Role of the 9-Methyl Group of Retinal in Cone Visual Pigments†

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ABSTRACT: In rhodopsin, the 9-methyl group of retinal has previously been identified as being critical in linking the ligand isomerization with the subsequent protein conformational changes that result in the activation of its G protein, transducin. Here, we report studies on the role of this methyl group in the salamander rod and cone pigments. Pigments were generated by combining proteins expressed in COS cells with 11-*cis* 9-demethyl retinal, where the 9-methyl group on the polyene chain has been deleted. The absorption spectra of all pigments were blue-shifted. The red cone and blue cone/green rod pigments were unstable to hydroxylamine; whereas, the rhodopsin and UV cone pigments were stable. The lack of the 9-methyl group of the chromophore did not affect the ability of the red cone and blue cone/green rod pigments to activate transducin. On the other hand, with the rhodopsin and UV cone pigments, activation was diminished. Interestingly, the red cone pigment containing the retinal analogue remained active longer than the native pigment. Thus, the 9-methyl group of retinal is not important in the activation pathway of the red cone and blue cone/green rod pigments. However, for the red cone pigment, the 9-methyl group of retinal appears to be critical in the deactivation pathway.

Visual pigment proteins are members of the superfamily of G protein-coupled receptors (GPCRs).[1] Rhodopsin, the photosensitive pigment in retinal rod photoreceptor cells, is arguably the best-studied GPCR and is the only member with a crystal structure (*1*). Its ligand, 11-*cis* retinal (A1 retinal, Figure 1), is covalently bound to the protein through a protonated Schiff base linkage to a lysine in the seventh transmembrane helix. Light activates rhodopsin by inducing isomerization of the 11-*cis* retinal chromophore to the all-*trans* form. This isomerization invokes a series of conformational changes in the protein, eventually forming the Metarhodopsin II (Meta II) state (*2*), which activates the G protein transducin. The cone photoreceptor opsins are also seven helical transmembrane proteins with the same photoactivatable A1 retinal ligand covalently bound.

The ligand of these opsins is critical to the control of both the absorption spectrum and activation state of these proteins. A1 retinal in the 11-*cis* form acts as an inverse agonist, holding these GPCRs in an inactive state. The all-*trans* form acts as an agonist, placing the protein in an active conformation. The interaction of the ligand with red rod opsin (rhodopsin), which is the most easily obtained of the opsins, has been a widely studied subject (for recent reviews, see refs *3* and *4*). A number of studies have examined the role of the 9-methyl group of retinal by using the retinal analogue, 9-demethyl (9-DM) retinal (Figure 1), in which the methyl group at the 9 position on the polyene chain has been eliminated (*5*−*10*). In vitro studies have shown that a stable pigment can be formed between this analogue and red rod opsin, although the absorption maximum of the pigment is blue-shifted (*5*) from that of the native pigment containing A1 retinal. Several groups have demonstrated that bovine rod opsin regenerated with 9-DM retinal is much less efficient at activating transducin than native rhodopsin (*6*, *10*, *11*). Han et al. (*8*) have proposed a "steric trigger" role for the 9-methyl group, where the conformational change to the active state was driven by steric interactions between this methyl group and specific amino acid residues on the rod opsin. However, removal of this methyl group did not abolish the ability of rhodopsin to activate transducin. Meyer et al. (*10*) and Vogel et al. (*9*) concluded that the decreased efficiency of activating transducin with 9-DM retinal regenerated rhodopsin was due to an inability to efficiently form

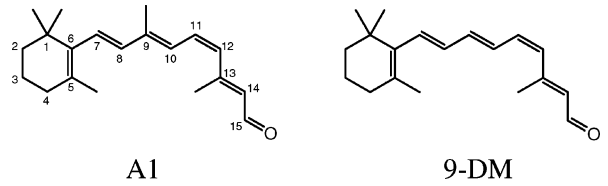

FIGURE 1: Structures of the 11-*cis* retinals used in this paper. A1 retinal is the native 11-*cis* retinal chromophore; 9-DM retinal is an analogue lacking the methyl group at the 9-carbon position along the polyene chain.

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[1] Abbreviations: GPCR, G protein-coupled receptor; A1 retinal, 11-*cis* retinal; 9-DM retinal, 11-*cis* 9-demethyl retinal; Meta II, metarhodopsin II; Meta I, metarhodopsin I; A2 retinal, 11-*cis* 3,4-dehydro retinal.

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the active Meta II species by stabilizing its precursor, Meta I.

We wanted to determine whether cone pigment activation was similarly inhibited by the absence of the 9-methyl group of retinal. We, therefore, generated A1 and 9-DM retinal pigments with all of the cloned salamander opsins and compared their spectral and activation properties. We found that all of the pigments containing 9-DM retinal are able to activate transducin in a light-dependent manner, but the efficiency in activation and deactivation varies depending on the opsin. In particular, the red cone pigment behaves very differently from rhodopsin. These results suggest that, in contrast to rhodopsin, there is no steric role of the 9-methyl group of the chromophore in forming the active Meta II intermediate in cone pigments.

EXPERIMENTAL PROCEDURES

Retinals. 11-*cis* A1 retinal was synthesized as previously described (*12*), purified by crystallization, and stored as a yellow crystalline solid under argon at −80 °C until use. The 11-*cis* 9-DM retinal was synthesized by the method of Kropf et al. (*5*), purified by high-pressure liquid chromatography, and stored as above. The structures of all of the compounds were confirmed by NMR.

Pigment Expression. The salamander cDNAs for red rod rhodopsin (*13*), blue cone (*14*), red cone (*14*) and UV cone (*15*) opsins were cloned as described previously. All four pigment cDNAs were inserted into the pMT3 expression vector (*16*) as an *Eco*RI−*Not*I cassette. The cDNAs were modified at the 3′ end with the addition of a sequence that encodes the carboxyl terminal eight amino acids of bovine rhodopsin (ETSQVAPA), the 1D4 epitope (*17*). Procedures for transfection of COS cells, reconstitution of the pigments with retinal, solubilization of the COS cell membranes with 1% dodecyl maltoside, and purification of the proteins by immunoaffinity chromatography on the 1D4-Sepharose 4B matrix have been described previously (*15*, *18*, *19*). The 1D4 antibody was purchased from the National Cell Culture Center (Minneapolis, MN). The only difference was the use of 10 mM MES buffer with 150 mM NaCl at pH 6.0 for solubilization and elution.

Pigment Absorbance Spectra. UV−visible absorption spectra were recorded using a Hitachi model U-3210, a Hewlett−Packard HP-8452A diode array, or a Varian Cary 300 spectrophotometer. All spectra were recorded on samples with a 1.0-cm path length in thermostated cell holders with the temperature maintained at 4 °C unless stated otherwise. All spectra were recorded with the pigments in 0.1% dodecyl maltoside and 10 mM MES buffer with 150 mM NaCl at pH 6.0.

Pigment Sensitivity to Hydroxylamine. Pigment sensitivity to hydroxylamine was measured as described by Ma et al. (*20*). Freshly prepared hydroxylamine was added to the pigment solutions such that the final concentration of hydroxylamine was 50 mM at pH 7.0 in 100 μL. Samples were kept in the dark, and the temperature was maintained at 4 °C. Spectra were recorded at several time intervals after the addition of hydroxylamine to follow the loss of pigment and formation of the oxime for all of the pigments except for the UV cone pigment. For the UV cone pigment, the protein was acid-denatured by adding concentrated hydrochloric acid (2 μL, 12 N) after incubation for 1 h with hydroxylamine, trapping the remaining bound chromophore as a protonated Schiff base free in solution and red-shifting the spectrum to about 440 nm. This absorbance was then compared with the absorbance of the same sample that was also acid-denatured but had never been incubated with hydroxylamine to determine the amount of pigment remaining after the incubation for 1 h with hydroxylamine to roughly estimate its resistance to hydroxylamine attack.

Transducin Activation Assay. Light-dependent activation of bovine rod transducin was assessed by following the binding of [^{35}S]GTPγS as described previously (*21*). Transducin was purified from bovine retina (W. L. Lawson, Lincoln, NE) essentially according to Wessling-Resnick and Johnson (*22*) and Baehr and co-workers (*23*). Concentration of the pigments was determined by comparing the absorbance at 440 nm of the acid-denatured spectrum of the pigments with 440 nm absorbance of the acid-denatured spectrum of a known amount of bovine rhodopsin. The reaction mixture consisted of 5 nM pigment, 2.5 μM transducin, and 3 μM GTPγS in 0.01% dodecyl maltoside, 10 mM Tris buffer, 100 mM NaCl, 5 mM $MgCl_2$, and 0.1 mM EDTA at pH 7.5. The 10-μL aliquots were spotted onto filter membranes on a vacuum manifold and washed three times with 4 mL of 10 mM Tris buffer, 100 mM NaCl, and 5 mM $MgCl_2$ at pH 7.5. The amount of GTPγS bound was determined by counting the radioactivity on the filters. Data were plotted as the number of moles GTPγS bound per mole of pigment as a function of time. All samples were bleached for 10 s with 300-W white light from a slide projector. Assays were conducted under dim red light conditions.

Meta II Decay. Meta II decay of the A1 and 9-DM retinal red cone pigments was determined by bleaching the pigments with long wavelength light (>500 nm) for 10 s in the presence of a 20-fold excess of A1 retinal and following the time course of A1 retinal pigment regeneration by monitoring at 560 nm. This procedure is a slight modification of the measurements made by Gross et al. (*24*), where the rate of bovine rhodopsin Meta II decay was determined by following the regeneration of rhodopsin upon addition of A1 retinal immediately after photobleaching. The rate-limiting step for rhodopsin regeneration under these conditions is the decay of Meta II and hydrolysis of the all-*trans* chromophore. In our measurements, we assumed that the rate-limiting step for red cone pigment regeneration was the same.

RESULTS

Spectral Properties of the Pigments. The salamander red rod (rhodopsin), red cone, blue cone/green rod, and UV cone opsins were expressed in COS-1 cells. The opsin in the salamander blue cone and green rod has previously been shown to be the same (*20*). Pigments from all four opsins were generated with both A1 and 9-DM retinals. The absorption spectra and maxima for the A1 retinal pigments are shown in Figure 2A. We have reported the absorption spectra of recombinant salamander UV cone (*15*) and blue cone/green rod (*20*) pigments containing A1 retinal. These results are in agreement with the spectral-sensitivity measurements of salamander red rod, red cone, and blue cone photoreceptor cells by Makino and co-workers (*25*). The spectra and absorption maxima for the 9-DM retinal pigments

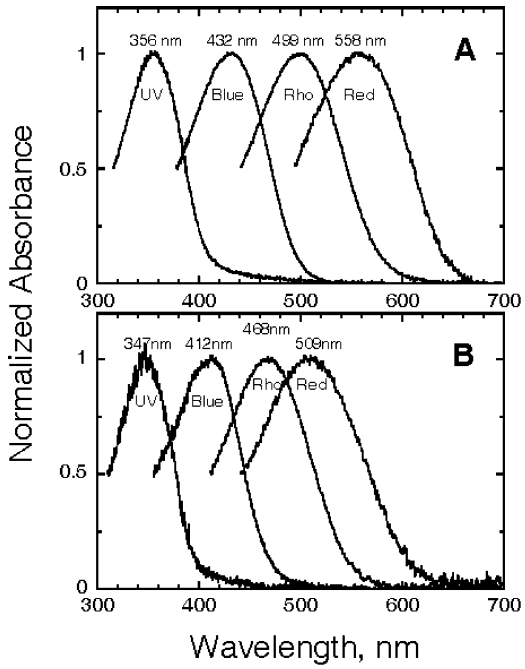

FIGURE 2: Absorption spectra of recombinant salamander rod and cone pigments. Pigments were generated with (A) 11-*cis* A1 retinal or (B) 11-*cis* 9-DM retinal. The absorption maximum of each pigment is printed above its respective spectrum, and the pigment type is also noted. The pigment denoted as the blue cone pigment has also been shown to be the same pigment as the green rod pigment in the tiger salamander (*20*). All pigments were acid-denatured to verify the formation of the covalent Schiff base linkage of the chromophore and opsin (not shown).

Table 1: Absorption Maxima of Recombinant Salamander Visual Pigments Regenerated with Various Retinals

	retinal		
pigment	A1 (nm)	9-DM (nm)	A2 (nm)
red rod	499	468	520[a]
red cone	558	509	nd[b]
blue cone/green rod	432[a]	412	440[a]
UV cone	356[c]	347	360[c]

[a] From ref *20*. [b] nd = not determined. [c] From ref *15*.

are shown in Figure 2B. They are all blue-shifted from those of the A1 retinal pigments. Covalent linkage of the retinal analogue to the protein was confirmed by acid denaturation for each opsin (data not shown). The absorption maxima for all of these pigments, as well as our data for the pure A2 (11-*cis* 3,4-dehydro retinal) pigments, are summarized for comparison in Table 1.

We have previously shown that the A1 retinal containing salamander rhodopsin pigment is stable in hydroxylamine; whereas, the blue cone/green rod pigment is not (*20*). Likewise, we find that the 9-DM retinal containing rhodopsin was quite stable in hydroxylamine, and the 9-DM retinal containing blue cone/green rod pigment was unstable with an exponential time constant for pigment loss of 10 min at 4 °C (data not shown). Both red cone pigments are also sensitive to hydroxylamine attack because the pigment was lost with exponential time constants of 18 and 10 min for the A1 and 9-DM retinal containing pigments, respectively (data not shown). Because of the spectral overlap between the UV cone pigments and oximes, we determined the amount of pigment remaining after incubation for 1 h with hydroxylamine by denaturing the samples with concentrated HCl. About 80−90% of the pigment remained after incubation for 1 h with hydroxylamine at 4 °C for UV cone pigments containing either chromophore (data not shown). The stability of the UV pigment was intriguing and could be due to the chromophore being buried in a protected environment such as in rhodopsin. This stability could also be due to the chromophore being bound as an unprotonated Schiff base because recent studies have suggested that retinal is bound as an unprotonated Schiff base in UV cone pigments (*15*, *26*−*29*).

Transducin Activation. All of the salamander pigments demonstrated the ability to activate transducin in a light-dependent manner. Light-dependent transducin activation was fit with a single exponential. The exponential rise is due to the exponential decay of Meta II from the pigment during the assay, and thus the rate constant from the fit should equal the rate of Meta II decay. We assumed a pseudo-first-order reaction where the transducin activation rate at any given time was dependent on a rate constant for transducin activation and the concentration of the active pigment, which is decaying with time. Integrating this results in an exponential rise function with an amplitude equal to the ratio of the rates of transducin activation and Meta II decay. This function also describes the data in Figure 3. Thus, from curve fits of Figure 3, the initial rate of transducin activation can be deduced by multiplying the amplitude by the Meta II decay rate constant, both of which are determined from a single exponential fit. The results are summarized in Table 2. The Meta II decay rate for the A1 salamander rhodopsin seems a little fast at 0.28 min^{-1}, but it is comparable to the decay rate of 0.27 min^{-1} for wild-type bovine rhodopsin found by Gross et al. (*24*), who noted that Meta II decay seems to be sensitive to the experimental conditions. Also shown in Table 2 are the Meta II decay rates determined for the red cone pigments by monitoring the rates of pigment regeneration (see below).

Activation by 9-DM retinal rhodopsin is lower than activation by A1 retinal rhodopsin (Figure 3A and Table 2). The smaller activity is similar to but not as dramatically reduced as the decreased activity seen with bovine rhodopsin regenerated with 9-DM retinal (*6*, *8*, *10*). In contrast to these results, transducin activation by the red cone pigment containing 9-DM retinal is not inhibited (Figure 3B and Table 2). Interestingly, the overall plot shows that much more transducin is activated by the 9-DM retinal pigment. This result is consistent with the fast formation of Meta II followed by an unexpected prolonged Meta II lifetime with the 9-DM retinal red cone pigment (see below and the Discussion).

Transducin activation by the blue cone/green rod pigment containing 9-DM retinal is neither inhibited nor prolonged (Figure 3C). We have previously shown that the UV cone pigment with A1 retinal is able to activate transducin, albeit at lower levels than rhodopsin, in a light-dependent manner (*15*). The 9-DM retinal UV cone pigment is also able to activate transducin and display the lowest levels of transducin activation among the pigments measured (Figure 3D).

Prolonged Meta II in the 9-DM Retinal Red Cone Pigment. Unlike with rhodopsin where the bleached 9-DM retinal pigment results in an inefficient formation of the Meta II intermediate (*9*, *10*), the red cone pigment containing 9-DM retinal readily forms a spectral Meta II (Figure 4A). At

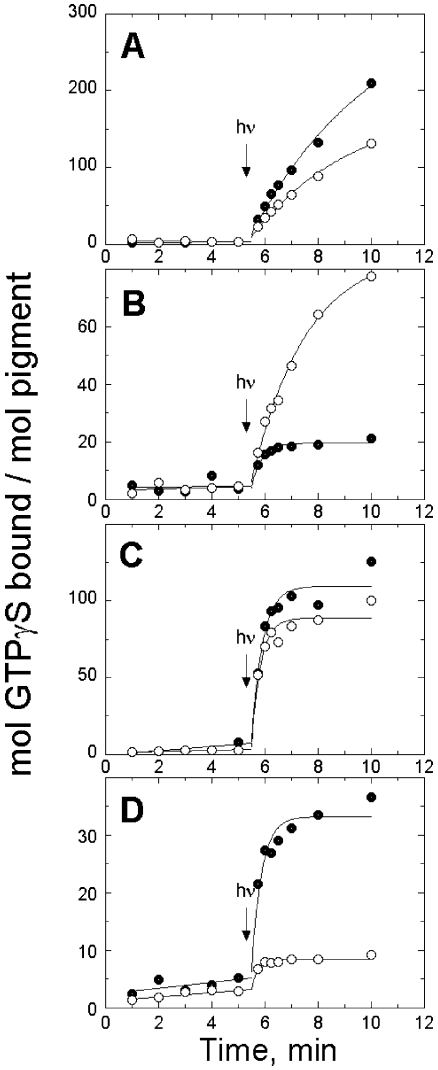

FIGURE 3: Transducin activation by salamander rod and cone pigments. A radioactive filter binding assay was used to follow the ability of salamander (A) rhodopsin, (B) red cone, (C) blue cone/green rod, and (D) UV cone pigments to activate bovine rod transducin in a light-dependent manner. (●) represents transducin activation by pigments containing A1 retinal. (○) represents transducin activation by pigments containing 9-DM retinal. The activity was normalized to the amount of pigment.

Table 2: Initial Transducin Activation and Meta II Decay Rates

	transducin activation rate (min^{-1})		Meta II decay rate (min^{-1})		Meta II decay rate (min^{-1})[a]	
	A1	9-DM	A1	9-DM	A1	9-DM
rhodopsin	70	44	0.28	0.34		
red cone	43	44	3.3	0.59	5.0	0.40
blue cone	200	206	2.5	3.3		
UV cone	58	10	2.5	2.5		

[a] Decay rates were determined by measuring the rate of pigment regeneration (see Figure 4).

20 °C, the rate constant for the decay of Meta II of the A1 retinal red cone pigment is 5 min^{-1}; whereas, the rate constant for the decay of Meta II of the 9-DM retinal red cone pigment is 0.4 min^{-1} (parts B and C of Figure 4). These values are in agreement with the Meta II decay rates estimated from the transducin activation assays (Table 2). As mentioned in the Experimental Procedures, we made the assumption that the rate of pigment regeneration after

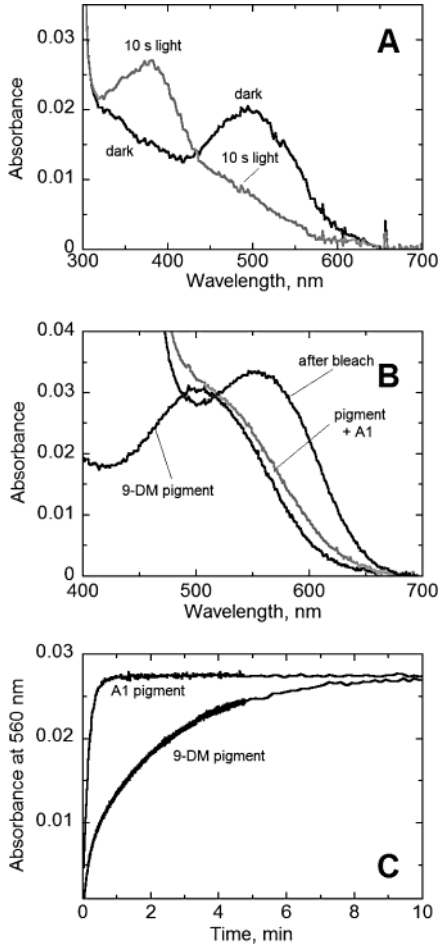

FIGURE 4: Meta II decay of the salamander red cone pigment containing either the A1 retinal or 9-DM retinal. (A) Absorption spectra for the dark and photobleached 9-DM retinal red cone pigment. The 9-DM red cone pigment readily forms Meta II. (B) Absorption spectra of the purified 9-DM retinal red cone pigment, the same pigment after the addition of an excess of A1 retinal, and the A1 regenerated pigment after photobleaching the 9-DM retinal red cone pigment in the presence of A1 retinal. (C) Time course of A1 pigment regeneration following a 10-s pulse of light (>500 nm) to 9-DM or A1 retinal red cone pigments in the presence of an excess of A1 retinal is shown as function of time after illumination as illustrated in B.

photobleaching reflected the rate of Meta II decay. Although we did not directly measure the rate that retinal binds to the opsin, A1 retinal binds to the photobleached product of both red cone pigments; the same pigment is being regenerated but with different rates. Thus, retinal binding is clearly not rate-limiting for the bleached 9-DM retinal red cone pigment. The pigments were bleached in the presence of A1 retinal because Meta II decay is so fast in cone pigments (*30*, *31*) and illuminated with long wavelength light (>500 nm) to photobleach the pigment and keep the free retinal untouched and able to regenerate an A1 red cone pigment. Because there is an excess of free chromophore, bleaches could be repeated several times in succession with the same sample. As illustrated in Figure 4B, we start with 9-DM retinal red cone pigment and add a 20-fold excess of A1 retinal. At this point, it is evident that some A1 retinal pigment is formed in the dark because the absorbance above 550 nm increases (Figure 4C). The A1 pigment formed is likely due to some chromophore exchange taking place in the dark (*32*, *33*). The sample is then illuminated with long wavelength light. When

the all-*trans* 9-DM retinal is released, the free A1 retinal binds to the opsin to form the A1 retinal red cone pigment. Regeneration of the A1 retinal pigment following the first photobleach can be monitored at 560 nm as a function of time (Figure 4C) and reflects the time course for the hydrolysis of all-*trans* 9-DM retinal as Meta II decays. Pigment regeneration after subsequent photobleaches reflects Meta II decay of the A1 retinal pigment and is much faster than the first regeneration. The kinetics for Meta II decay could essentially be fit with a single exponential. A double exponential fit of the full time course of red cone pigment regeneration from bleaching the 9-DM retinal red cone pigment resulted in a better fit because of the contribution from the small amount of A1 retinal pigment that had regenerated in the dark. This was not observed when the red cone pigment was photobleached in the presence of an excess of the identical chromophore.

DISCUSSION

We have successfully purified all four of the tiger salamander rod and cone visual pigments containing the 11-*cis* forms of A1 and 9-DM retinal. The 9-DM retinal chromophore does not greatly alter the stability of any of the pigments in the dark. The role of the 9-methyl group of retinal has its most profound effect after photoisomerization. All of the pigments containing either chromophore are able to activate transducin in a light-dependent manner (Figure 3). The amount of transducin activated is a function of how fast Meta II forms, how long it remains, and how efficiently it activates transducin. The presence or absence of the 9-methyl group on the chromophore effects the formation and/or decay of the active Meta II species of rhodopsin and the red cone pigment.

The most intriguing results in this paper are those that involve the red cone pigment. The absence of the 9-methyl group of retinal does not inhibit the formation of Meta II with the red cone pigment, as judged by the initial rate of transducin activation (Figure 3), unlike with rhodopsin. We see no evidence for an equilibrium shift favoring the Meta I intermediate with the red cone pigment containing 9-DM retinal (Figure 4A). What did change was the lifetime of the active intermediate of the red cone pigment. 9-DM retinal in the red cone pigment dramatically prolongs the active Meta II species. Because Meta II remains for several minutes, transducin continues to be activated (Figure 3). In a separate experiment, we confirmed the presence of the longer-lived active pigment by determining how fast the all-*trans* chromophore is released from the protein by following the regeneration of the 11-*cis* pigment after photobleaching (Figure 4). These results suggest that the 9-methyl group may play a steric role in the efficient hydrolysis of the Schiff base in the red cone pigment.

Our in vitro data on the red cone pigments are consistent with data from single red cone cells regenerated with both A1 and 9-DM retinal (*34*). Single-cell current recordings by Corson and Crouch (*34*) reported that the current changes after a flash of light were indistinguishable between red cone cells regenerated with A1 and 9-DM retinal. These results suggested that activation and inactivation of the red cone cells containing pigments regenerated with either chromophore were the same. This conclusion does not contradict our findings because in an inactivated red cone cell the pigment protein can remain in a Meta II conformation. For example, in rhodopsin, the kinase and transducin both recognize the Meta II intermediate (*35*, *36*), and it is highly likely the same is true in red cones. Phosphorylation of the pigment is followed by arrestin binding, which prevents the pigment from activating more transducin. This is the simplest explanation why the single red cone cell containing 9-DM retinal pigments inactivates quickly like the A1 retinal containing red cone despite our results that show the 9-DM retinal red cone pigment remains in an active conformation longer.

Unlike the red cone pigment, the UV and blue cone/green rod pigments bound with 9-DM retinal do not appear to display a longer-lived Meta II intermediate. We infer a lack of prolonged Meta II because transducin activation by these 9-DM retinal pigments leveled off very quickly consistent with the fast decay of Meta II and therefore no more transducin was being activated. This is reflected in the calculated values for the decay rates of Meta II in Table 2. Furthermore, like the red cone pigment, the 9-DM retinal blue cone/green rod and UV pigments did not form a stable Meta I photoproduct as judged by absorption spectroscopy. We saw an immediate shift in the absorbance to about 380 nm upon illumination (not shown). The lower ability of the 9-DM retinal UV cone pigment to activate transducin could be due to its blue-shifted spectrum and hence not being fully bleached by our white light source.

Consistent with previous results with bovine rhodopsin (*6*, *8*, *10*), salamander rhodopsin containing 9-DM retinal activated transducin less efficiently than A1 rhodopsin because less Meta II is present because of an equilibrium shift favoring the Meta I intermediate (*9*, *10*). The transducin activation rate of the 9-DM salamander rhodopsin relative to A1 rhodopsin is higher at 63% than previously published reports with bovine rhodopsin (*6*, *8*, *10*). We did not exhaustively follow up on this point because overall our data are not in disagreement with others and upon closer examination of the literature, the relative activities vary greatly. Transducin activation by 9-DM bovine rhodopsin relative to activation by A1 rhodopsin has ranged from 8 (*6*) to 43% (*8*) upon bleaching with orange light. We assayed salamander rhodopsin, not bovine rhodopsin, and bleached the pigment with white light in order to light activate all of the pigments with the same light source. Interestingly, Ganter et al. (*6*) reported that the relative activity of 9-DM rhodopsin increased more than 3-fold (27%) when pigments were bleached with white light instead of orange light. Even within Han et al. (*8*), the relative activity varied from ~18 to 100% depending on environmental factors such as the detergent concentration and assay temperature. Thus, we are not concerned with our higher relative activity from 9-DM salamander rhodopsin because the activity seems highly sensitive to the experimental conditions.

Why does the 9-methyl group of retinal affect rhodopsin activation but not cone pigment activation? The inefficient activation of rhodopsin regenerated with 9-DM retinal might be related to the requirements of the rod for low dark noise. The chromophore is held in place in its resting state by the transmembrane helices and buried by the extradiscal β-stranded cap, and activation would require a steric push from many parts of the ligand upon isomerization on residues surround-

ing the ring and methyl groups. On the other hand, for the red cone pigment, which operates under photopic conditions, maintaining low dark noise is not as critical, but rather the fast activation and deactivation is of importance. The dark noise in the salamander red cone pigment is higher than in rods (*37*); 11-*cis* retinal is not bound as tightly as it is in rhodopsin (*32*, *33*); and the pigment containing A1 retinal, which has the 9-methyl group, deactivated much faster than the pigment containing 9-DM retinal. Perhaps, the overall conformation of the cone pigments are poised to activate transducin, and therefore cone pigments do not require the steric contributions of the 9-methyl group of retinal to form an active Meta II conformation. In rhodopsin, Fritze et al. (*38*) showed that the Meta I/Meta II equilibrium of 9-DM bovine rhodopsin could favor Meta II with key mutations on the cytoplasmic side of the protein at the end of helix 3 or within a highly conserved motif in helix 7. Kim et al. (*39*) have shown that the helix 3 mutations themselves result in the cytoplasmic loops having a Meta II-like conformation despite the dark-mutant holoprotein still being inactive. While retinal isomerization is not uncoupled to activation, the steric contribution from the 9-methyl group of retinal might be for these rhodopsin mutants and the cone pigments.

Despite the high homology among all visual pigment opsins, the interactions between the respective opsin and its ligand leading to the activated state appear to be quite different for the various opsins. The 9-methyl group of retinal is not part of a steric trigger for activation for all pigments because it appears to be with rhodopsin. Its role in the UV cone pigment is not clear although it does not appear to affect the Meta I/Meta II equilibrium. This methyl group seems to have no role in either activation or deactivation of the blue cone/green rod pigment. In the red cone pigment, the 9-methyl group has no effect on activation, but the presence of the methyl group accelerates deactivation of the pigment, suggesting that the 9-methyl group might have a steric effect on efficient hydrolysis of the Schiff base linkage of the chromophore.

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