Activation of *Chlamydomonas* Rhodopsin in Vivo Does Not Require Isomerization of Retinal†

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ABSTRACT: The unicellular eukaryote *Chlamydomonas reinhardtii* is a phototactic alga that swims toward or away from light, using rhodopsin as the photopigment. The activity of retinal analogues was tested in the mutant FN68, which has high phototactic sensitivity only after incubation with retinal or analogues of retinal. Analogues prevented from isomerizing about the 7-ene, 9-ene, 11-ene, 13-ene, or 15-ene ($C{=}N^{+}H$) bonds retained full activity. Also, bleaching, protonation of the N, and a stable geometrically altered chromophore are not required for full activity. An attractive hypothesis is that charge redistribution in the excited state of retinal directly triggers the activity of rhodopsin.

In the first step of vision, a photon is absorbed by retinal, which lies in the regulatory site of rhodopsin. Upon photoexcitation, charge is redistributed as the electron density shifts toward the imine (C=N) end of retinal. The charge redistribution triggers double-bond isomerization of retinal, as well as bleaching (release of retinal from its binding site), changes in the apparent pK_a of the imine nitrogen, proton motion in opsin, and potentially direct excitation of rhodopsin. Those phenomena known to occur lead by post hoc reasoning to reasonable hypotheses that one or another of these events activated rhodopsin to initiate the visual cascade. Geometrical cis–trans isomerization, discovered by Hubbard and Wald (1952), has been the most popular hypothesis since it was put forward by Hubbard and Kropf (1958), and much evidence has been amassed that isomerization normally occurs (Hubbard & Kropf, 1958; Akita et al., 1980), including spectroscopic evidence of resonance Raman (Eyring & Mathies, 1979; Aton et al., 1980) and differential FTIR (Rothschild et al., 1983; Bagley et al., 1985).

To the best of our knowledge, however, no experiment has been done on any rhodopsin that provides clear evidence that cis–trans isomerization activates rhodopsin. Unfortunately, absorption spectroscopic techniques monitor retinal in the regulatory site in the middle of rhodopsin and cannot determine the state of activation of the enzymatic site on the cytoplasmic surface of rhodopsin. For this purpose one must have a measure of the actual activation, such as the in vivo test system we have used in this study.

This system provided by the FN68 retinal-synthesis mutant of the unicellular alga *Chlamydomonas* (Foster et al., 1984; Foster & Smyth, 1980) allow us to apply the advantages of a microbial assay to the study of rhodopsin. The photoreceptor pigment is in the plasma membrane of the *Chlamydomonas* cell. The receptor pigment of mutant FN68, which is blocked in retinal synthesis, can be activated within 10 min by incubation with small quantities of retinal analogues. The assay requires no biochemical preparation and only a small amount of material. In fact, over 80 retinal analogues (Balogh-Nair & Nakanishi, 1982; Derguini & Nakanishi, 1986) have been tested with our assay. An in vivo system has the additional advantage that it guarantees physiological conditions.

To begin the process of distinguishing the role of different mechanisms in rhodopsin activation, we tested whether isomerization of retinal is required to activate the sensory function of rhodopsin. We did this by measuring the activity of retinal isomers that are blocked from isomerizing. With the same analogues we were also able to test whether the geometric rigidity of an analogue or the brevity of its photocycle of less than 100 ps (Buchert et al., 1983) would affect response. Several experiments have been carried out in other systems in which retinal isomerization has been blocked, but with inconclusive results with respect to the question of rhodopsin activation. The four retinal isomers **7**–**10** (Figure 1) have been incorporated into bovine and other opsins. These analogues have seven-membered rings in the side chain that prevent the 11-cis double bond from isomerizing, although small transient rotations may occur (Akita et al., 1980). These pigments do not bleach in vitro (Akita et al., 1980; Mao et al., 1981), but bleaching does not necessarily measure activation. The electroretinogram does measure activation, but the 11-cis isomer **7** did not produce detectable activity upon intraperitoneal injection into rats and frogs (Crouch et al., 1984). However, this might have been due to lack of sensitivity in the assay. As we show below, **7** had only 2% of the sensitivity of 11-*cis*-retinal in *Chlamydomonas*. Its relative insensitivity could be due to a cause other than lack of isomerization, such as improper orientation of the chromophore with the protein. The above results did not provide a clear-cut answer as to whether isomerization plays a role in activating the visual function of rhodopsin.

Materials and Methods

Culture Conditions and Cell Preparation. The mutant (FN68) of *Chlamydomonas reinhardtii*, culture conditions, and cell preparation are the same as described previously (Foster et al., 1988). For action spectrum determination most retinal analogues were added 4–5 h after transfer of the cells to a nitrogen-free minimal medium that does not support growth (Foster et al., 1984) and incubated with the cells overnight. Exceptions were analogues **14** and **15**, which were added 1 h before the phototaxis assay, and analogue **5**, which was added 30 min before assay. After an antioxidant, *d*-α-

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tocopherol acetate (Foster et al., 1988), was added, a 25 mM solution of each analogue in HPLC-grade methanol was added to give a final concentration of 25 μM. This concentration does not generally saturate the receptor. Saturation occurs at a sensitivity (1/threshold) of about 3000.0×10^{-18} m^2 s/photon, which compares to 300.0×10^{-18} m^2 s/photon with 25 μM 11-*cis*-retinal. Nonsaturating conditions were used so as not to emphasize minority impurities or small metabolic conversions if they should exist relative to the majority analogue.

Phototaxis Assay. Rhodopsin activation was assayed by measuring the rate at which cells swam away from light (negative phototaxis). Threshold action spectra were generated from the threshold irradiance determined at each wavelength. This threshold was obtained by extrapolation of a linear plot of the rate of phototaxis versus the logarithm of irradiance to zero rate of phototaxis. The sensitivity given in Figure 1 for each analogue is the reciprocal of threshold. These values range from 10×10^{-18} to $2800. \times 10^{-18}$ m^2 s/photon. We can confidently distinguish the activity of an active analogue with a sensitivity of 0.1×10^{-18} m^2 s/photon from an inactive one. With the exception of analogue **5**, the details for the assay are precisely as described recently (Foster et al., 1988) and similar in principle to that used previously (Foster et al., 1984). For analogue **5**, 0.75 mL of cell suspension was added to a rectangular quartz cell with a glass stopper. The action spectrum of **5** (Figure 3) was fitted by eye rather than as the others to a standard absorption curve (Knowles & Dartnell, 1977). In this study complete action spectra were carried out two or three times on each analogue with different batches of cells on different days.

Analogue Synthesis. Retinal analogues **2–4** locked about the 13-ene were made according to the method of Fang et al. (1983). The four retinal isomers **7–10** prevent 11-cis isomerization (Akita et al., 1980) and the analogues **11** and **12** (Kolling et al., 1983) block isomerization between C-9 and C-12. The two naphthalene analogues **13** (Akhtar et al., 1982) and **14** block 9-ene isomerization; **14** was synthesized by condensation of *trans*-3-methyl-3-β-naphthylpropenal (Akhtar et al., 1982) with the anion of (trimethylsilyl)acetone *tert*-butylimine, elongation of C_{18} ketones with diethyl cyanomethyl phosphonate, DIBAL reduction, and HPLC. The azulene analogue **15** synthesis is described by Zarrilli (1984). The acid fluoride analogue **5** was synthesized according to the method of Wong and Rando (1984). On binding, it leads to the formation of an amide bond in the binding site rather than an imine bond. Retinonitrile (CN instead of CHO in **1**) (Liu & Asato, 1984) was tested because it cannot bind to a lysine.

Given the very high sensitivity of our assay, which is greater than that provided by current chemical tests of purity, one could expect that we would be able to detect impurities in samples thought pure. This is indeed the case, so that in addition to absolute HPLC purity (no shoulders or seemingly insignificant bumps) and checking for correctness of compound by NMR, we required that the fit of the phototaxis action spectrum to the standard Dartnell rhodopsin curve be such as to imply the presence of only a single peak of analogue. The one exception is the acid fluoride **5**, in which the absorption spectrum in bovine rhodopsin (Calhoon & Rando, 1985) and the action spectrum in *Chlamydomonas* show the same nonstandard shape, presumably due to the vibrational coupling of the oxygen.

Results

As a step in determining the intraprotein pathway of activation of rhodopsin from the regulatory site, where retinal is bound, to the enzymatic site, where G protein is activated, we have explored the effect of blocking the hypothesized geometrical isomerization step. We have done this by measuring the ability of retinal analogues that are blocked from isomerizing at specific places (Figure 1) to activate rhodopsin. The analogues are further interesting due to their geometric rigidity and the brevity of the photocycles they undergo.

Analogues **2–4**, which cannot isomerize about the 13-ene bond, restored phototaxis when incubated with the *Chlamydomonas* mutant. Therefore, rhodopsin can function as the photoreceptor for phototaxis without 13-ene isomerization.

Analogues **7–10**, locked in the cis-11-ene conformation, restore phototaxis, showing that isomerization of the cis-11-ene bond is not essential. These seven-membered ring compounds differ in activity. Analogue **8** was fully active and exhibited a similar irradiance-response curve as *all-trans*-retinal (Figure 2), while **7**, **9**, and **10** were less active. The similarity between irradiance-response curves of **1** and **8** indicates that **8** functions as an agonist and not as a partial agonist. Correspondence of double-bond geometry with native retinals does not appear to be the important consideration. Analogue **7** has the double-bond geometry of **6** (11-*cis*-retinal) but low activity, whereas **8** is 13-cis and has high activity. Analogues **9** and **10** differ from **7** in being 9-cis; both have low activity. It is conceivable that the spatial distribution of the bond system as dictated by the geometry of the seven-membered ring and the other double bonds accounts for the differences in activity.

The fact that even **11** or **12**, each with a phenyl ring, was fully active also shows that isomerization around C-9–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 analogues **13** and **14** [see also Foster et al. (1987)]. The azulene analogue **15** also gave a positive result.

To summarize, isomerization about a particular double bond, specifically the 7-ene, 9-ene, 11-ene, or 13-ene, is not essential for activation of *Chlamydomonas* rhodopsin. The only other specific double bond that could be involved is the terminal C=N (syn/anti). However, this bond is reported not to isomerize during bathorhodopsin formation, the stage at which it should have occurred if it were to occur (Bagley et al., 1985; Palings et al., 1987), which means that syn–anti isomerization cannot be specifically necessary. Bleaching can also be ruled out as being specifically necessary, because acid fluoride **5** (which forms an amide bond in the protein) restored phototaxis. The resulting action spectrum (Figure 3) resembles the absorption spectrum of bovine rhodopsin derived from **5** (Wong & Rando, 1984); however, this retinal analogue was reported to be inactive in an in vitro assay of bovine opsin activation (Calhoon & Rando, 1985).

Figure 3 also shows that imine nitrogen in the rhodopsin of *Chlamydomonas*, as in other rhodopsins, is protonated. Since the proton is primarily responsible for the red shift of rhodopsin, the action spectrum of the acid fluoride **5** that cannot protonate is strongly blue shifted.

The relative sensitivities of the analogues [compared to 11-*cis*-retinal (**6**)] show that the presence of a ring in the analogue does not reduce the sensitivity per se. Thus, **8** (seven-membered ring), **11** and **12** (six-membered ring), and **3** (five-membered ring) all have normal activity. Analogues **7**, **9**, **10**, **2**, and **4** all have lower sensitivity, showing that the precise geometry of the compound is important. These analogues probably have lower extent of incorporation or lower quantum efficiency. Analogues **13** and **14** with planar dou-

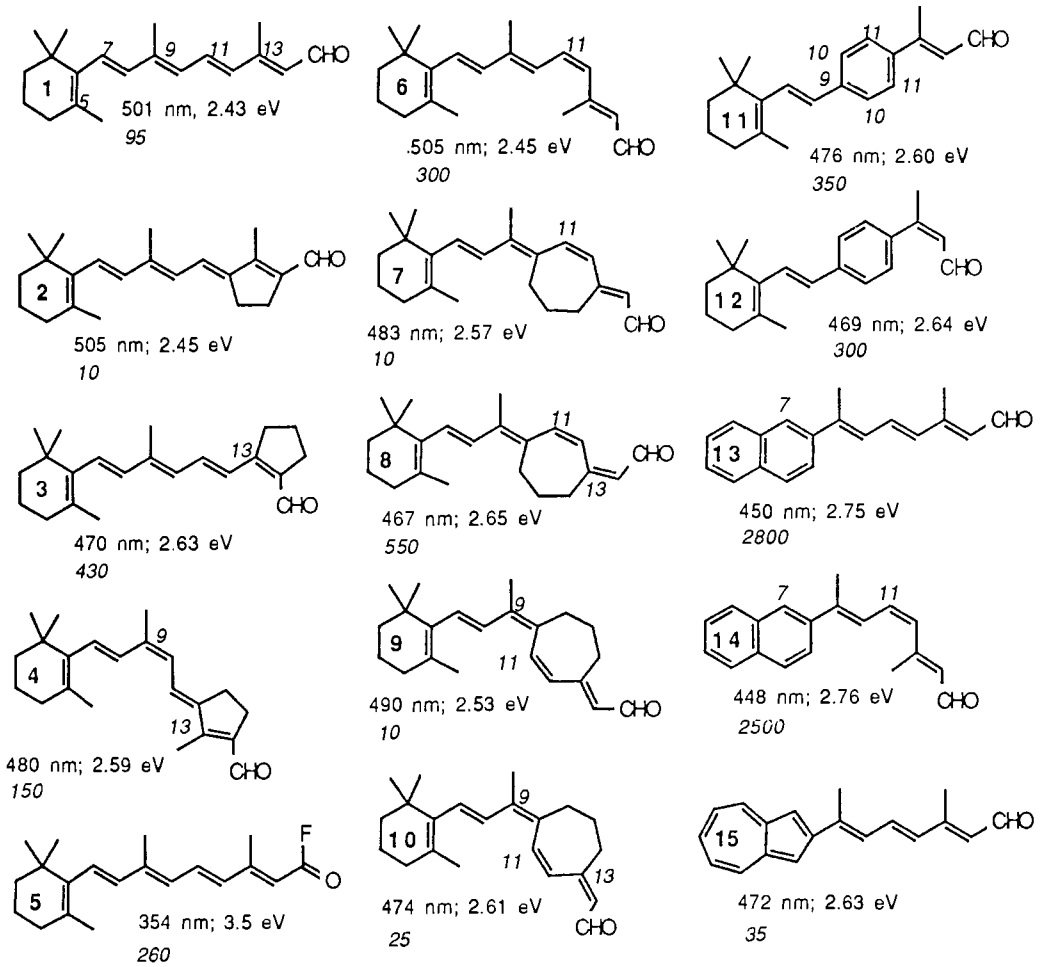

FIGURE 1: Retinal analogues incorporated into *Chlamydomonas* strain FN68. Below each structure, values are given for the location of the phototaxis action spectrum peak in units of nanometers (nm) and electron volts (eV) [wavelength in nm = 1239.85/(energy in eV)]. The maximal sensitivity, shown in *italics* on the second line below each structure, is the reciprocal of the threshold for phototaxis (see text) and is in units of 10^{-18} m^2 s/photon. Bovine rhodopsin absorption spectrum maxima for some of the analogues were as follows: 498 nm (2.49 eV) for **6**; 490 nm (2.53 eV) for **7**; 488 nm (2.54 eV) for **8**; 489 nm (2.54 eV) for **9**; and 483 nm (2.57 eV) for **10** (Akita et al., 1980).

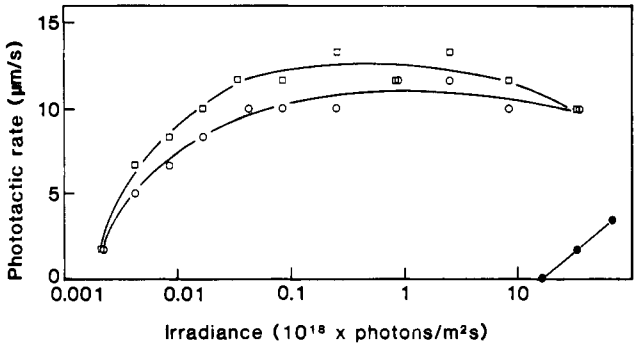

FIGURE 2: Stimulus irradiance-response curves obtained from measurement of phototaxis rate of *Chlamydomonas* FN68 as a function of irradiance of 546-nm light. With the same batch of cells, FN68 was incorporated with 10 μM of *all-trans* retinal (**1**) (□) or 11,13-dicis 7C-ring-locked retinal **8** (○) for 3 h. Before the measurement, cells were spun down and resuspended in analogue-free solution. Phototaxis rate was measured by starting at the lowest irradiance and then increasing stepwise over the range. The curves were drawn by eye. The control (●) was the same batch of cells without analogue added.

ble-ring systems [see also Foster et al. (1987)] show higher than normal sensitivity, perhaps because rigid planarity or higher hydrophobicity facilitated entry of the analogue into the binding site. As already noted, the 10 000-fold dynamic range of our assay allowed accurate measurement of the analogues with low sensitivity.

We made a series of observations to test the specificity of our assay system. The mere presence of a chromophore in the binding site does not restore activity. We tested this by incubating cells with retinonitrile (CN instead of CO in **1**), which cannot form a bond with a lysine amino group. The compound occupied the site, as indicated by reduced rate of incorporation of *trans*-retinal in its presence, but was itself inactive.

Unlike starved cells, vegetative cells of the mutant FN68 show no trace of phototaxis after addition of *trans*-retinal, presumably because the vegetative cells lack opsin. Whatever nonspecific binding of retinal to cell proteins occurs under these conditions does not restore phototaxis.

In some photosystems, such as in *Fusarium* (Lang-Feulner & Rau, 1975) and corn (Britz et al., 1979), methylene blue (a photosensitizer) can be incorporated as an additional functional photoreceptor. We incubated *Chlamydomonas* cells (which we knew would become fully phototactic when incubated with retinal) with 25 μM methylene blue; no phototaxis occurred. We also tested 1,8-diphenyloctatetraene, a pigment with high absorption that incorporates into the membrane; again no phototaxis occurred.

The results of these specificity experiments strengthen the conclusion that recovery of phototaxis is a consequence of binding of retinal analogues to a specific site on a specific retinal-binding protein which causes a "visual" type of response (i.e., opsin).

When using an in vivo assay, it is important to consider the possibility of metabolic alteration of the analogue before it

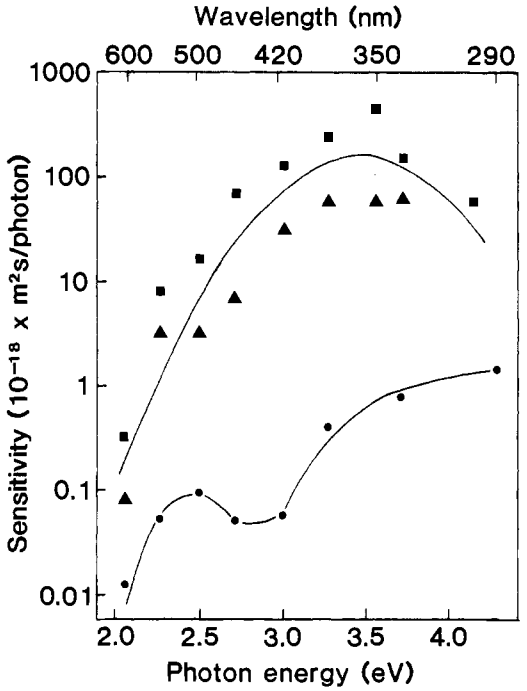

FIGURE 3: Phototaxis action spectrum following incorporation of 25 μM of *all-trans*-15-fluororetinal (**5**) into FN68 for 30 min (■, ▲); the different symbols represent two data sets obtained with different batches of cells. A typical control action spectrum (●) obtained from a different batch with no analogue added is also shown. In this case, for high irradiance, a 150-W xenon lamp was used for wavelengths below 380 nm. Curves were drawn by eye.

becomes incorporated into the protein. A number of compounds we know are converted to retinal in other systems, including retinyl acetate, retinyl palmitate, retinol, and β-carotene. These compounds were effective in restoring phototaxis, presumably as a result of the metabolic activity of the cell. The similarity of the action spectra of **1** and **6** is probably due to an isomerase (Foster et al., 1984). In fact, partial isomerization of analogues **1**, **5**, **12**, **13**, and **15** is quite possible. However, the arguments of this paper would be not affected if the actual isomer was different from that drawn, since each analogue would still be blocked from isomerization about the same point. The other analogues, however, appear not to be substantially altered because the phototaxis action spectra measured in *Chlamydomonas* are very similar to the absorption spectra of bovine rhodopsin measured in vitro (Akita et al., 1980), as shown in the legend of Figure 1. Our prior experience (Foster et al., 1984) has also been close correspondence between the absorption maxima of in vitro bovine rhodopsin and in vivo *Chlamydomonas* rhodopsin.

Discussion

Our results led to the following conclusions with respect to the rhodopsin in *Chlamydomonas*:

(a) Blocking of individual double-bond isomerizations does not prevent the activity of rhodopsin. Therefore, if isomerization is required at all, it must not matter at what position along the chromophore it occurs.

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

(c) Changes in molecular geometry of the chromophore may be unnecessary for protein activation. Palings et al. (1987) found that Raman intensities of hydrogen out-of-plane wags and lack of change in the C=N bond in bovine bathorhodopsin suggest that the 10–11, 12–13, and 14–15 bonds are all rotated about 20° following excitation; this model suggests that excitation involves no major change in the geometric shape of the molecule but does not exclude some transient rotation of the N from activating rhodopsin.

(d) Incorporation of the seven-membered ring analogue **7** into bovine rhodopsin blocked bathorhodopsin formation and subsequent bleaching. However, transiently excited species were present for 100 ps (Buchert et al., 1983). Given the nonisomerizable nature of the chromophore and the similarity of the environment of the chromophores in bovine and *Chlamydomonas* rhodopsin, the lifetime of excited species in *Chlamydomonas* is likely to be very similar to that in bovine rhodopsin. Activity in *Chlamydomonas* implies that changes in the protein must be triggered within the 100-ps period before the chromophore returns to its original ground state. In the case of bacteriorhodopsin, charge separation is initiated in less than 30 ps (Groma et al., 1984). The nanosecond or longer period that had been suggested to be needed to trigger the protein (Mathies & Stryer, 1976) evidently is not required.

This work suggests that the hypothesis that the rhodopsin of *Chlamydomonas* is activated as a direct result of the charge redistribution in the excited state of the chromophore (Salem & Bruckmann, 1975) is more probable than the hypothesis that activation occurs via isomerization. Stabilization by subsequent retinal structural alteration (Lewis, 1978) would not seem to be required, although it could play a complementary role. Certainly it can no longer be assumed that activation of rhodopsin necessarily occurs via cis–trans isomerization. Another alternative that we consider less likely than direct activation is that whatever partial rotation or change in pK_a at the N that occurs as a consequence of excitation is sufficient to trigger activation. The fact that the precise degree of rotation or the state of protonation does not matter argues against such a possibility but does not completely exclude it. We consider it improbable for a protein to be designed to pick up a shape change anywhere along the chromophore with equal efficiency when normally it expects a specific change in one place.

If these results can be applied to other rhodopsins, as we believe, then the popular view regarding rhodopsin activation must be markedly changed. Our results suggest that there are two photocycles to be distinguished, that of the chromophore and that of opsin, and two distinct activities of opsin that should be distinguished, namely, activation of the enzymatic site and its role in chromophore recycling (bleaching). Since changes in both are triggered by the charge redistribution of the photoexcited chromophore, charge redistribution should probably be considered as the primary and critical step in vision.

One aspect of cis–trans isomerization that was awkward to interpret if it played a role in activation was its relative nonspecificity (Thomson, 1975). Theoretical papers by Salem and Bruckman (1975), Lewis (1978), and Warshel (1978) have also suggested that geometrical isomerization might not play a critical role. Certainly the effect of charge redistribution is large and well documented, as measured by Mathies and Stryer (1976). They used electric field spectra to measure the change in the dipole moment on excitation of *all-trans*-retinal, of its unprotonated imine with *n*-butylamine, and of the chloride salt of the protonated imine. They found that the directions of the ground- and excited-state dipole moments are nearly parallel to the long axis of these molecules and that the change in the dipole moment corresponds to the net movement of electron density corresponding to one unit charge by 0.32,

0.21, and 0.25 nm (15.6, 9.9, and 12 D, respectively) toward the carbonyl or imine end.

We anticipate that while the precise sequence of amino acids in the rhodopsin in *Chlamydomonas* will likely be less similar to mammalian rhodopsin than that of *Drosophila*, for example, the overall structure and mechanism of activation will remain the same. The rhodopsin in *Chlamydomonas* closely resembles animal rhodopsin in its properties. Previously we showed that retinal is the active chromophore for phototaxis in *Chlamydomonas*, according to three lines of evidence: (a) the virtually perfect fit of the action spectrum to the standard curve of animal rhodopsin, including the highly distinctive slope of the low energy tail; (b) the requirement for either retinal synthesis or exogenously added retinal for activity; and (c), most importantly, the characteristic spectral shifts introduced when retinal analogues were substituted for retinal (Foster et al., 1984). The spectral absorption profile of retinal and its analogues shifts to lower energy upon incorporation into the opsin protein. The reasons are the specific polar charge distribution within a relatively nonpolar binding site and the protonation of the imine bond that binds retinal to an opsin lysine. The similarity of the shifts produced by each analogue in *Chlamydomonas* and bovine rhodopsin indicates that the binding sites are very similar (Foster et al., 1984). The similarity of shifts and the strongly blue shifted peak for acid fluoride analogue **5** also implies that the imine is protonated in *Chlamydomonas* as it is in bovine rhodopsin. As in other rhodopsins, the chromophore remains stably bound to the opsin in the dark and can be removed by hydroxylamine, the removal being facilitated by light (Hegemann et al., 1988). This behavior is characteristic of imine bonds in rhodopsins. Further, sequence similarity between bovine rhodopsin and a putative rhodopsin gene in *Chlamydomonas* has been suggested by hybridization studies (Martin et al., 1986).

From the prospective of evolution and results of molecular biology these similarities are more expected than surprising. The rhodopsin signaling system of animals probably arose early in the evolutionary line leading to animals, since *Dictyostelium*, which branched much earlier than *Chlamydomonas* from this line, has a membrane receptor system for cAMP that initiates a similar pathway involving GDP–GTP exchange activity of the receptor and activation of adenylate and guanylate cyclase (Janssens & vanHaastert, 1987), suggesting a receptor probably homologous to rhodopsin. Rhodopsin signaling systems of organisms such as *Chlamydomonas* that branched from animals much later (Hori et al., 1985) would be expected to have rhodopsins and related receptors essentially similar in overall structure and mechanism to that found in animals.

Similar information on activation can be obtained for other rhodopsins by using an in vitro enzyme assay of the visual cascade. Pigment bleaching is not a suitable assay, because it presumes an activation role for bleaching. Because of the technical difficulties and the expense of in vitro assays, an abbreviated protocol has been used that gives a dynamic range of about 20-fold (Fukada et al., 1984; Calhoon & Rando, 1985). We would suggest our traditional protocol that, though requiring more measurements, gives at visible wavelengths an approximately 10 000-fold range between the normal response and the minimum measurable response (Foster et al., 1988). A wide dynamic range makes detection of low levels of activation more reliable, thus minimizing the potential for false negative results. The avoidance of false negative results is important, because from negative results one cannot draw any conclusions since an experiment with an analogue might not work for many reasons.

This study introduces a nonbleachable analogue that fully activates rhodopsin. It is now possible with assurance of biological relevance to do all the experiments with ciliary rhodopsins (e.g., from *Bos* or *Chlamydomonas*) previously only possible with bacteriorhodopsin. Solution of this technical problem with 11,13-dicis seven-member-locked analogue **8** should prove of enormous benefit in the study of rhodopsin activation. The analogue should be sensitive as an antenna to all electronic events during protein activation that are in the neighborhood of the chromophore without being more than transiently excited or displaced from the retinal binding or regulatory site of rhodopsin. Repetitive stimulation of ciliary rhodopsins without the need of reincorporation of new chromophore is now conveniently accomplished.

A more extensive analysis of the biophysical principles underlying the conclusions of this paper is in preparation. This will include analysis of more recent results from a series of analogues with conjugated length varying from seven double bonds to one double bond (*n*-hexanal).

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Registry No. Retinal, 116-31-4.

References

Akhtar, M., Jallo, L., & Johnson, A. H. (1982) *J. Chem. Soc., Chem. Commun. 1*, 44–46.

Akita, H., Tanis, S. P., Adams, M., Balogh-Nair, V., & Nakanishi, K. (1980) *J. Am. Chem. Soc. 102*, 6370–6372.

Aton, B., Doukas, A. G., Narva, D., Callender, R. H., Dinur, U., & Honig, B. (1980) *Biophys. J. 29*, 79–94.

Bagley, K. A., Balogh-Nair, V., Croteau, A. A., Dollinger, G., Ebrey, T. G., Eisenstein, L., Hong, M. K., Nakanishi, K., & Vittitow, J. (1985) *Biochemistry 24*, 6055–6071.

Balogh-Nair, V., & Nakanishi, K. (1982) *Methods Enzymol. 88*, 496–506.

Britz, S. J., Schrott, E., Widell, S., & Briggs, W. R. (1979) *Photochem. Photobiol. 29*, 359–365.

Buchert, J., Stefancic, V., Doukas, A. G., Alfano, R. R., Callender, R. H., Pande, J., Akita, H., Balogh-Nair, V., & Nakanishi, K. (1983) *Biophys. J. 43*, 279–283.

Calhoon, R. D., & Rando, R. R. (1985) *Biochemistry 24*, 3029–3034.

Crouch, R., Nodes, B. R., Perlman, J. I., Pepperberg, D. R., Akita, H., & Nakanishi, K. (1984) *Invest. Ophthalmol. Visual Sci. 25*, 419–428.

Derguini, F., & Nakanishi, K. (1986) *Photobiochem. Photobiophys. 13*, 259–283.

Eyring, G., & Mathies, R. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 33–37.

Fang, J.-M., Carriker, J. D., Balogh-Nair, V., & Nakanishi, K. (1983) *J. Am. Chem. Soc. 105*, 5162–5164.

Foster, K. W., & Smyth, R. D. (1980) *Microbiol. Rev. 44*, 572–630.

Foster, K. W., Saranak, J., Patel, N., Zarrilli, G., Okabe, M., Kline, T., & Nakanishi, K. (1984) *Nature (London) 311*, 756–759.

Foster, K. W., Saranak, J., van der Steen, R., & Lugtenburg, J. (1987) *Invest. Ophthalmol. Visual Sci. 28*, S253.

Foster, K. W., Saranak, J., & Zarrilli, G. R. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 6379–6383.

Fukada, Y., Shichida, Y., Yoshizawa, T., Ito, M., Kodama, A., & Tsukida, K. (1984) *Biochemistry 23*, 5826–5832.

Groma, G. I., Szabo, G., & Varo, Gy. (1984) *Nature (London) 308*, 557-558.
Hegemann, P., Hegemann, U., & Foster, K. W. (1988) *Photochem. Photobiol. 48*, 123-128.
Honig, B., Dinur, U., Nakanishi, K., Balogh-Nair, V., Gawinowicz, M. A., Arnaboldi, M., & Motto, M. G. (1979) *J. Am. Chem. Soc. 101*, 7084-7086.
Hori, H., Lim, B.-L., & Osawa, S. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 820-823.
Hubbard, R., & Wald, G. (1952) *Science (Washington, D.C.) 115*, 60-63.
Hubbard, R., & Kropf, A. (1958) *Proc. Natl. Acad. Sci. U.S.A. 44*, 130-139.
Janssens, P. M. W., & vanHaastert, P. J. M. (1987) *Microbiol. Rev. 51*, 396-418.
Knowles, A., & Dartnell, H. J. (1977) in *The Eye* (Davson, H., Ed.) Vol. 2B, p 76, Academic, New York.
Kolling, E., Gartner, W., Oesterhelt, D., & Ernst, L. (1984) *Angew. Chem., Int. Ed. Engl. 23*, 81-82.
Lang-Feulner, J., & Rau, W. (1975) *Photochem. Photobiol. 21*, 179-183.
Lewis, A. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 549-553.
Liu, R. S. H., & Asato, A. E. (1984) *Tetrahedron 40*, 1931-1969.
Mao, B., Tsuda, M., Ebrey, T. G., Akita, H., Balogh-Nair, V., & Nakanishi, K. (1981) *Biophys. J. 35*, 543-546.
Martin, R. L., Wood, D., Baehr, W., & Applebury, M. L. (1986) *Science (Washington, D.C.) 232*, 1266-1269.
Mathies, R., & Stryer, L. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 2169-2173.
Palings, I., Pardoen, J. A., van den Berg, E., Winkel, C., Lugtenburg, J., & Mathies, R. A. (1987) *Biochemistry 26*, 2544-2556.
Rothschild, K. J., Cantore, W. A., & Marrero, H. (1983) *Science (Washington, D.C.) 219*, 1333-1335.
Salem, L., & Bruckmann, P. (1975) *Nature (London) 258*, 526-528.
Thomson, A. J. (1975) *Nature (London) 254*, 178-179.
Warshel, A. (1978) *Proc. Natl. Acad. U.S.A. 75*, 2558-2562.
Wong, C. G., & Rando, R. D. (1984) *Biochemistry 23*, 20-27.
Zarrilli, G. R. (1984) Ph.D. Thesis, Columbia University.

Simultaneous Optical Measurement of Osmotic and Diffusional Water Permeability in Cells and Liposomes†

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ABSTRACT: A quantitative description of transmembrane water transport requires specification of osmotic (P_f) and diffusional (P_d) water permeability coefficients. Methodology has been developed to measure P_f and P_d simultaneously on the basis of the sensitivity and rapid response of the fluorophore aminonaphthalenetrisulfonic acid (ANTS) to solution H_2O/D_2O content. Cells loaded with ANTS in an H_2O buffer were subjected to an inward osmotic gradient with a D_2O buffer in a stopped-flow apparatus. The time courses of cell volume (giving P_f) and H_2O/D_2O content (giving P_d) were recorded with dual photomultiplier detection of scattered light intensity and ANTS fluorescence, respectively. The method was validated by using sealed red cell ghosts and artificial liposomes reconstituted with the pore-forming agent gramicidin D. At 25 °C, red cell ghost P_f was 0.021 cm/s with P_d 0.005 cm/s (H_2O/D_2O exchange time 7.9 ms). P_f and P_d were inhibited by 90% and 45% upon addition of 0.5 mM $HgCl_2$. The activation energy for P_d increased from 5.1 kcal/mol to 10 kcal/mol with addition of $HgCl_2$ (18-35 °C). In 90% phosphatidylcholine (PC)/10% cholesterol liposomes prepared by bath sonication and exclusion chromatography, P_f and P_d were 5.1×10^{-4} and 6.3×10^{-4} cm/s, respectively (23 °C). Addition of gramicidin D (0.1 μg/mg of PC) resulted in a further increment in P_f and P_d of 7×10^{-4} and 3×10^{-4} cm/s, respectively. These results validate the new methodology and demonstrate its utility for rapid determination of P_f/P_d in biological membranes and in liposomes reconstituted with water channels.

While the mechanism of many biologically important membrane transporters is understood at the biochemical and molecular levels, the description of water movement across biological membranes remains empirical. The working definition of a water channel has been derived from data obtained in human red cells (Macey, 1984; Mlekoday et al., 1983; Solomon et al., 1984), the kidney proximal tubule (Verkman & Wong, 1987; Meyer & Verkman, 1987), and vasopressin-sensitive tight epithelia (Harmanci et al., 1978; Levine et al., 1984; Verkman et al., 1988). Compared with pure phospholipid membranes, membranes containing water channels generally have a higher osmotic water permeability coefficient ($P_f > 0.01$ cm/s), an osmotic to diffusional permeability ratio (P_f/P_d) > 1, a low activation energy ($E_a < 5$ kcal/mol), and partial inhibition of P_f by mercurials (Fettiplace & Haydon, 1980; Finkelstein, 1987).

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