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Regeneration of Bovine and Octopus Opsins in Situ with Natural and Artificial Retinals†

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ABSTRACT: We consider the problem of color regulation in visual pigments for both bovine rhodopsin (λ_{max} = 500 nm) and octopus rhodopsin (λ_{max} = 475 nm). Both pigments have 11-*cis*-retinal (λ_{max} = 379 nm, in ethanol) as their chromophore. These rhodopsins were bleached in their native membranes, and the opsins were regenerated with natural and artificial chromophores. Both bovine and octopus opsins were regenerated with the 9-*cis*- and 11-*cis*-retinal isomers, but the octopus opsin was additionally regenerated with the 13-cis and all-trans isomers. Titration of the octopus opsin with 11-*cis*-retinal gave an extinction coefficient for octopus rhodopsin of 27 000 ± 3000 M^{-1} cm^{-1} at 475 nm. The absorption maxima of bovine artificial pigments formed by regenerating opsin with the 11-cis dihydro series of chromophores support a color regulation model for bovine rhodopsin in which the chromophore-binding site of the protein has two negative charges: one directly hydrogen bonded to the Schiff base nitrogen and another near carbon-13. Formation of octopus artificial pigments with both all-trans and 11-cis dihydro chromophores leads to a similar model for octopus rhodopsin and metarhodopsin: there are two negative charges in the chromophore-binding site, one directly hydrogen bonded to the Schiff base nitrogen and a second near carbon-13. The interaction of this second charge with the chromophore in octopus rhodopsin is weaker than in bovine, while in metarhodopsin it is as strong as in bovine.

Rhodopsin is an integral membrane protein participating in the light-transduction process that takes place in the photoreceptor cells of higher organisms. Current models of the process propose that incoming photons excite the light-sensitive rhodopsin, inducing protein conformation changes that initiate an enzymatic cascade resulting in the electrical excitation of the photoreceptor cell (Stryer, 1986; Pugh & Cobbs, 1986; Koutalos & Ebrey, 1986; Tsuda, 1987). In this vein the photochemistry of rhodopsin is of fundamental importance for understanding the visual process.

Rhodopsin is composed of a light-insensitive protein moiety and a light-sensitive chromophore, linked to it by a protonated Schiff base. The use of retinal (vitamin A aldehyde) as the chromophore by rhodopsins is almost universal, the few exceptions using closely related retinals. Even the light-sensitive proteins of some unicellular organisms, like Halobacteria (Oesterhelt & Stoeckenius, 1971) and Chlamydomonas (Foster et al., 1984, 1988; Nakanishi et al., 1989), use retinal.

Accordingly, the widely varying enzymatic and spectral properties of rhodopsins are to be explained by differences in the protein moiety and its interaction with the chromophore. Studies substituting artificial chromophores for the natural one in order to probe the chromophore-binding site are very useful. The type of artificial chromophores used in the present study are the dihydro series, which have already been used to

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study bacteriorhodopsin (Nakanishi et al., 1980; Balogh-Nair et al., 1981), and, in a preliminary way, vertebrate (bovine) rhodopsin in detergents (Arnaboldi et al., 1979; Honig et al., 1979). In the latter studies, 9-cis analogues were used due to ease of their synthesis. However, the 11-cis series of retinals have now been synthesized and have been bound to bovine rhodopsin in addition to octopus rhodopsin, in order to obtain a clearer picture of the alignment of charges within the binding site. This octopus study is the first to be done with these chromophores on an invertebrate rhodopsin. Invertebrate rhodopsins not only have quite different absorption maxima from vertebrate rhodopsins but different photochemistries as well. The initial photochemical event, the 11-cis to 11-trans isomerization, is the same for both. But, while the photoproducts of vertebrate rhodopsin are unstable and eventually hydrolyze to retinal and opsin, the final photoproduct of invertebrate rhodopsin is stable and the Schiff base remains unhydrolyzed.

A major experimental concern in studies with artificial chromophores is the testing of the legitimacy of the artificial pigments formed. Thus, the development of stringent criteria is important for studies with artificial chromophores and pigments and is presented in this work along with their application to some of the pigments.

Rationale. In brief, the rationale for using the dihydro series is that they provide a series of chromophores sterically similar to retinal but with different numbers of double bonds (Arnaboldi et al., 1979; Honig et al., 1979; Kakitani et al., 1985). This number determines the chromophore's absorption maximum. The change in the chromophore's absorption maximum in going from a protonated Schiff base in solution to the reconstituted pigment is a measure of the protein–chromophore interaction energy. This difference in energy is the opsin shift (Nakanishi et al., 1980). By collecting the data for different lengths of double bonds, the interactions can be localized around the chromophore structure.

Preliminary accounts of this work have been presented at the Annual Meeting of the Biophysical Society (Koutalos et al., 1988).

Materials and Methods

Membrane Preparation. Frozen bovine retinae were from Hormel (Austin, MN). Frozen octopus (*Paroctopus defleini*) eyeballs, dissected from octupi collected near Hokkaido, Japan, were shipped to Urbana for further processing. Bovine rod outer segment membranes were purified according to Papermaster and Dreyer (1974). Rhodopsin is the only detectable chromophore-containing pigment of this preparation. Octopus microvillar membranes were prepared according to Tsuda et al. (1986). The major contaminant of this preparation is retinochrome which has an all-trans chromophore (Hara & Hara, 1982). Chromophore extraction followed by quantitative HPLC analysis showed that the rhodopsin content of our preparation was between 93% and 95%. For this, a slight modification of Tsuda's (1982) method was employed: after extraction of chromophores as oximes, they were eluted from the μ-Porasil column with 2% isopentyl alcohol in hexane at a flow rate of 2.0 mL/min.

Bleaching. Slight modifications of standard procedures for bovine (Ebrey, 1982) and cephalopod (Seki et al., 1980) rhodopsins were employed. Membranes from either bovine or octopus were suspended in a 38% sucrose solution in 66 mM sodium/potassium phosphate buffer with 0.2 M hydroxylamine, pH = 6.5. For bovine membranes dithiothreitol was added to a final concentration of 10 mM. The rhodopsin concentrations were between 0.5 and 1.0 OD. The bleaching was carried out in an ice-cold bath in a dark room, using light of wavelengths longer than 480 nm for octopus and 520 nm for bovine (Corning cutoff filters 3-70 and 3-68, respectively). With a 400-W projector, bleaching was complete in 30 min for bovine and 60 min for octopus. The suspension was magnetically stirred throughout the procedure, and spectra were taken at regular time intervals. The membranes were then washed, at 4 °C, four times (JA-20 rotor in a J2-21 Beckman centrifuge, at 20*K* rpm for 30 min) with 66 mM sodium/potassium phosphate buffer, pH = 7.0, to remove the hydroxylamine. The pellet was then suspended to the same final sample volume as during bleaching, in 38% sucrose in the same buffer used for washes. This was used for regeneration with or without further dilution.

Chromophores. The all-trans series of 5,6-*trans*-dihydro-, 7,8-dihydro-, and 11,12-dihydroretinals were synthesized according to published procedures (Arnaboldi et al., 1979). The 11-*cis*-dihydroretinal series were obtained by condensation of the corresponding trans C_{15} aldehyde with the anion of (trimethylsilyl)acetone *tert*-butylimine (Croteau & Termini, 1983), elongation of the 11-cis and trans C_{18} ketones with diethyl (cyanomethyl)phosphonate, and reduction of the nitrile group with dibal followed by HPLC purification. The 11-*cis*-3,4-dehydroretinal was prepared from 3,4-dehydro-β-ionone which was synthesized according to Surmatis and Tommen (1967). 3,4-Dehydro-β-ionone was converted to all-trans and 9-*cis* C_{15} aldehyde through conventional Emmons reaction with triethyl phosphonoacetate (Wadsworth & Emmons, 1961), dibal reduction, MnO_2 oxidation, and flash chromatographic separations of isomers. The all-trans C_{15} aldehyde isomer gave the 11-*cis*-3,4-dehydroretinal according to the procedure described for the 11-*cis*-dihydroretinal series. The 11-*cis*-retinal was a gift from Professor R. Crouch; 9-cis, all-trans, and 13-cis were from Sigma. The purity of all chromophores used was confirmed with HPLC.

Spectral Measurements. The spectrophotometers used were a Cary 118 with opal glass for scattering reduction and an Aviv 14DS without opal glass but with an end on photomultiplier close to the sample cuvettes. The reference cuvette contained just buffer during the recording of spectra of the bleached sample and contained the same opsin solution as the sample cuvette for regeneration spectra.

Regeneration. Chromophores in ethanol, having a maximum volume of 20 μL, were added to the sample cuvette (2 mL). Since the dilution was less than 1%, spectra were not corrected for this small effect. Control experiments showed that at these concentrations ethanol does not significantly affect opsin regeneration capacity. The spectra of the regenerated opsins were recorded after sufficient time had elapsed for changes not to occur within the scanning time interval. Apart from the extinction coefficient measurements and the 11,12-dihydrorhodopsins, all regeneration spectra shown were recorded after the addition of 20 μL of 2.0 M hydroxylamine. All regeneration experiments were performed at room temperature.

Results

Criteria for Judging Artificial Pigment Legitimacy. Since the objective for the use of artificial pigments is to probe the native chromophore's binding site, a legitimate pigment is one where the artificial chromophore is occupying this particular site. Five criteria were developed to test this requirement:

(A) The pigment should have good regeneration efficiency. This is to ascertain that the peak is not due to minor impurities in the chromophore. In this vein it is particularly helpful to use the same opsin solution in both reference and sample

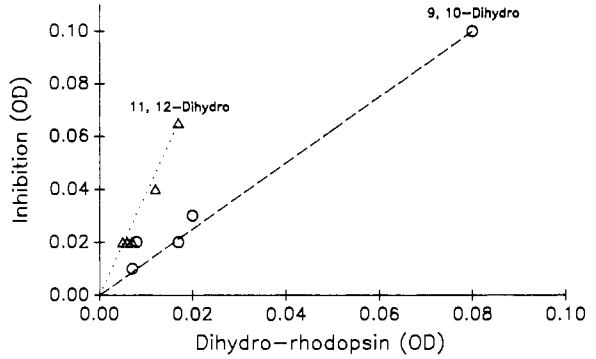


FIGURE 1: Inhibition of 11-cis bovine rhodopsin formation plotted vs the amount of dihydrorhodopsin formed: Approximate proportionality is exhibited between inhibition of 11-cis bovine rhodopsin formation and the amount of dihydro pigment present. The amount of dihydro pigment is measured as the absorbance (at 300 nm for the 11,12- and at 350 nm for the 9,10-dihydro) of the difference between the spectrum recorded immediately after dihydro chromophore addition and the spectrum recorded 2 h afterwards. Different amounts of dihydro chromophore were added, so that different amounts of pigments were formed within the 2-h periods. After the end of the 2-h period, 11-*cis*-retinal was added in excess (the OD ratio to opsin was about 2:1), and the amount of rhodopsin formed was measured at 500 nm after hydroxylamine addition. This was compared to the amount of rhodopsin formed when no artificial chromophore was added before the 11-cis addition, and the inhibition due to the artificial pigment formation was obtained.

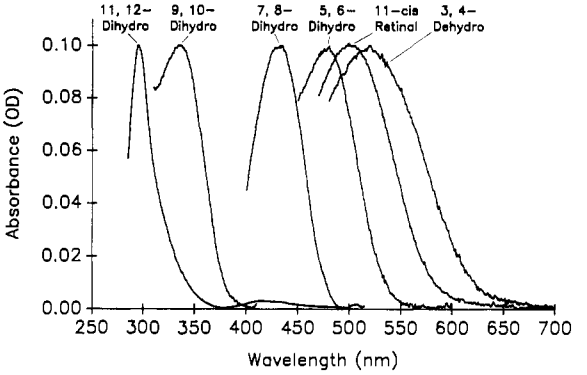


FIGURE 2: Spectra of bovine artificial and natural rhodopsins obtained by opsin regeneration in membranes with 11-cis chromophores (see text for details). The spectra have been normalized to the same maximum absorbance.

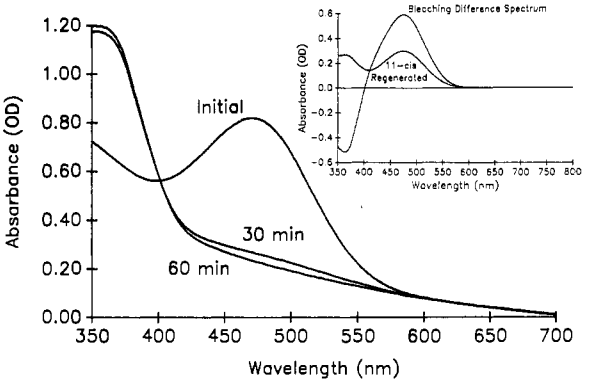


FIGURE 3: Octopus rhodopsin bleaching, carried out by using light of wavelengths longer than 480 nm. The labels of the spectra refer to the time duration of the light exposure ("Initial" is the rhodopsin spectrum before bleaching begins). Bleaching was complete within 60 min. The inset compares the bleaching difference spectrum (initial minus the 60-min spectrum) with the fully 11-cis regenerated opsin spectrum. The latter was obtained after the addition of excess 11-*cis*-retinal to opsin solution (the OD ratio to opsin is about 1:1), 1-hour incubation, and addition of hydroxylamine. The regeneration efficiency is 50%.

cuvettes so that minute spectral changes occurring during regeneration, but not due to added chromophore, are properly recorded. This criterion was satisfied by all the regenerated artificial pigments of this study.

(B) Relative resistance to hydroxylamine (as measured by the recording of the pigment spectra after the addition of hydroxylamine). This is to check for the contribution of random Schiff bases to the assigned peak. All regenerated pigments satisfied the criterion with the exception of the bovine and octopus 11,12-dihydrorhodopsins. The criterion is inapplicable for the 11,12 pigments because the absorbance of the products of the reaction of excess chromophore with hydroxylamine covers the region of interest.

(C) Under light exposure the pigment peak should shift (bleachability). This is inapplicable for the 11,12 pigments because their chromophore cannot isomerize around the 11,12 bond, and hence they cannot bleach (Gawinowicz et al., 1977). This criterion tests the chromophore-based as well as the protein-interactive character of the assigned peak. The bovine and octopus 9,10-dihydrorhodopsins were tested for and found to satisfy the criterion.

(D) No pigment formation, as judged by the appearance of the assigned peak, should be observed when artificial chromophore is added to unbleached membranes or membranes fully regenerated with 11-*cis*-retinal. This is an obvious requirement if the pigment is due to occupation of the native chromophore-binding site by the artificial one. This was tested for the bovine 9,10- and 11,12-dihydrorhodopsins and was found to be satisfied.

(E) Formation of 11-*cis*-rhodopsin should be proportionately inhibited by increasing amounts of artificial pigment formed. This is the most stringent criterion testing the occupation of the native chromophore site by the artificial one. This was tested for the bovine 9,10- and 11,12-dihydrorhodopsins and was found to hold (Figure 1).

It is evident that the emphasis of the application of the criteria was on the 9,10- and 11,12-dihydro pigments. The reason is that they are the most difficult to regenerate and detect (they absorb in the UV) and they are the crucial ones for simple point-charge models of the binding site.

Bovine Pigments. A comparison of the bleaching difference spectrum (i.e., the initial unbleached minus the final bleached) superimposed on the fully 11-*cis*-retinal regenerated opsin spectrum of samples having the same initial concentration of rhodopsin shows the regeneration efficiency to be 90%.

Figure 2 shows the spectra of the pigments formed with bovine opsin by six retinals of different "chromophore" length. They were all recorded after hydroxylamine addition (except for 11,12, to which no hydroxylamine was added). Hydroxylamine destroys random Schiff base formation (unrelated to rhodopsin's Schiff base binding site), which might broaden the pigment peaks. Table I gives the chromophore protonated Schiff base absorption maxima (the protonated *n*-butylamine Schiff base, with chloride anion as counterion, in methanol), the pigment maxima, and the opsin shifts.

Octopus Pigments. Figure 3 shows the spectra from the bleaching procedure for octopus rhodopsin. The inset compares the octopus bleaching difference spectrum with the fully 11-*cis*-retinal regenerated opsin spectrum. The regeneration efficiency in this experiment is 50%, though the maximum efficiency achieved is 60% (data not shown).

The incorporation of 11-*cis*-retinal into opsin, accompanied by Schiff base formation, is expected to be essentially irreversible. With this in mind, known quantities of retinal were

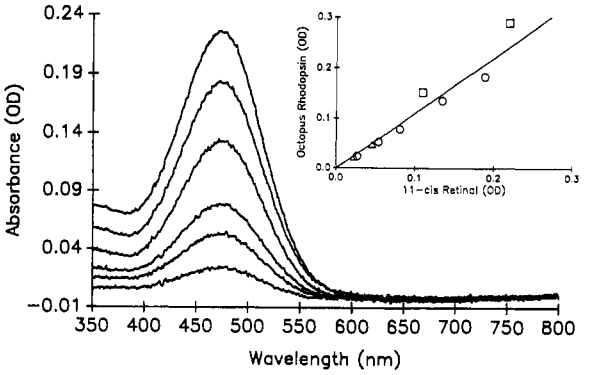


FIGURE 4: Titration of octopus opsin with 11-*cis*-retinal. 11-Cis was added in ethanol, the sample was incubated for 30 min (enough for stabilization), and the spectrum was recorded. The process was repeated until random Schiff base formation was detected from the shift of the pigment peak. The inset shows accumulated data from titration experiments. The slope of the straight line is the extinction coefficients' ratio of rhodopsin over retinal. Different symbols refer to different experiments.

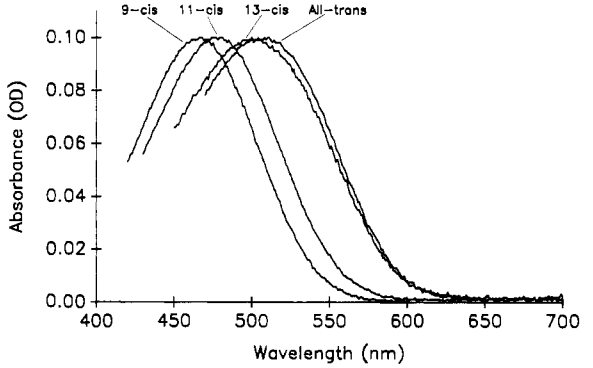


FIGURE 5: Octopus opsin regeneration in membranes with 9-*cis*, 11-*cis*, 13-*cis*, and *all-trans*-retinals. The spectra have been normalized to the same maximum absorbance.

added to an opsin sample stepwise, and the amount of pigment formed after each addition was recorded (Figure 4). The incubation times between additions were sufficiently long for all the chromophore to be incorporated. By plotting the amount of pigment formed versus the amount of 11-*cis*-retinal added, the ratio of the extinction coefficient of rhodopsin to retinal is obtained (inset of Figure 4). By use of an extinction coefficient of 25 000 M^{-1} cm^{-1} for 11-*cis*-retinal in ethanol (Hubbard et al., 1971), the rhodopsin coefficient is calculated to be 27 000 ± 3000 M^{-1} cm^{-1} at 475 nm. The error has been estimated from the scatter of the points in the plot in the inset of Figure 4.

Octopus opsin regenerations with the 9-*cis*-, 11-*cis*-, 13-*cis*-, and *all-trans*-retinal isomers are shown in Figure 5. The 9-cis isomer forms isorhodopsin (λ_{max} = 460 nm) while the all-trans isomer gives the stable photoproduct of rhodopsin, acid metarhodopsin [λ_{max} = 510 nm; since the pH is 7.0 and the p*K* of the acid–alkaline transition is about 9.5 (Cooper et al., 1986)]. It is unclear whether the 13-cis pigment (λ_{max} = 502 nm) is actually acid metarhodopsin or not (see Discussion). Nevertheless, in contrast to 11-cis, both 13-cis and all-trans do not regenerate very well, and a great excess of chromophore is required in order to incorporate them into the opsin within a few hours. The pigment spectra of the four isomers as well as the spectra of the dehydro and dihydro ones (except the 11,12-dihydro) were recorded after the addition of hydroxylamine, in order to eliminate the contribution of random Schiff bases. In the case of the all-trans pigments this resistance to hydroxylamine excludes the possibility of contribution from contaminating retinochrome, since the latter is hydroxylamine-labile (Hara & Hara, 1982).

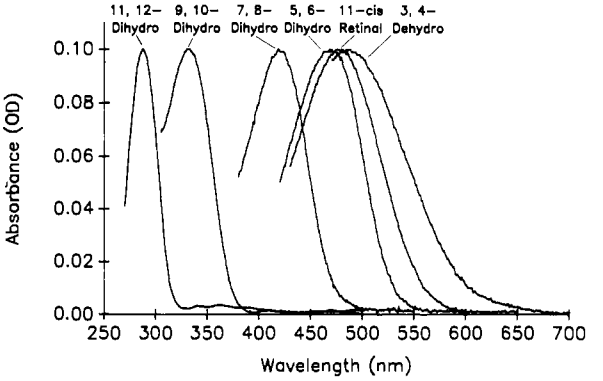


FIGURE 6: Octopus artificial and natural rhodopsins obtained by opsin regeneration in membranes with 11-cis chromophores (see text for details). The spectra have been normalized to the same maximum absorbance.

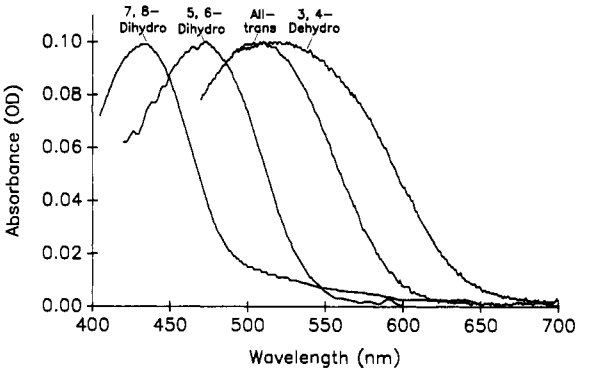


FIGURE 7: Octopus artificial and natural acid metarhodopsins obtained by opsin regeneration in membranes with all-trans chromophores. The spectra have been normalized to the same maximum absorbance.

Figure 6 shows the 11-cis dehydro and dihydro pigment spectra for octopus and Table II their opsin shifts. Figure 7 has the all-trans pigment spectra and Table III their opsin shifts. Though the 11,12-dihydro chromophore is free to isomerize around the 11,12 bond, the 11,12-dihydro pigment has been classified along with the 11-cis pigments. The reason for this is that 11-cis isomers regenerate with the octopus opsin far better than all-trans isomers, indicating that the preferred conformation for the 11,12-dihydro chromophore in the protein would be 11-cis.

Discussion

Extinction Coefficient of Octopus Rhodopsin. The method used to determine the extinction coefficient is ridden with two systematic errors for scattering samples that would lead to overestimation. The first, the high scattering background, was corrected by using opsin in the reference cuvette. The second, increase of the path length due to scattering inside the sample, cannot be corrected. But, since the samples were rather dilute, the overestimation is not expected to be more significant than the random errors (about 10%). The value of 27 000 M^{-1} cm^{-1} is in close agreement with Tsuda et al.'s (1982) value of 30 000 M^{-1} cm^{-1}, obtained for octopus rhodopsin in digitonin by a different method. It is interesting that the octopus extinction coefficient is much smaller than bovine's 40 000 M^{-1} cm^{-1} (Wald & Brown, 1953), though the biological significance of the ensuing difference in photon-capturing efficiency is not known.

13-Cis and All-Trans Pigments of Octopus. The incorporation of the all-trans isomer, the relative resistance to hy-

Table I: Bovine Rhodopsin Based Pigments (11-Cis Isomer)

chromophore	protonated Schiff base (nm)	pigment (in membranes) (nm)	opsin shift (cm^{-1})
3,4-deh, 11-cis	471	520	2000
11-cis	440	498	2700
5,6-dih(t), 11-cis	433	473	1950
7,8-dih, 11-cis	392	428	2200
9,10-dih, 11-cis	320	336	1600
11,12-dih	270	294	3000

droxylamine, and the 510-nm absorption maximum lead us to identify this pigment as metarhodopsin; nevertheless, the existence of different pigment conformers that share these properties cannot be ruled out. In the context of vertebrate photochemistry the regeneration of an opsin with *all-trans*-retinal is rather striking. For octopus, an invertebrate, it is a rather expected result, since there is a stable, all-*trans*-containing photoproduct, the metarhodopsin.

It is unclear whether the similarity in the spectra of the 13-*cis*- and *all-trans*-retinal-based pigments formed with octopus opsin is due to their being actually the same pigment or not. The 13-cis isomer is very stable and so it is being incorporated as such. Thus, any 13-cis to all-trans isomerization would have to take place in the binding site. A 13-cis pigment has been reported for octopus (Ohtsu & Kito, 1985), but its absorption spectrum is unknown. To decide on the matter, correlation of pigment spectra with chromophore extractions is required and this is technically very complicated.

The Chromophore-Binding Site. It is of interest to consider models that might account for the various opsin shifts shown in Tables I–III. Three general mechanisms for opsin shifts have been identified in previous studies of bacteriorhodopsin (Nakanishi et al., 1980; Harbison et al., 1985; Spudich et al., 1986; Lugtenburg et al., 1986): (a) weakened interaction (compared to the one in methanol) between the Schiff base and a negatively charged counterion positioned nearby; (b) charges or dipoles placed along the chain and near the β-ionone ring; (c) a planarized ring–chain conformation.

These explanations that have been proposed for pigment spectra were based on the simplest models that could account for the existing data. As new evidence has become available from spectroscopic measurements, structural information, and site-directed mutagenesis experiments, it has become necessary to refine or abandon earlier concepts. One important example is the idea of a negatively charged counterion which stabilizes the positive charge on the Schiff base. The counterion is usually assumed to correspond to a carboxylate which hydrogen bonds directly to the Schiff base and is the source of a negative electrostatic potential. Decreasing this potential should both red shift the spectrum and decrease the affinity of the site for a proton, that is, decrease the apparent pK of the Schiff base.

However, the Schiff base may interact with an environment that is more complex than a single carboxylate group (Birge et al., 1988; Deng & Callender, 1987; Gilson & Honig, 1988). For example, it has been proposed that the nitrogen is hydrogen bonded to a polar but neutral group [such as a bound water (Hildebrandt & Stockburger, 1984; Birge et al., 1988)] and that a nearby carboxylate stabilizes the Schiff base but does not hydrogen bond to it directly. Indeed, it may be possible that in some pigments there is no carboxylate near the nitrogen and that rather a group of dipoles, or possibly the end of an α-helix (Honig, 1987), stabilizes the positive charge on the Schiff base. Recent evidence from mutagenesis studies of bacteriorhodopsin indicates that several different charged groups have relatively small (less than 1000 cm^{-1}) spectroscopic effects, but no single dominant interaction has been identified (Mogi et al., 1988). This could be taken to imply that charged groups play only a secondary role or that several of them act in concert or that one group can substitute for another. In either case, the effect of mutating a single group would be smaller than expected from simple arguments.

Table II: Octopus Rhodopsin Based Pigments (11-Cis Isomer)

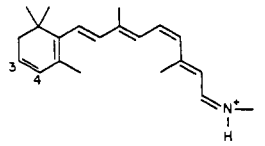
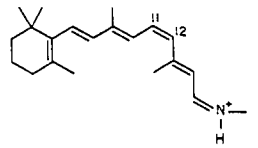
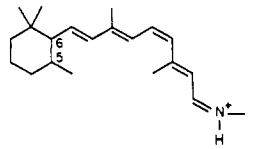
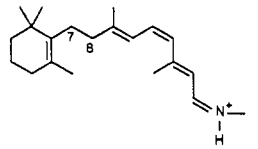
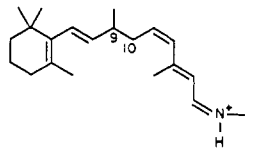
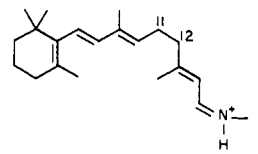
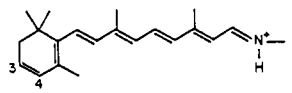
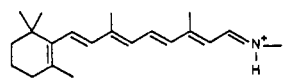
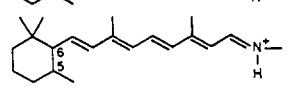
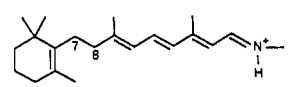
chromophore		protonated Schiff base (nm)	pigment (in membranes) (nm)	opsin shift (cm^{-1})
	3,4-deh, 11-cis	471	486	700
	11-cis	440	475	1700
	5,6-dih(t), 11-cis	433	464	1500
	7,8-dih, 11-cis	392	417	1500
	9,10-dih, 11-cis	320	332	900
	11,12-dih	270	286	2000

Table III: Octopus Rhodopsin Based Pigments (All-Trans Isomer)

chromophore		protonated Schiff base (nm)	pigment (in membranes) (nm)	opsin shift (cm^{-1})
	3,4-deh, 11-trans	471	520	2000
	11-trans	445	510	2900
	5,6-dih(t), 11-trans	430	471	2100
	7,8-dih, 11-trans	392	433	2400

Complexities such as these must be kept in mind in analyzing the data presented in the tables.

An implicit assumption for drawing inferences about the chromophore-binding site from the data in Tables I–III is that each of the chromophores has the same conformation as the natural one and they have kept the protein structure the same. This is reasonable for bovine, since resonance Raman data (Mathies et al., 1976) suggests that the rhodopsin-bound chromophore is as unstrained as in solution, as far as C–C stretches are considered. Similar experiments for octopus (Pande et al., 1987; Bagley et al., 1989) suggest a strained chromophore with respect to C–C stretches, questioning the validity of the assumption in this case.

For bovine 11-cis pigments, it is evident from Table I and Figure 8 that the opsin shifts are all approximately 2000 cm^{-1}. The largest shifts correspond to those of 11-*cis*-retinal itself (2700 cm^{-1}) and the 11,12-dihydro. The simplest, but probably incorrect, interpretation of the data is that in all bovine 11-cis pigments there is a weakened interaction of the Schiff base with a negative electrostatic potential (weakened with respect to the Schiff base in methanol), which accounts for the nearly uniform opsin shifts. If this model were correct, the somewhat

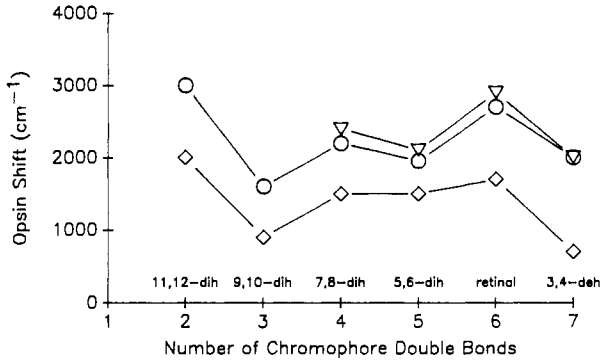


FIGURE 8: Plot of the opsin shifts from Tables I–III vs the number of chromophore double bonds. This way of plotting facilitates comparisons between the different pigments (see text for details). (○) Bovine rhodopsin; (◇) octopus rhodopsin; (▽) octopus metarhodopsin.

larger shift of the native chromophore relative to the dihydro chromophores might be attributed to a more planar ring-chain conformation for the former than the one that exists in solution [the effect could not be very large, since NMR data suggest that the conformation of the ring in bovine rhodopsin is still 6-s-cis (Smith et al., 1987)]. However, there are a number of reasons to suggest that a weakened electrostatic interaction is not the dominant effect in bovine 11-cis pigments and, rather, that a charged group near the middle of the polyene chain is responsible for the bulk of the opsin shift.

It should first be pointed out that, in its simplest form, the idea of a weakened protonated Schiff base–carboxylate interaction can be ruled out by the Raman data which show that the Schiff base in rhodopsin is strongly hydrogen bonded (Baasov et al., 1987; Deng & Callender, 1987; Gilson & Honig, 1988). However, the Raman data are consistent with two other, more complex, possibilities: (a) The Schiff base is hydrogen bonded to a neutral group, and the opsin shift is due to a weak electrostatic interaction with a non-hydrogen-bonding carboxylate. (b) The Schiff base is strongly hydrogen bonded to a neutral group but does not interact with a carboxylate. In this case, electrostatic stabilization of the positive charge is provided by one or more oriented dipoles [e.g., from an α-helix (Honig, 1987)]. The opsin shift, as in case a, is due to a weak electrostatic interaction of the Schiff base, in this case with the surrounding dipoles.

As noted above, the simplest interpretation of the Raman data requires a second negative charge electrostatic interaction to explain the opsin shift. Other evidence for such an interaction along the polyene chain comes from the opsin shift of the 11,12-dihydro, which is about 3000 cm^{-1}, significantly larger than the shifts of the other dihydroretinals. The magnitude of this shift suggests the presence of an interaction in the middle of the chain, close to carbon-13. ^{13}C NMR studies have recently provided supporting evidence for the existence of an electrostatic interaction in this region (Mollevanger et al., 1987; Smith et al., 1988), as have the model compound studies of Baasov et al. (1987).

Finally, another kind of evidence that a negative charge along the chain is an important determinant of wavelength regulation comes from a comparison of the 11-cis pigment with the 9-cis pigment. Purely on the basis of the slightly lower Schiff base frequency and the smaller deuterium shift (Palings et al., 1987), one might expect that the 9-cis pigment would absorb at the same or longer wavelengths than the 11-cis pigment, but bovine (and octopus) isorhodopsin absorbs at shorter wavelengths (485 nm; 460 nm for octopus). The simplest explanation for this difference is that in the 11-cis pigment the second negative charge is closer to the polyene chain that in the 9-cis, and hence the 9-cis pigment spectrum is blue shifted.

Thus, the current data are in agreement with the preliminary results for opsin shifts for 9-cis pigments in detergent (Honig et al., 1979), and the original external point charge model, which places a negative charge near the middle of the polyene chain, appears to provide the simplest means of accounting for them. This negative charge could be due to a single carboxylate but might also represent the effect of oriented dipoles such as the potential due to an α-helix. With regard to the environment around the Schiff base, it is clear that the nitrogen is strongly hydrogen bonded, but whether this is due to a carboxylate or to a dipolar group is uncertain. In any case, it appears that, in contrast to bacteriorhodopsin, the Schiff base interacts with a negative potential similar to that felt by model compounds in methanol.

For octopus pigments there have been far fewer studies than for bovine and so the amount of data is significantly limited. Nevertheless, simple point-charge models for color regulation may be constructed along similar lines as for bovine. The 1655-cm^{-1} stretching frequency of the C=N bond in octopus rhodopsin and acid metarhodopsin (Kitagawa & Tsuda, 1980; Pande et al., 1987) is about the same as in bovine (Oseroff & Callender, 1974), implying that the Schiff base is strongly hydrogen bonded in both octopus pigments.

The pattern of the opsin shifts for the 11-cis octopus pigments is similar to bovine's (Figure 8), though their absolute magnitude is significantly less. Thus, the simplest model for octopus rhodopsin would have a Schiff base strongly hydrogen bonded to the counterion, and the opsin shift would be due to the interaction with a negative charge near the middle of the polyene chain. Furthermore, this interaction should be weaker than in bovine rhodopsin because the opsin shift is smaller. Finally, the small opsin shift for the 3,4-dehydroretinal suggests a perturbation near the 5,6 bond, but the effect is not big enough to evoke a positive charge in order to account for it.

For the all-trans octopus pigments the available data indicate a similar pattern and a remarkably similar magnitude of opsin shifts as for the bovine 11-cis pigments (Figure 8). So, the simplest model for octopus metarhodopsin would have a Schiff base strongly hydrogen bonded to the counterion, and the opsin shift would be due to the interaction with a negative charge near the middle of the polyene chain. This interaction should be approximately as strong as in bovine rhodopsin since the opsin shifts are almost the same.

Similar studies on the chicken cone pigment iodopsin (λ_{max} = 563 nm) show an opsin shift pattern similar to bacteriorhodopsin's (Chen et al., 1989). This indicates that a color regulation model for iodopsin may include electrostatic interactions close to the ionone ring, as in bacteriorhodopsin.

Though the simplicity of the external point-charge model makes it particularly appealing, it is unlikely that in the long run it will be able to explain all of the data about the protein–chromophore interaction. Its main weakness seems to be the assumption of a simple (i.e., the same as in methanol) Schiff base–counterion interaction, which permits direct comparisons of the pigments with their chromophores in methanol. Nevertheless, for bovine rhodopsin the large deuterium shift of the C=N stretch suggests a different interaction than the one in methanol (Palings et al., 1987). Furthermore, the different p*K*s of octopus rhodopsin and acid metarhodopsin (Koutalos and Ebrey, unpublished results) hint toward a similar conceptual problem there, too. In the case of bacteriorhodopsin, interpretation of the dihydro chromo-

phore and Raman data according to this simple point-charge model (i.e., with the counterion directly hydrogen bonded to the Schiff base nitrogen) suggests a weakened Schiff base nitrogen–counterion interaction. This predicts a Schiff base pK lower than the one in solution, whereas the experimentally measured one is 6 pH units higher (Sheves et al., 1986).

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