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11-*cis*-retinal, a molecule uniquely suited for vision

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Abstract- The chromophores of all visual pigments, the rhodopsins, is the 11-cis form of retinal and its analogs bound covalently to opsin, the receptor protein through a protonated Schiff base bond. Similarly, the pigments present in *Halobacterium halobium,* namely, bacteriorhodopsin (proton pump), halorhodopsin (chloride pump), and the two sensory rhodopsins (phototaxis receptor) contain the all-trans isomer of retinal bound to the apoprotein by a protonated Schiff base linkage. The chromophores are indeed uniquely designed to perform such vital functions. Why was retinal chosen as the chromophore of pigments, why is the chromophore a protonated Schiff base, and why 11-cis in some cases and all-trans in other cases?

INTRODUCTION

The family of retinal proteins (refs. 1,2) comprises rhodopsins (visual pigments), four pigments present in the archaebacterium *Halobacterium halobium* (Fig.1), and possibly the pigment responsible for the phototaxis of the unicellular alga *Chlamydomonas.* They are 28-40 kDa transmembrane proteins consisting of seven α-helices to which is bound retinal, a C_{20} terpenoid derived from the C_{40} β-carotene.

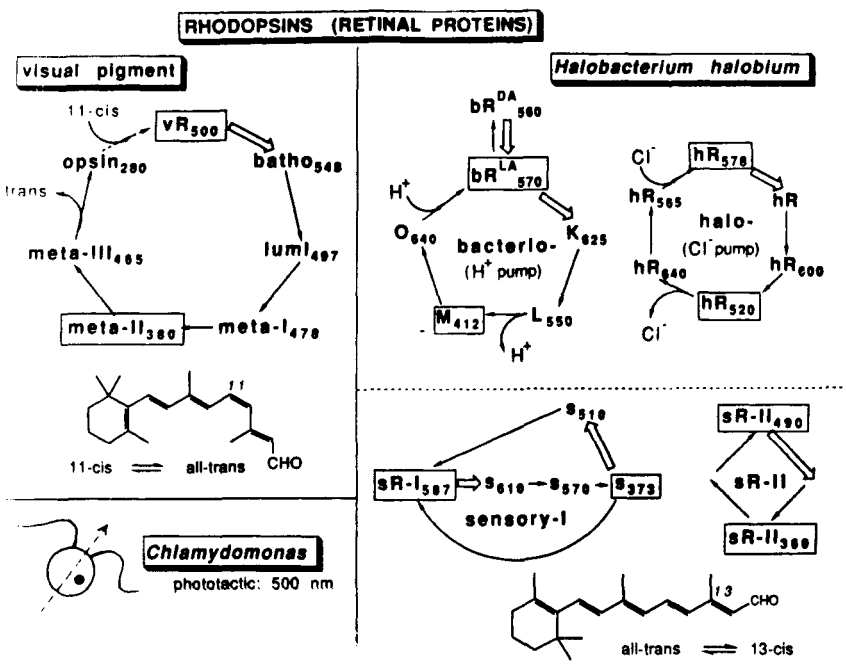
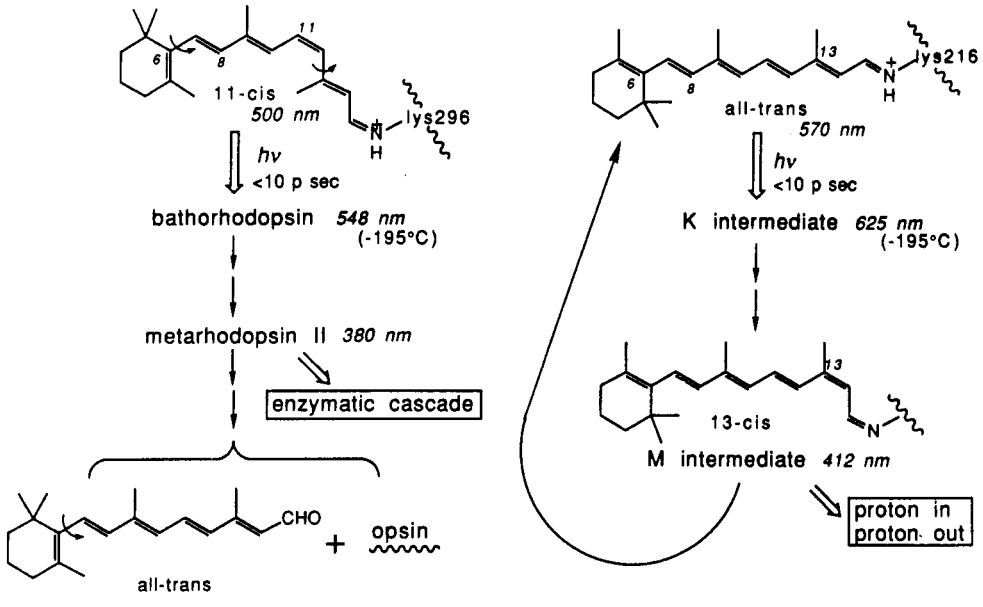


Fig. 1. The retinal proteins. The isomeric forms of retinals involved in the visual cycle of rhodopsin and the four pigments of *H. halobium* are, respectively, 11-*cis* / all-*trans* and all-*trans* / 13-*cis*. Neither the protein nor the chromophore of the *Chlamydomonas* photoreceptor is known at this stage. The wide arrows in each cycle denote photoactivation steps.

The human retina contains about a hundred million rod cells responsible for scotopic (dim light) vision, and about three million cone cells responsible for photopic (bright light) color vision. The human rod cell has its sensitivity maximum at 500 nm, whereas the three kinds of cone cells absorb maximally at 440 nm (blue), 530 nm (green) and 580 nm (red). Since solar energy is strongest in the region defined as the visible region of 400-700 nm, humans take full advantage of the solar energy distribution. The integrated area of the absorption spectra (oscillator strength) of the visual pigments is close to the maximal value attainable by organic molecules. The ratio of rod to cone cells is characteristic for the animals that have adapted to the various photic environments, e.g., 4000 for the nocturnal rat, 20 for humans, 15 for goldfish and 1 for frog (ref. 3).



rhodopsin *visual transduction* **bacteriorhodopsin** *proton pump*

Fig. 2. Outline of the bleaching of rhodopsin and the photocycle of bR, leading to enzymatic cascade and proton translocation, respectively. Curved arrows indicate nonplanarity.

The chromophore of the majority of rhodopsins is 11-*cis*-retinal, although 11-*cis*-retinal derivatives, i.e., 3-dehydro-(salmon, eel, tadpole), 3-hydroxy- (insects) and 4-hydroxy-retinals (squid) are also found as native chromophores (see Fig. 10). Most animals, including cats and dogs lack color vision. Cattle also have only one visual pigment which absorbs at 500 nm; in this case the 11-*cis*-retinal is bound to the terminal amino group of Lys-296 of the opsin *via* a protonated Schiff base linkage (Fig.2).

Visual transduction is initiated by photons which isomerize the 11-*cis* chromophore to all-*trans*; this changes the conformation of the rod and cone rhodopsins, which in turn results in an enzymatic cascade culminating in the hydrolysis of cyclic GMP and transmission of a neural signal to the brain. The absorption of light by rhodopsin isomerizes rhodopsin in a few picoseconds into the primary photoproduct, bathorhodopsin (λmax 548 nm), the chromophore of which is the protonated Schiff base of a "distorted" *trans*-retinal. Microcalorimetric measurements show that bathorhodopsin has energy 35 kcal higher than rhodopsin, and it is this high chromophore/protein distortion energy which results in thermal relaxation of the protein conformation and final expulsion of the chromophore from opsin as all-*trans*-retinal. Thus, a photon energy is translated into motion of the photoreceptor cell, which in turn results in nerve impulse and visual transduction. The cleavage of retinal and opsin is called bleaching because the initial orange-red color of the pigment is discolored into a pale yellow mass. The empty opsin regenerates rhodopsin by taking up another molecule of 11-*cis*-retinal, whereas the all-*trans*-retinal moves to the outside of the retina into the pigment epithelium, is reduced and esterified to retinol fatty acid ester, the hydrolysis of which regenerates 11-*cis*-retinol (see Fig. 9).

The first step in the critical enzymatic cascade leading to visual transduction is activation of G protein or transducin (ref. 4), which is triggered by the conformation adopted by the intermediate metarhodopsin II, λmax 380 nm (Figs. 2, 3). The active form of transducin activates PDE (phosphodiesterase), which rapidly hydrolyzes cGMP to GMP; the resulting blockage of the Na channel results in membrane hyperpolarization and leads to nerve impulse. One photon activates ca. 100 molecules of transducin and 100,000 molecules of cGMP. The activated metarhodopsin II is eventually deactivated by rhodpsin kinase, which phosphorylates the serine and threonine residues in the carboxyl terminal (Fig. 3).

Whereas rhodopsin serves as a light detector for the sensory system, the pigment bacteriorhodopsin (bR) present in *Halobacterium halobium*, an ancient 1.3 billion-years-old bacterium, functions as a photosynthetic energy source (Figs. 1, 2). This halophilic prokaryotic cell lives in salt water. Under anaerobic conditions it begins to develop patches of purple membrane, which contains bR as its sole protein; bR absorbs solar energy and converts it into chemical energy. As with other rhodopsins, the tertiary structure of bR, composed of 248 amino acids of known sequence, has yet to be elucidated (ref. 5). The chromophore of light-adapted bR, λmax 570 nm (purple region), is all-*trans*-retinal which is attached to Lys-216 *via* a protonated Schiff base linkage . ^{13}C NMR results (ref. 6) indicate that the ring structure adopts the 6-s-*trans* conformation, as opposed to rhodopsin where it is 6-s-cis; see Section (V) for discussion on 6-*s-cis versus* 6-*s-trans*. Under irradiation, the transient bR^{LA} immediately converts to the primary photoproduct, intermediate K, λmax 625 nm which, according to vibrational spectral data, has a "distorted" 13-*cis*-retinal protonated Schiff base chromophore. Relaxation of the stored energy in K induces a series of intermediates to reconvert the pigment back to the original bR^{LA}. Around the M intermediate, λmax 412 nm, this photo-induced cycle results in the transport of two protons from the cytoplasm to the external medium. In contrast to the process of rhodopsin bleaching in vertebrates, the retinal is not detached from the opsin in this photocycle.

In addition to bR, *H. halobium* contains the chloride pump halorhodopsin hR, λmax 578 nm, and two sensory rhodopsins responsible for its phototaxis (Fig. 1)(ref. 7). During the early exponential growth

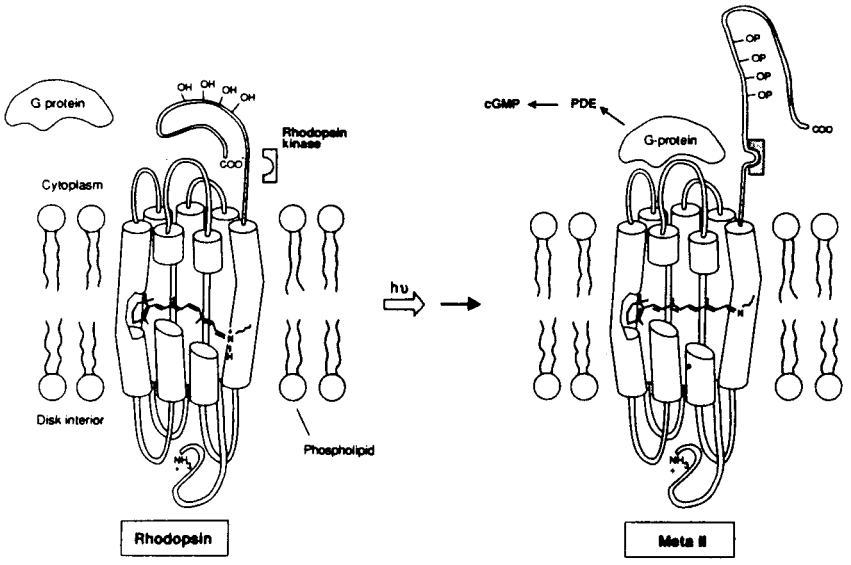


Fig. 3. When rhodopsin adopts the conformation of metarhodopsin II, the G protein or transducin in the cytoplasmic domain of the disk (or intradiskal space) is activated by binding to the loop connecting the helices and initiates the enzymatic cascade which hydrolyzes cGMP. Rhodopsin kinase phosphorylates the serine and threonine residues in the carboxyl terminal of metarhodopsin II; phosphorylated meta II binds to the inhibitory protein, arrestin, that inactivates the rhodopsin by blocking the binding of transducin.

phase, the bacterium is aerobic and grows in the dark; the major sensory pigment is sR-II (or phoborhodopsin) with λmax 490 nm, which induces light-repellent flagellar movement to avoid solar radiation. In contrast, the late exponential growth phase is anaerobic and requires light; at this stage, the pigment is sR-I_{587} which leads to light-attractant flagellar motion upon irradiation with 587 nm light; however, in order to protect the bacterium from moving towards the dark, this irradiation gives an intermediate S_{373}, which leads to a repellent movement when irradiated with 373 nm light.

POINT CHARGE MODELS

Although bovine rhodopsin absorbs at 500 nm (20,000 cm^{-1}), the protonated Schiff base formed from retinal and n-butylamine only absorbs at 440 nm (22,700 cm^{-1}), the difference between the two maxima clearly being caused by factors operating within the protein binding cavity (Fig. 4). The absorption maxima of the natural pigments vary from the UV (insects) to 725nm. The cause of such wavelength regulation had been a central issue in vision science and several theoretical models had been proposed. The difference in λmax, in cm^{-1}, between the protonated Schiff base formed from a retinal analog with n-butylamine and the

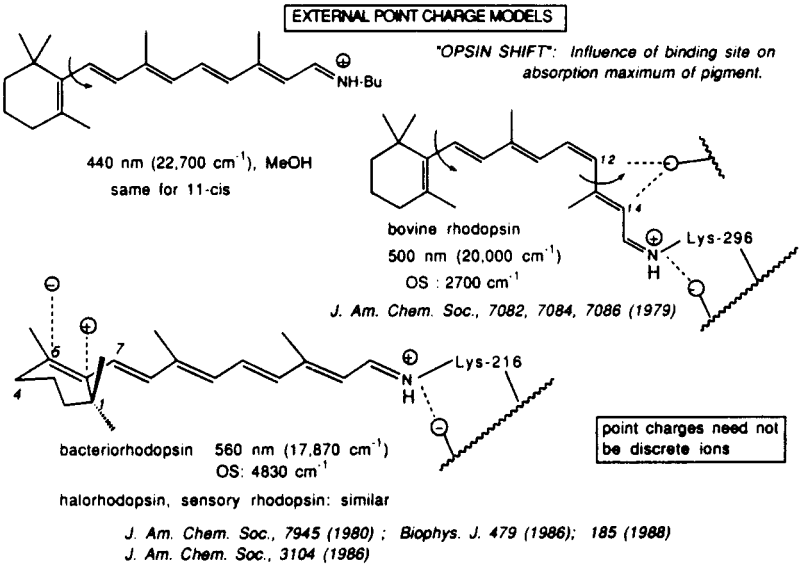


Fig. 4. The external point charge models proposed for bovine rhodopsin and bR. Curved arrows indicate nonplanarity.

pigment regenerated from the corresponding retinal analog is called the "opsin shift" (OS)(ref. 8), which reflects the influence of the protein-binding site on the absorption maximum of the pigment. Experimental measurements and theoretical calculations of the OS of a series of bovine rhodopsin analogs regenerated from dihydroretinals led to the external point charge model (ref. 9). According to this model, "negative charges" reside near the protonated nitrogen and in the vicinity of C-12 and C-14 in bovine rhodopsin. The maxima of visual pigments are regulated in a subtle manner by charged groups from amino acids; however, the point charges need not necessarily be discrete ions, they could, for example, be a cluster of water molecules that would neutralize the charges by hydrogen bonding. Recent site-specific mutation experiments performed with human pigments have shown that the counterion in bovine rhodopsin is Glu-113. (ref. 10).

Similar consideration of the OS of bR reconstituted from a series of *trans*-dihydroretinals, coupled with extensive model studies (ref. 11) led to the external point charge model shown in Fig. 4; the initial model (ref. 8) lacked the positive charge above the ionone ring. This additional charge was added in a revised model in which, in addition to the counter anion, an ion pair resides close to the ring (ref. 12, 13). Incorporation of the 5-membered-ring analog **2** (Fig. 8) inhibits proton pumping (ref. 14, 15) and phototaxis (ref. 16). It is thus clear that an all-*trans*/13-*cis* isomerization of the retinal chromophore is essential for these photochemical reactions. These results support the conclusion that the photoactivation processes which trigger the conformational changes of bR (proton pump), hR (chloride pump), sR-I (dual attractant/repellent phototaxis receptor) and sR-II (repellent phototaxis receptor) are conserved despite the different biological functions of their photoactivation.

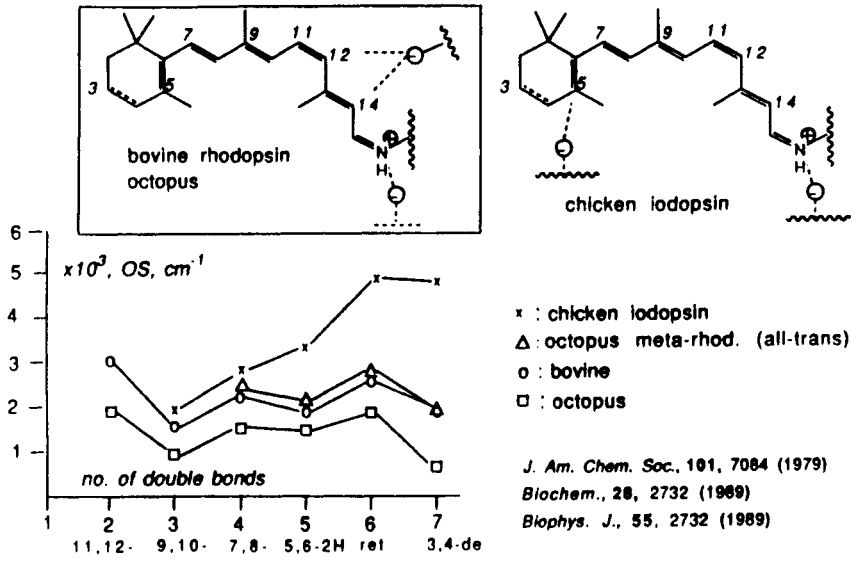


Fig. 5. Plot of opsin shift and number of conjugated double bonds of retinal analogs incorporated into various visual pigments. Two point charge models are also shown.

The OS values obtained from experiments with octopus pigments (ref. 17) and chicken iodopsin (ref. 18), a cone pigment, are plotted against the number of conjugated double bonds of the incorporated retinal analogs in Fig. 5. A plot like this readily tells that the distribution of charges within the binding sites of bovine and octopus rhodopsins is similar, but that it differs in iodopsin. In the case of chicken iodopsin, the OS increases monotonically as the number of conjugated double bonds increases from 3 to 6 (retinal); 3,4-dehydroretinal is always a special case in OS because of the presence of a homoannular diene system. The monotonic increase seen in chicken iodopsin leads to a simple interpretation depicted in Fig. 5. Namely, since the magnitude of the shift induced by external charges would depend on their proximity to the conjugated π-electron system, the monotonic increase strongly suggests the presence of a negative point charge near the vicinity of the ring. This charge would have a maximal effect on the native chromophore with six double bonds and the effect would successively decrease as the location of the double bond which was saturated approaches the side-chain terminus (ref. 18).

Again a plot of OS against the number of double bonds in the retinal analog (Fig. 6) shows that the environments within the binding sites must be similar for sR-I (ref. 19), hR (ref. 20) and bR (ref. 12). However, this is different in phoborhodopsin (sR-II) (ref. 21). Indeed, unlike other *H. halobium* pigments, the absorption spectrum of the native pigment has a blue-shifted maximum at 487 nm with a pronounced shoulder at 460 nm, which has been shown to reflect vibrational levels. The fine-structured and blue-shifted features of sR-II spectra suggest that the chromophore adopts *a more rigid conformation,* presumably due to a closer distance between the counter-anion and N^+, lack of interacting charges near the ring, and a planar 6-*s*-*trans* conformation (see next paragraph).

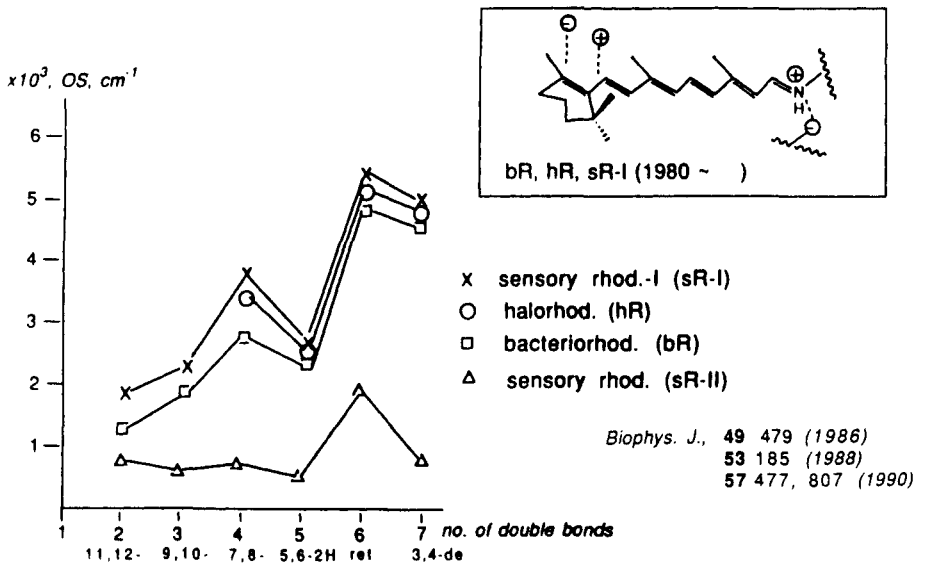


Fig. 6. Plot of opsin shift and number of conjugated double bonds of retinal analogs incorporated into various pigments of *H. halobium*.

Three factors, distance between the counter-anion and N^+, planarity of its conjugated system and external point charges, can conceivably contribute to the opsin shifts. In the case of bR, the OS of ca. 5000 cm^{-1} (Figs. 3, 5) can be broken down as follows (Fig. 7):

(1) Counter-anion / N^+ distance. In 9,10-dihydroretinal I, the OS is 1910 cm^{-1}, or ca. 2000 cm^{-1} (ref. 12). Neither the planarity of the ring 5,6-ene nor the external charges are participating in this OS because they are separated from the terminal dienal chromophore by the 9,10-single bond. Therefore, 5000 cm^{-1} minus 2000 cm^{-1}, or *3000 cm^{-1}*, is attributable to this distance factor. Namely, if the negative charge were farther removed than in the case of bR (3 Å, ref. 8), the pigment would absorb more in the red (or the OS would be larger) because of greater delocalization of the positive charge, and *vice versa*.

(2) Planar structure (6-*s-trans*) *vs.* nonplanar structure (6-*s-cis*). The ionone ring can adopt a nonplanar 6-s-cis conformation as depicted in Fig. 4 for bovine rhodopsin, as shown by solid-state ^{13}C NMR

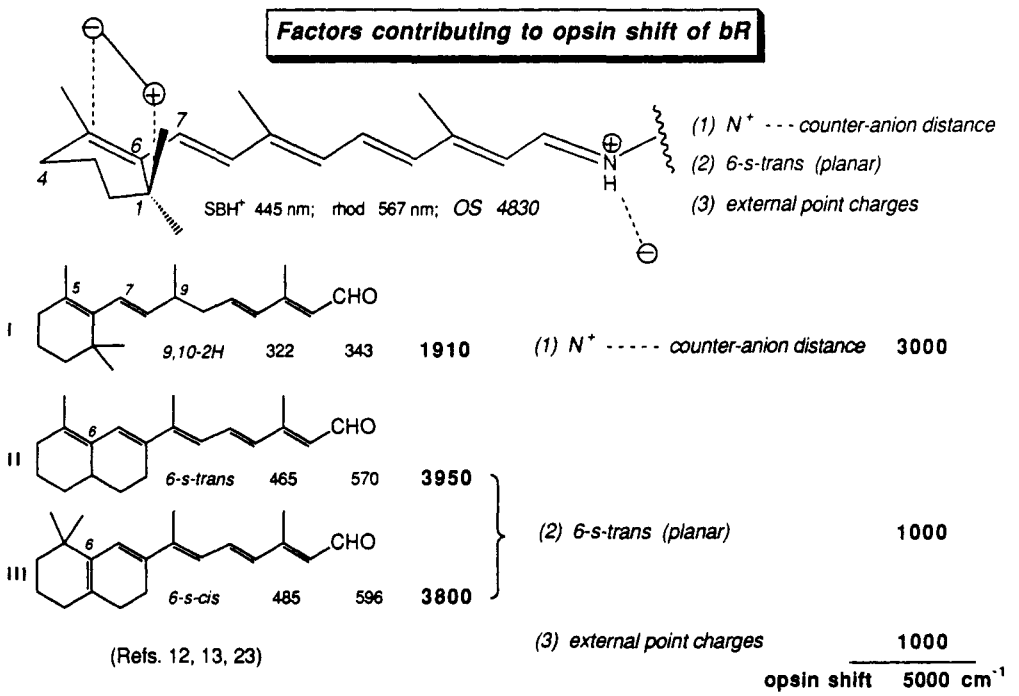


Fig.7. Factors contributing to the opsin shift in bacteriorhodopsin.

of rhodopsin (ref. 22); alternatively, it can be in the 6-s-trans conformer as in bR (Fig.4), also shown by ^{13}C NMR to be the case (ref. 6). Lugtenburg and coworkers incorporated hexahydronaphthylretinals II and III, containing, respectively, a 6-*s-trans* locked and 6-*s-cis* locked diene system into bR (ref. 23). The OS in these two derivatives with locked planar structures was around 4000 cm^{-1} in both cases. Since the OS is the difference between the maxima of the pigment and the corresponding protonated Schiff base, planarity / nonplanarity of the ring is not contributing to the OS in II and III. Therefore, the difference, 5000 cm^{-1} minus 4000 cm^{-1}, or *1000 cm^{-1}*, represents the OS arising from the change of the nonplanar ionone ring in retinal to the planar 6-*s-trans* conformation in bR (ref. 23).

(3). External point charge contribution in bR. The remaining 1000 cm^{-1} should be attributed to the influence of the negative and positive charges near the ring (ref. 12).

THE PHOTORECEPTOR OF *CHLAMYDOMONAS REINHARDTII*

Chlamydomonas is an aquatic organism of 10 μm diameter with a single eye spot and two flagella which guide the swimming cell either toward or away from light (Fig. 1). This unicellular eukaryote is estimated to have separated from the vertebrate evolutionary line at least a billion years ago (see Fig. 12). A blind mutant of *Chlamydomonas reinhardtii* lacks retinal because of blockage in the biosynthesis of β-carotene, the precursor of vitamin A and retinal. It therefore does not undergo phototaxis, but this property is readily restored upon incubation of the microorganism in media containing retinal analogs (ref. 24). Incubation with over 90 retinal analogs led to a restoration of phototaxis with *action spectrum maxima* that could be accounted for by the retinal structures. However, these *in vivo* behavioral experiments led to most unexpected findings (ref. 25, 26).

Figure 8 lists the induced phototaxis maxima upon incubation of the blind mutant with various retinal analogs. The italicized numerals denote approximate sensitivities on a relative scale. The native *Chlamydomonas* has its action maximum around 503 nm but several attempts to isolate the natural chromophore have not been successful owing to the minute size of the eye spots. The first surprise was that both all-*trans*-retinal **1** and 11-*cis* **5** smoothly led to restoration of phototaxis with maxima close to those of the wild species; it is well-known that all-*trans*-retinal cannot be incorporated into rhodopsin and hence we suspected the presence of an isomerase. However, restoration of phototaxis by seven-membered analogs **6** and **7** cannot be accounted for by isomerization. Because the 11-*cis* → *trans* isomerization is blocked, incorporation of these into bovine opsin yields nonbleachable pigments (ref. 27), and injection into rats or administration to retina give nonfunctional rhodopsin (ref. 28). Phototaxis is even induced by the phenyl analog **8** in which the phenyl ring blocks most isomerizations. These results demonstrate that isomerizations of specific double bonds are not required !

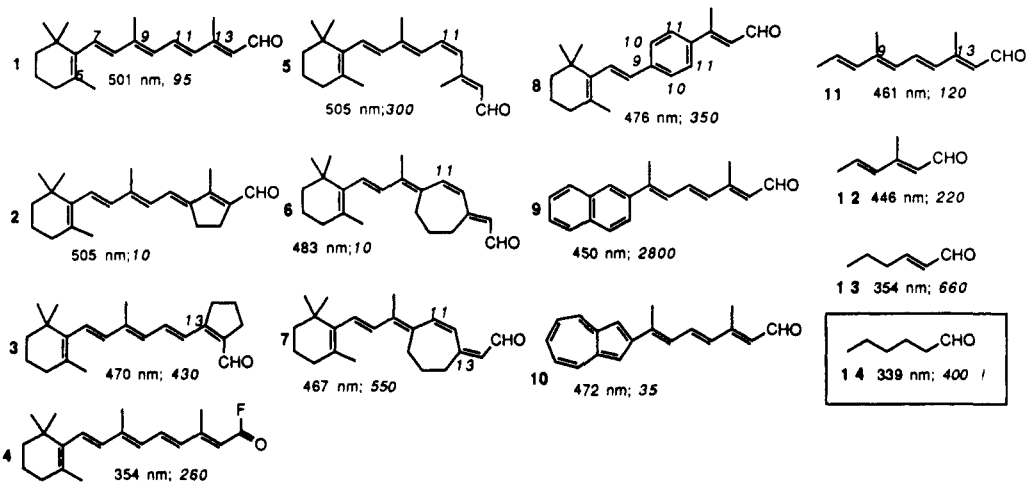


Fig. 8. Induced action spectral maxima upon incorporation of the various retinal analogs into the blind mutant of *Chlamydomonas reinhardtii*. Italicized numerals denote relative sensitivity (or efficiency) of incorporated retinal.

The blind *Chlamydomonas* mutant was also systematically incubated with acyclic compounds **11-13** having different numbers of conjugated double bonds; this not only resulted in restoration of phototaxis but the induced phototaxis action spectral maxima are comparable with the maxima obtained when retinal analogs containing the same number of conjugated double bonds are incorporated *in vitro* into bovine rhodopsin. Thus, whatever the native chromophore in this unicellular alga, it is likely to be a retinal bound to the photoreceptor protein through a protonated Schiff base. However, the positive results obtained with the short acyclic aldehydes, dienal **12**, hexenal **13**, and particularly the saturated hexanal **14** are most unexpected ! The sequential blue shifts accompanying the shortening of conjugation, **11** → **13**, suggest that the length of conjugation is playing a role. However, in the case of hexanal **14**, even consideration of the bathochromic shift resulting from protonation of a Schiff base cannot account for the 339 nm maximum (we have so far not been able to prepare the protonated Schiff base of hexanal). Except for the possibility that these short

aldehydes restore phototaxis through a mechanism differing from that with the retinal analogs and longer acyclic series, these results cannot be rationalized. Moreover, the fact that acid fluoride 4, which should form an amide rather than a protonated Schiff base with lysine, is another unexpected finding. It is essential that a sufficient quantity of the photoreceptor protein be obtained, by genomic and / or affinity labeling techniques, in order to perform *in vitro* experiments, particularly spectroscopic measurements.

THE GENESIS OF 11-*cis*-RETINAL

The source of 11-*cis*-retinal is vitamin A, which we have to obtain from carotenoids (Fig.9). Bleaching of rhodopsin yields all-*trans*-retinal, a portion of which is recycled back to 11-*cis*-retinal. But how is 11-*cis*-retinal biosynthesized ? This central and fascinating problem is being clarified mostly through recent studies performed by Robert Rando and co-workers (ref. 29). They discovered in 1987 that exogenous vitamin A was converted into 11-*cis*-retinol by membranes of the retinal pigment epithelium (ref. 30).

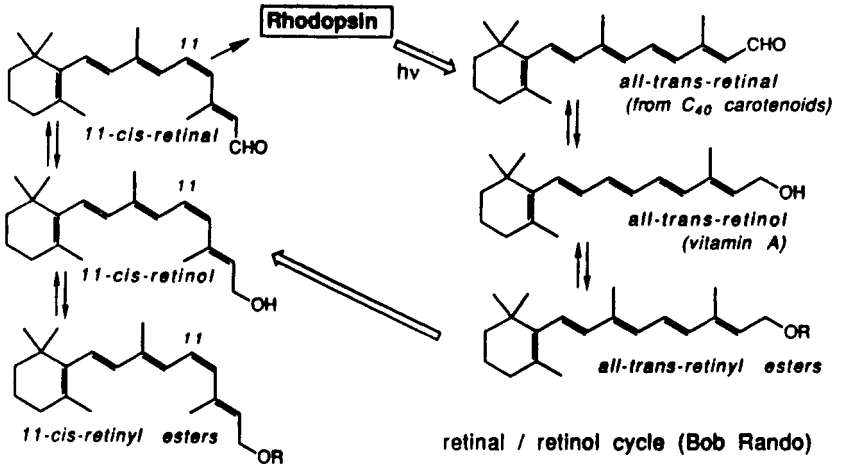


Fig. 9. The visual cycle of retinoids.

However, a key question was the source of energy to drive the isomerization of all-*trans*-retinol to 11-*cis*-retinol, which is higher in energy by ca. 4 kcal / mol. It is now understood that vitamin A is esterified to retinyl palmitate and other esters, and that these esters are transformed directly into free 11-*cis*-retinol, the uphill 4 kcal / mol being provided by the 5 kcal / mol gained from ester hydrolysis occurring in the lipid membrane (ref. 29, 31). It is known that full conjugation of the retinol molecule is necessary for efficient trans → cis isomerization (ref. 32), but the mechanism of the crucial isomerization step remains to be solved.

WHY RETINAL AND WHY 11-*cis*-RETINAL?

Ranging from insects, which have UV and visible vision, to humans to fish, some of which have vision extending to the near infrared, animal vision covers the wide range from 300 nm to 725 nm; the characteristics of the respective visual pigments match the spectral characteristics of the habitat. The chromophore of most visual pigments is 11-*cis*-retinal, but some aquatic animals have 3,4-dehydroretinal as an additional chromophore, while insects have 3-hydroxyretinal and the fire-fly squid (ref. 33) has 4-hydroxyretinal as the chromophore (Fig. 10). By subtle differences in the extent of bleaching of the three

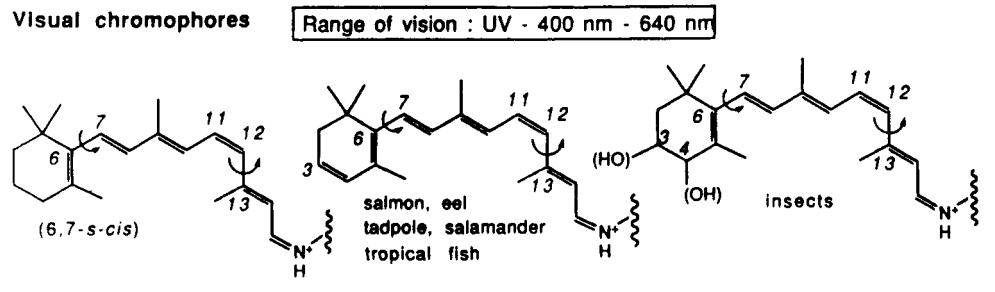


Fig. 10. Various pigments. Curved arrows in 11-*cis*-retinal denote nonplanarity.

cone pigments peaking at 440 nm, 530 and 570 nm, the human is capable of distinguishing monochromatic light that differs by only 1 nm, and can also differentiate thousands of of hues. The maxima of the three cone pigments in goldfish are at 455, 530 and 625 nm.

This extremely wide variation in color perception ranging from UV through 725 nm is caused by the *interaction between the rhodopsin binding site and the 11-cis-retinal chromophores* (or its simple analog). How can the protonated Schiff base of 11-*cis*-retinal cover such a wide range ? First, the chromophore is a protonated Schiff base which carries a positive charge on an extended conjugated system. Secondly, as depicted in Fig. 11, the 6-*s-cis* conformation of 11-*cis*-retinal indeed gives a molecule most cleverly designed to be able to adopt an infinite number of conformations through rotations around the 6,7- and 12,13-single bonds. Moreover, the positive charge can be delocalized throughout the long conjugated polyene system; dictated by the necessity of animals to adapt to their environments, the delocalized positive charge can interact with charges and polar neutral groups within the opsin binding site in numerous manners, namely, rotation around the 6-7 / 12-13 single bonds coupled with positive charge delocalization enables *the same chromophore* to cover a wide range of the light spectrum; no other single chromophore could achieve such a feat. The three cone pigments of humans have not yet been isolated but their genes have been cloned and sequenced and have been shown to possess about 50% homology with rhodopsin (ref. 34, 35). The pigments with maxima at 530 nm (green) and 580 nm (red) are encoded by two closely linked genes on the X chromosome, a fact which accounts for the high incidence of green-red color blindness caused by defects in these closely related genes; the gene for the 440 nm absorbing pigment is on an autosome that separated from the two other at a much earlier stage in evolution (ref. 36). Although the human green and red pigments differ only in 15 amino acid residues, the difference in protein environment causes the 50 nm shift in absorption maxima. The extra double bond in the 3,4-dehydro chromophore leads to further shifts towards the red, whereas the influence of the hydroxyl groups could be variable.

For the pigments of *Halobacterium* such fine tuning is unnecessary. The function of the pigments is simply to translocate hydrogen or chloride ions, or to induce positive or negative phototaxis. A more rigid and simple planar structure suffices, and hence it was not necessary to develop the sophisticated system for producing the less stable 11-*cis* form from the all-trans form. Since the retinal molecule in its free state can adopt either the 6-*s-cis* or 6-*s-trans* conformation, in this case the receptor apoproteins dictate the conformation around the 6-single bond to be the planar 6-*s-trans* (the 7-H is trapped between the two methyls at C-1 and keeps this portion of the molecule planar). In order that the flat molecule can still adjust its absorption maxima to 570 / 580 nm (bR), 590 / 370 nm (sR-I) or 490 nm (sR-II), the chromophore is also a protonated Schiff base (Fig. 10).

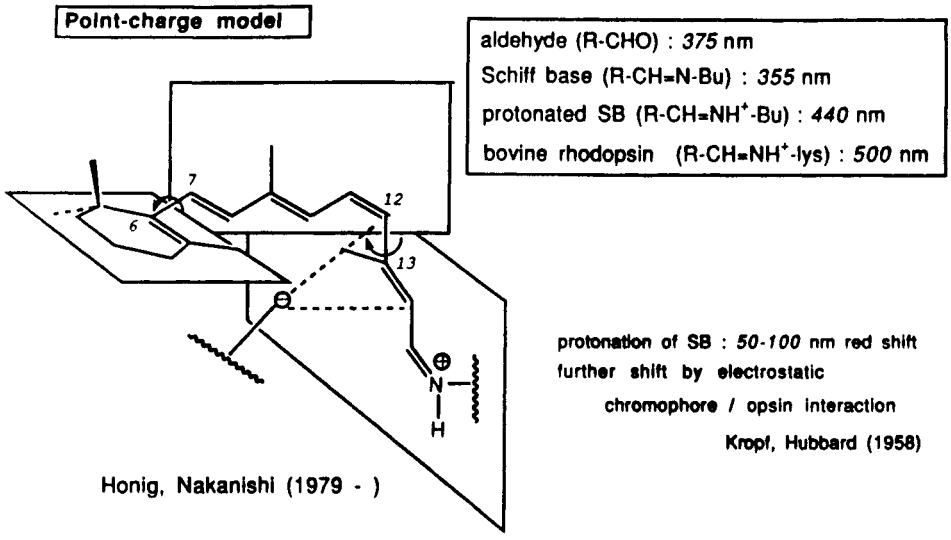


Fig. 11. The conformationally flexible 11-*cis*-retinal contained in visual pigments. Curved arrows indicate nonplanarity.

As mentioned above, the chromophore of visual rhodopsin is the *less stable* 11-*cis*-retinal. Irradiation isomerizes the double bond to the *more stable* trans form, the all-trans-retinal is expelled from the system, and then reconverted to the unstable 11-*cis* form in the pigment epithelium, the energy being provided by ester hydrolysis (ref. 29). In contrast, the chromophore of *Halobacterium* is the *more stable* all-*trans*-retinal. In this case, light isomerizes it to the *less stable* 13-cis form, which spontaneously reconverts to the more stable *trans*-form.

Through evolution, the higher animals and insects came to use the subtle 11-*cis*-retinal as their transduction chromophore, while the archaebacterial *H. halobium* adopted the more primitive all-*trans*-retinal for its transduction and sensory pigments (Fig.12). It will be interesting to see what the photoreceptor chromophore of the eukaryote *Chlamydomonas* is, because it is probably responsible only for phototaxis and need not differentiate fine shades of color. Will it be 11-*cis*-retinal, or all-*trans*-retinal, or some other molecule ?

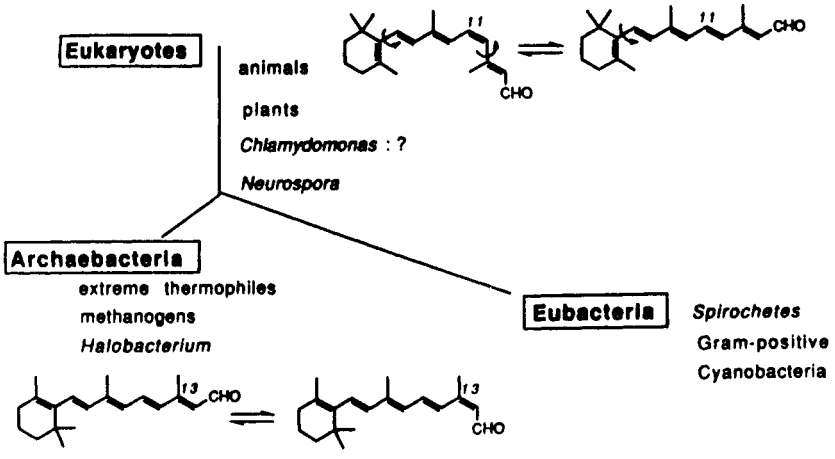


Fig.12. Evolutionary relation of retinal pigments.

Finally, the extended linear conjugation of the C_{20} retinal molecule is crucial for wavelength regulation covering a wide range. An ideal and economic source to provide this unique molecule having a trimethylcyclohexene moiety and methyl substituents for hydrophobic binding and other purposes, is to break the C_{40} β-carotene, a pigment occurring abundantly in Nature. Thus retinal became the ideal chromophore.

Acknowledgements

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