamichi and Okada, 2006) with isomerization-locked analogs, as we have used in comparison with native chromophores. Given our results, we would anticipate that the protein changes conformation without the need of isomerization.

A question is to what degree is this proposed mechanism generalized to all three classes of rhodopsins. The in vivo experiments reported here are with respect to a class 2 *Chlamydomonas* rhodopsin. Some relevant in vivo experiments were previously reported for a class 3 rhabdomeric-type rhodopsin of *Allomyces*, in which a short chromophore without a β-ionone moiety formed from n-hexenal was sufficient to restore phototaxis (Saranak and Foster, 1997). Because all known class 1 (Gordeliy et al., 2002) and class 3 rhodopsins (Kikuchi and Suzuki, 1997; Murakami and Kouyama, 2008) have similar structures around the retinal binding pocket and similar amino acid sequences from the fifth through seventh helices including the critical polarizable amino acids and either flexible Gly or helix-breaking Pro residues, it seems likely that a similar mechanism holds for all rhodopsins. Of course there are differences, particularly with respect to the details of chromophore-protein interaction at longer time scales following initial photoactivation. As an example of a difference, with respect to the proton pumping of bacteriorhodopsin, the initial charge redistribution is followed by isomerization required to complete the pumping function. Similarly, activation of class 2 and 3 rhodopsins, which have a full-length chromophore, is facilitated, not initiated, by isomerization.

Summary of Proposed Initial Activation Mechanism

We have presented two sets of experimental data. One set shows that no geometric change of the chromophore is necessary during activation of *Chlamydomonas* rhodopsin (Figure 6), and the other set with naphthalene analogs shows a clear and strong interaction of the chromophore excitation with the neighboring polarizable residues of the opsin protein (Figures 4 and 5). As already noted, chromophores interact with these polarizable amino acids as seen by analysis of the resulting absorption spectra (Houjou et al., 1998), the tuning of the absorption wavelength by this environment in bacteriorhodopsin (class 1) (Singh and Hota, 2007), the action spectra reported here for *Chlamydomonas* (class 2), and by circular dichroism (Pescitelli et al., 2008) in mammalian rhodopsins (class 3). Based on these observations and the fact that the chromophore environments of all rhodopsins are similar (Figure 7), we suggest that all rhodopsin receptors, including those of animals, activate via these known unique interactions between the photoexcited chromophore and the protein.

In brief, charge separation in the photoexcited chromophore "kicks" the protein by coupling with the polarizability of the binding site. Although we show here that this initial activation is both necessary and sufficient for *Chlamydomonas* rhodopsin physiological function, it may not be sufficient for all rhodopsins. In some cases a subsequent chromophore shape change may remove inverse agonist activity that suppresses spontaneous activation, subtly modify activity, or potentially stabilize the active conformation of the protein.

SIGNIFICANCE

The function of retinal analogs not previously incorporated and designed specifically to demonstrate effects of charge redistribution in the retinal binding site have been tested with an in vivo physiological assay for rhodopsin photoactivation (Foster et al., 1988, 1989, 1991; Nakanishi et al., 1989). The results support the hypothesis that the charge separation in the excited state couples directly to nearby polarizable amino acid residues and the hydrogen-bonding network of the protein. This finding is consistent with the theoretical suggestion (Birge and Zhang, 1990; Lewis, 1978, Salem and Bruckmann, 1975) that the electron redistribution resulting from photon absorption could be responsible for the bond rotation and rearrangement of amino acid residues leading to protein activation. If allowed to occur, the subsequent chromophore shape change due to isomerization completes proton pumping in some cases (class 1, and apparently some putative class 2 rhodopsins), removes the antagonistic activity of the β-ionone moiety toward completion of rhodopsin activation in other cases, and may influence later steps in the visual photocycle. We further found that the chromophore-binding site is highly sensitive to the charge separation in the chromophore. The large shift in the action spectra of the two-naphthaldehyde series in *Chlamydomonas* and the absorption spectrum for bacteriorhodopsin shows interaction of the excitation of these chromophores with the nearby polarizable amino acids. Hence, the light-induced consequences of charge separation are necessary and sufficient to initiate the phototransduction process. Given that class A GPCRs have similar activation mechanisms (Gouldson et al., 2004) and similar structures surrounding the ligand as the rhodopsin chromophore, the induced polarization of polarizable amino acids (Trp and Try) in the binding site and changes in the hydrogen-bonding network may collectively trigger their receptor activation as a result of electronic changes in the binding site.

EXPERIMENTAL PROCEDURES

Rhodopsin Action Spectra

Phototaxis threshold was obtained with the eukaryotic cell, *Chlamydomonas reinhardtii*, which uses rhodopsin to track a light source. Recovery of negative phototaxis of the carotenoid mutant, FN68, following incorporation of different chromophores into the cell's opsin was used to assay for the activity of the different chromophores. Phototaxis assays and action spectrum parameters are described in detail in the Supplemental Experimental Procedures. In brief, a 1.5 ml aliquot of cell suspension in a small plastic Petri dish was tested for phototaxis sensitivity (1/threshold) by observing the phototaxis distance of the cell population in response to three different intensities (near threshold) of each wavelength of light. Log sensitivity was plotted against the wavelength (photon energy) to obtain an action spectrum.

In addition to the action spectral peaks ± uncertainty, we present the degree of recovery of phototaxis sensitivity for each chromophore analog in two ways: the s.s., which is the fold increase in sensitivity upon addition of the analog; and the absolute p.s. in units of nm^2 s/photon. The s.s. is the relative occupancy multiplied by the relative efficacy of the exogenous versus the endogenous chromophore. Because s.s. is large in each case, the p.s. is just the occupancy of the exogenous chromophore multiplied by its efficacy. Action spectra were plotted on the same relative sensitivity scale so that these geometrically and substituent similar chromophores can be easily compared as to whether they have the same range of sensitivity, regardless of forming an imine or amide bond with the lysine N.

Compound Synthesis

Compounds **1**, **2**, **5**–**7** (Foster et al., 1989), and **9**–**14** (Johnson, 1991) were synthesized as has been reported. Synthesis of analogs **3**, **4**, and **15**–**17** is described in Supplemental Experimental Procedures. Compound **8** was from Aldrich.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.chembiol.2011.04.009.

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