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Theory of rhodopsin activation: probable charge redistribution of excited state chromophore

Koji Nakanishi, Fadila Derguini, V. Jayathirtha Rao, Gerald Zarrilli, Masami Okabe, Thoai Lien, Randy Johnson

Department of Chemistry, Columbia University, New York, NY 10027

Kenneth W. Foster and Jureepan Saranak

Department of Physics, Syracuse University, Syracuse, NY 13244-1130

Abstract - The unicellular eukaryote *Chlamydomonas reinhardtii* is a phototactic alga which swims toward or away from light by using rhodopsin as its photoreceptor. The blind mutant FN68 lacks retinal and therefore does not undergo phototaxis, but this property is readily restored upon incubation of the microorganism in media containing retinal analogs. Incorporation of chromophores having fixed double bond geometry indicates that specific isomerizations of the 7-ene, 9-ene, 11-ene, 13-ene and 15-ene ($C{=}N^{+}H$) are unnecessary. Phototaxis is also restored by numerous retinal analogs including short acyclic aldehydes. Phototaxis is even restored by hexenal and hexanal. An attractive hypothesis is that charge redistribution in the excited state of the chromophore triggers the activation of rhodopsin.

In the first step of vision a photon excites the retinal chromophore in the regulatory site of rhodopsin; this change triggers isomerization of retinal and activation of rhodopsin, the first protein of the visual cascade. We have asked whether or not double bond isomerization of retinal is a prerequisite for this activation. The chromophore of the visual pigment rhodopsin is 11-cis-retinal while those of bacteriorhodopsin are all-trans and 13-cis retinal; in *Chlamydomonas* (ref. 1) the geometry is still unknown, but it is likely all-trans **1** or 11-cis **6** (ref. 2). The retinal/apoprotein link is a protonated Schiff base, and double bond isomerization has been considered essential for activity (ref. 3). In the following we present evidence that isomerizations of specific double bonds in the retinal moiety is unnecessary for the phototaxis of the unicellular algae *Chlamydomonas reinhardtii*.

The native action spectral maximum for positive and negative phototaxis of *Chlamydomonas* is at 503 nm (2.46 eV)(ref. 2). However, the photoreceptor of the blind mutant FN68 does not respond to light since it lacks retinal due to blocking of carotenoid biosynthesis; upon incubation with retinal analogs phototaxis is restored in minutes, the action spectra maxima closely paralleling the *in vitro* absorption spectra of corresponding bovine rhodopsin analogs (ref. 2). This led to the conclusion that *Chlamydomonas* and bovine photoreceptors are similar (ref. 2), a finding corroborated by gene hybridization studies (ref. 4). The *in vivo* assay with *Chlamydomonas* has several distinct virtues. We have found that, over a 10,000 fold range, the threshold sensitivity for phototaxis increases linearly with the amount of retinal incorporated. In bovine rhodopsin, the measurable range of sensitivity is only about 20 for the assay based on cGMP hydrolysis (ref. 5). In contrast to an *in vitro* assay, the *in vivo* assay eliminates the question of whether the conditions are physiological or not and whether the binding is specific or nonspecific. The assay requires no biochemical preparation, and moreover, the economy in time and material has made it possible to test over 80 retinal analogs that we have prepared (ref. 6).

Figure 1 depicts analogs incorporated in current studies (ref. 7), together with action spectral maxima and sensitivity (1/threshold). The similarity in phototaxis action spectral maxima and corresponding rhodopsin absorption spectra (ref. 2) suggests that, as in other retinal proteins, retinal is linked to rhodopsin via a protonated imine. The receptor of *Chlamydomonas* functions with a wide variety of analogs including acyclic analogs of varying lengths **16-20**.

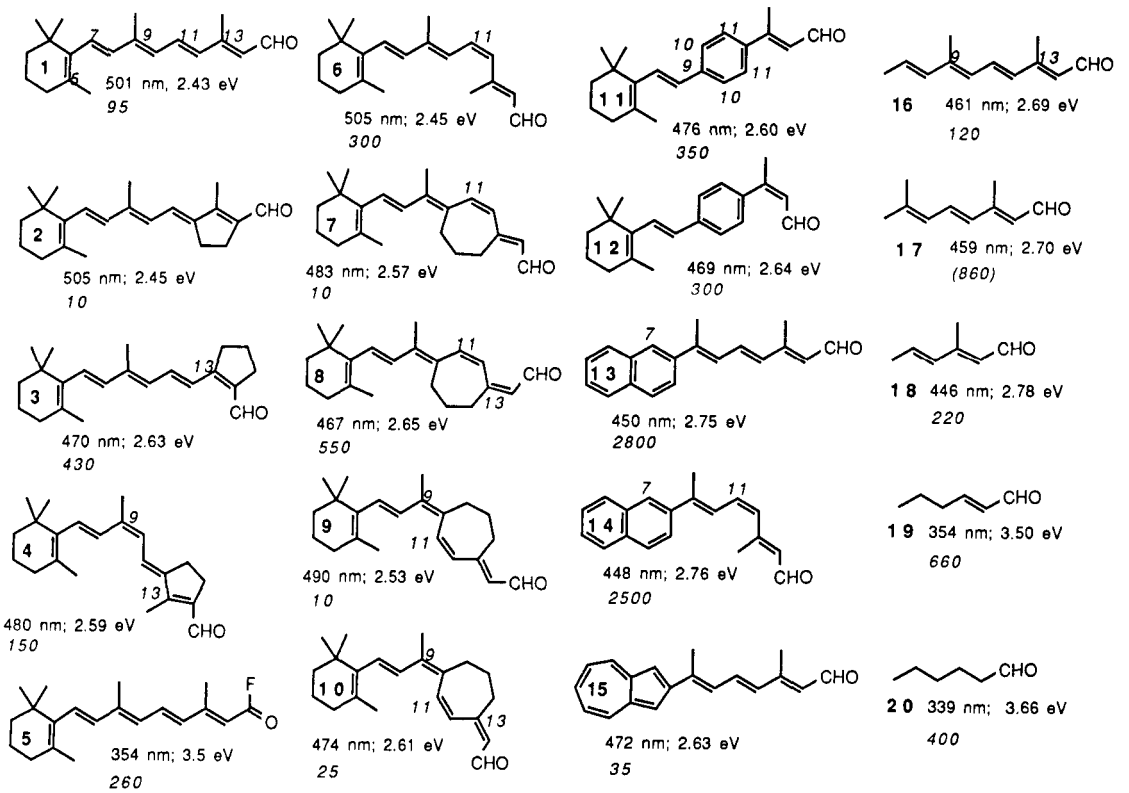

Fig. 1. Retinal analogs and phototaxis action spectrum peaks in nanometers and electron volts (wavelength in nm = 1239.85/the energy in eV). Each analog, 25 μM, was incorporated into *Chlamydomonas* FN68 overnight (similar results were obtained for 10 min - overnight incorporations); results for **5** are for 30 min. The sensitivity, shown in *italics*, is defined as the reciprocal of threshold at the peak of each analog, and is in units of m^2s/E photon ($E=10^{18}$).

Bacteriorhodopsin incorporated with five-membered ring analogs **2** and **3** where isomerization of the 13-ene is blocked do not lead to detectable proton translocation (ref. 8). Similarly, the four isomers **7-10** have seven-membered rings in the side-chain that prevent the 11-cis double bond from isomerizing, although small transient rotations may occur. Although all four were incorporated into bovine and other opsins, the pigments did not bleach *in vitro* (refs. 9, 10); furthermore, **7** led to no detectable change in the electroretinogram b-wave sensitivity upon intraperitoneal injection into rat and frog deprived of vitamin A (ref. 11). Although it is well-known that double bond isomerizations accompany proton pumping and visual transduction in the native system (ref. 3), the described results do not exclude the possibility that mechanisms other than isomerization may also be responsible for activating rhodopsin. Furthermore, pigment bleaching may not be required for visual transduction.

In contrast, the phototaxis of *Chlamydomonas* incubated with analogs **2-4** (fixed 13-ene) is restored. Therefore, isomerization of 13-ene is not a necessary factor in phototaxis. Analog **8** is fully active and exhibits the same irradiance-response curve as all-trans retinal, while **7**, **9**, and **10** are less active. Analogs **7** and **8** apparently correspond to 11-cis isomers whereas **9** and **10** correspond to 9-cis isomers. However, the decisive factor that determines their incorporation must be the shape of the nonplanar and bulky 7-membered ring as dictated by location of double bonds, rather than the apparent correspondence of double bond geometry with retinals. Results with **7-10** indicate that phototaxis can occur without isomerization of the cis-11-ene.

The fact that **11** and **12**, both with a phenyl ring (ref. 12), are fully active further shows that isomerization around C-9 to C-12 is not required. The 7-ene isomerization is also ruled out because of the very efficient response resulting from incorporation of the two naphthalene analogs **13** (ref. 13) and **14** (ref. 14; also ref. 15). The azulene analog **15** also gave a positive result (ref. 16). To summarize, specific isomerizations of the 7-ene, 9-ene, 11-ene and 13-ene are not essential for recovery of phototaxis. The only other specific double bond that could be involved is the terminal C=N (syn/anti) although this does not seem to occur during visual transduction (refs. 17, 18). This syn/anti

isomerization can also be ruled out together with pigment bleaching because acid fluoride **5** (ref. 19) (leading to an amide bond formation in the protein) restores phototaxis. Furthermore, the resulting action spectrum resembles the absorption spectrum of bovine rhodopsin derived from **5** (ref. 19); however, this rhodopsin analog was reported to be inactive (ref. 20).

The acyclic aldehydes also appear to be linked to the photoreceptor via a protonated Schiff base linkage $C=N^+H$ (to a lysine amino group) as in other retinal proteins, since (i) the maxima undergo progressive blue shifts with shorter conjugation, and (ii) the trend of phototaxis maxima restored by the 11-cis-dihydro series is similar to that of absorption maxima of dihydrobovine rhodopsins (ref. 6). Hexenal **19** and hexanal **20** incorporate readily and are as sensitive to low levels as the other retinals! The normal activity of n-hexenal and n-hexanal was unexpected since these structures lack resemblance to retinals. The action spectrum maximum for hexanal is at 339 nm, a shorter wavelength than that caused by hexenal **19**; this together with its bathochromic shift compared to the "transparent" hexanal clearly shows that hexanal is also bound through $C=N^+H$.

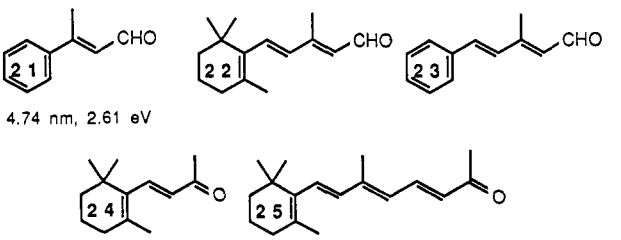

Fig. 2. Aromatic aldehyde and ketone retinal analogs.

The short aromatic aldehyde **21**, a moiety present in **11**, also restores phototaxis. However, compounds **22-25** are inactive (**Fig.2**). The negative response to ketones **24/25** is not unexpected because they may not bind to the retinal binding site lysine. However, since aldehydes **2/8/11** with rings near the terminal aldehyde readily incorporate and yield normal action spectra, it is surprising that aldehydes **22, 23** with similar structural features did not bind, i.e., they neither restored phototaxis nor affected the incorportation of trans-retinal. An explanation for their lack of incorporation could be constriction in the binding site about 5 or 6 carbons removed from the $C=N^+H$ link. The majority of retinals giving positive results must fit through the constriction. In short acyclic enals, the chromophores are properly positioned for energy transduction although only bound through $C=N^+H$. Presumably aldehydes **2/8/11** span the constriction and **20** fits in the pocket adjacent to the $C=N^+H$ bond, but in **22/23**, formation of $C=N^+H$ is blocked due to steric interference between the rings and the constricted region.

Because these experiments have been performed in living cells, it is possible that other colored pigments might lead to phototaxis. The cells were therefore incubated with 25 mM of methylene blue, a photosensitizer which in *Fusarium* (ref. 21) and corn (ref. 22) can substitute as a photoreceptor; the results with *Chlamydomonas* were negative. Retinonitrile (CN instead of CHO in **1**) (ref. 23) which also cannot bind to a lysine amino group was found to reduce the rate of incorporation of retinal; it was however inactive, a result showing that the mere presence of a chromophore in the binding site is insufficient for recovery of phototaxis. Clearly, all analogs play a functional role as the light-absorbing chromophore in rhodopsin, because the resulting action spectra were shifted from their corresponding absorption maxima as well as from that of the native pigment. Formation of a chromophore/protein covalent bond is also borne out from the data with dihydroretinals and acyclic retinal analogs, where shorter conjugation leads to successive higher energy or blue shifts in action spectra.

While our measure of sensitivity is approximate because it depends on a combination of factors such as degree of incorporation, extinction coefficient of pigment and quantum efficiency, it is nevertheless useful. Three out of four of the analogs with 7-membered rings (**7, 9, 10**) have between 2-5% of normal sensitivity (although **8** exhibits full sensitivity). Analogs **11** and **12** are in the normal range while those with 5-membered rings (**2, 3, 4**) range from 2% to normal. Analogs **13** and **14** with a planar ring (also ref. 15) were extraordinarily sensitive implying that planarity or higher hydrophobicity may facilitate entrance into the hydrophobic site. If the values for bovine rhodopsin were less than 5% of normal sensitivity in the *in vitro* reconstitution experiments, pigment formation would be difficult to detect. Because of the high sensitivity of the *Chlamydomonas* assay, it is possible in instances of low activity that the phototaxis gives positive results, whereas other assays give negative results.

In summary, the results described above lead to the following conclusions. (a) Blocking of individual double bond isomerizations still result in phototaxis. Therefore, if isomerization is indeed required, it must be non-regiospecific.

(b) The positive activity of acid fluoride **5**, in particular, shows that neither detachment of chromophore from the binding site (bleaching) nor protonation of a C=N bond is required for restoration of phototaxis.

(c) Changes in molecular geometry of the chromophore may be unnecessary for protein activation. Raman intensities of the hydrogen out-of-plane wags and lack of change in the CN bond in bovine bathorhodopsin suggest that the 10-11, 12-13 and 14-15 bonds are all rotated by 20° following excitation; this model suggests that excitation involves no major change in the geometry of the molecule (ref. 18).

(d) Incorporation of the 7-membered analog **7** into bovine rhodopsin blocked bathorhodopsin formation and subsequent bleaching. However, the transiently excited species were present for 100 ps (ref. 24). This result implies that changes in the protein must be triggered within the 100 ps period before the chromophore returns to its ground state. In the case of bacteriorhodopsin, proton motion is initiated in less than 30 ps (ref. 25). The nanosecond or longer period (ref. 26) anticipated to trigger the protein must not be required.

(e) Phototaxis is restored by the short aldehydes hexenal and even hexanal, which has no C=C bond.

An attractive hypothesis is that activation of rhodopsin in *Chlamydomonas* is triggered by charge redistribution in the excited state of the chromophore (ref. 27). Stabilization by subsequent retinal structural alteration (ref. 28) would not seem to be required. A mechanism similar to that occurring in Chlamydomonas may be involved in triggering the activity of other rhodopsins. These aspects are currently under investigation.

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