Role of the C_9 Methyl Group in Rhodopsin Activation: Characterization of Mutant Opsins with the Artificial Chromophore 11-*cis*-9-Demethylretinal†

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ABSTRACT: Activation of the visual pigment rhodopsin involves both steric and electrostatic interactions between the chromophore and opsin within the retinal-binding site. Removal of the C_9 methyl group of 11-*cis*-retinal inhibits light-dependent activation of the G protein, transducin, suggesting a direct steric contact. More recently, we have shown that steric interactions lead to receptor activation when Gly^{121} in the middle of transmembrane helix 3 is replaced by larger hydrophobic residues. In order to understand in more detail the role of the C_9 methyl group of retinal in the structure and function of rhodopsin, we first studied the properties of recombinant 9-dm-Rho (opsin reconstituted with 11-*cis*-9-demethylretinal). The 9-dm-Rho pigment displayed a blue-shifted λ_{max}, increased hydroxylamine reactivity, and decreased ability to activate transducin. These properties are consistent with the hypothesis that the C_9 methyl group is a crucial structural anchor for the correct docking of the chromophore in its binding site. Next, we investigated the possible interaction between Gly^{121} of opsin and the C_9 methyl group of retinal by characterizing recombinant pigments produced by combining mutant opsins (G121A, -V, -I, -L, and -W) with 11-*cis*-9-demethylretinal. Mutant opsins G121I, -L, and -W failed to bind the chromophore. However, the double mutant G121L/F261A bound 11-*cis*-9-demethylretinal to form a stable pigment with a λ_{max} of 451 nm. When activity was assayed in membranes, the reduction in transducin activation by 9-dm-Rho caused by the lack of a C_9 methyl group on the chromophore could be partially restored by replacing Gly^{121} with a bulky residue (leucine, isoleucine, or tryptophan). These results support a model of receptor activation that involves steric interaction between the C_9 methyl group of the chromophore and the opsin in the vicinity of Gly^{121} on transmembrane helix 3.

Rhodopsin (Rho)[1] is the visual photoreceptor responsible for dim-light vision. It is a member of the large family of related G protein-coupled receptors (*1*). The main structural feature shared among these receptors is the presence of seven transmembrane (TM) helices. Rho contains a retinylidene chromophore, 11-*cis*-retinal, which is bound in the interior of the seven-helix bundle via a protonated Schiff base linkage to a specific lysine residue on TM helix 7. The positive charge of the Schiff base is neutralized by a carboxylic acid counterion, Glu^{113} (*2*−*5*). Photon absorption by rhodopsin (λ_{max} = 500 nm) leads to chromophore isomerization and receptor activation. The metarhodopsin II (MII) intermediate catalyzes guanine-nucleotide exchange by the heterotrimeric G protein transducin. Several structural changes in the transition from Rho to MII appear to be concomitant with receptor activation: (1) the *all-trans*-retinal chromophore Schiff base deprotonates (*6*), (2) Glu^{113} becomes protonated (*7*), (3) TM helices 3 and 6 move apart with respect to the helical bundle (*8*−*10*), and (4) Glu^{134} at the cytoplasmic border of TM helix 3 becomes protonated (*11*). Still, the mechanism by which chromophore isomerization initiates these conformational changes leading to the active state of the receptor is not well understood.

The retinal chromophore is a linear conjugated polyene composed of an unsaturated carbon backbone terminated at one end by a protonated imine linkage to Lys^{296} and at the other end by a six-membered ring. Extending from both the conjugated chain and the ring are methyl groups that contribute to the complexity of the van der Waals surface of the retinal and have been shown to influence the photochemistry of rhodopsin. Early studies of 9-demethylrhodopsin (9-dm-Rho), obtained by regenerating opsin with 11-*cis*-9-demethylretinal (11-*cis*-9-dm-Ret),[2] yielded a number of interesting findings. The MII-like state of 9-dm-Rho is protonated and fails to activate transducin efficiently (*12*). Studies using salamander rods show that 11-*cis*-9-dm-Ret causes prolonged excitation and a lowered quantal response

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[1] Abbreviations: 9-dm-Rho, artificial pigment composed of opsin and 11-*cis*-9-demethylretinal; 11-*cis*-9-dm-Ret, 11-*cis*-9-demethylretinal; DM, *n*-dodecyl β-D-maltoside; DTT, dithiothreitol; MI and MII, metarhodopsin I and II; Rho, rhodopsin; R*, light-activated rhodopsin; TM, transmembrane.

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(*13*). The 11-*cis*-9-dm-Ret is also capable of relieving the desensitization of bleached rod cells (*14*). Related biochemical studies show that the prolonged lifetime of the active state of 9-dm-Rho (9-dm-R*) is due to reduced light-dependent phosphorylation by rhodopsin kinase (*15*). Finally, resonance Raman and Fourier transform infrared spectroscopy have demonstrated that the chromophore in the 9-dm-Rho bathorhodopsin photoproduct is not strained compared to that in native bathorhodopsin (*12*, *16*).

While the C_9 methyl group of the retinal chromophore clearly plays a crucial role in rhodopsin activation, the C_5 and C_{13} methyl groups seem to be less important. Removal of the C_5 methyl group results in a pigment (5-dm-Rho) with a λ_{max} of 480 nm (*17*) and a bathorhodopsin-like photolysis intermediate with a twisted *all-trans* chromophore (*16*). This implies that the C_5 methyl group is probably not crucial for correct ligand binding and receptor activation, although transducin activation by this artificial pigment has not yet been tested. The C_{13} methyl group is not important for either ligand docking or receptor activation. The 13-dm-Rho pigment displays a λ_{max} of 498 nm (*18*) and it can interact with rhodopsin kinase with the same efficiency as native Rho (*19*). Interestingly, numerous studies demonstrate that the C_{13} methyl group, rather than the C_5 methyl or C_9 methyl groups, is most important in the function of the light-driven proton pump bacteriorhodopsin (*20*) and sensory rhodopsin (*21*, *22*), where photochemical isomerization occurs about the C_{13}═C_{14} double bond.

One strategy in elucidating the molecular mechanism of rhodopsin photoactivation is to identify key functional opsin−chromophore interactions. Previously, we showed that Gly^{121}, a highly conserved amino acid residue in the middle of TM helix 3, plays a key role in defining the functional retinal-binding site of Rho (*23*). On the basis of site-directed mutagenesis and computer modeling, we proposed a possible interaction between the C_9 methyl group of retinal and Gly^{121} (*23*, *24*). In the present study, we investigate the interaction between position 121 of opsin and the C_9 methyl group of retinal by characterizing recombinant pigments produced by combining mutant opsins (G121A, -V, -I, -L, and -W) with synthetic 11-*cis*-9-dm-Ret. We report spectral and biochemical properties of the artificial pigments in detergent solution and in COS cell membranes. The results confirm the important role of the C_9 methyl group in rhodopsin function. In addition, the observation that the defect in transducin activation by 9-dm-Rho can be partially restored by substituting a bulky side chain in place of Gly^{121} is consistent with the presence of a steric interaction between the C_9 methyl group of the chromophore and TM helix 3 of the protein in the vicinity of Gly^{121}.

EXPERIMENTAL PROCEDURES

Materials. *n*-Dodecyl β-D-maltoside (DM) detergent was obtained from Anatrace, Inc. [^{35}S]GTPγS (Du Pont−New England Nuclear) and BA-85 nitrocellulose filters (Schleicher & Schuell) were used for the radionucleotide filter-binding assay. Other nucleotides were from Boehringer Mannheim. Sources of other materials have been previously reported (*2*, *3*, *9*, *25*).

Organic Synthesis of 11-cis-9-Demethylretinal. All chemicals were purchased from Aldrich. 11-*cis*-9-dm-Ret was synthesized according to Broek et al. (*26*) with slight modifications. Briefly, commercially available β-ionone was reacted with sodium and ethyl formate in diethyl ether. After 24 h, the resulting salt was reacted with sulfuric acid and dry methanol to give acetyl ketone. Reduction of the ketone with $LiAlH_4$, followed by dehydroxylation of the resulting secondary alcohol and deprotection of the aldehyde with phosphoric acid, leads to a conjugated aldehyde. The aldehyde was converted into an isomeric mixture of 9-dm-Ret by the Horner−Emmons reaction with triethyl 4-phosphono-3-methylcrononate, followed by $LiAlH_4$ reduction and MnO_2 oxidation. The 11-*cis* isomer was obtained by irradiation of the synthetic mixture in dry acetonitrile (1 mg/mL) and HPLC separation of the isomers. The identity of the compound was confirmed by mass spectrometry and ^{1}H NMR analysis (*26*).

Expression and Preparation of Rhodopsin Mutants. Opsin genes were expressed in COS-1 cells as previously described (*23*, *25*, *27*).

UV−Visible Absorption Spectroscopy of C_9-Demethyl Pigments. UV−visible absorption spectroscopy was performed at 25 °C on purified samples unless otherwise specified. Illumination of the pigment was carried out using a 150-W projector lamp equipped with a 495-nm long-pass filter.

Reaction of Mutant Pigments with Hydroxylamine. The rates of hydroxylamine reaction with mutant pigments were determined in the dark as previously described (*28*). The conditions for the reaction at 25 °C were 25 mM hydroxylamine, 50 mM Tris-HCl, pH 6.9, 100 mM NaCl, and 0.1% DM.

Transducin Activation Assay. A filter-binding assay, which monitors the light-dependent guanine-nucleotide exchange by transducin, was described previously (*23*, *29*).

RESULTS

UV−Visible Spectroscopy of 9-Demethylrhodopsin. Opsin expressed in COS cells was regenerated with 11-*cis*-9-dm-Ret, and the resulting 9-dm-Rho pigment was purified in DM. UV−visible spectra of the 9-dm-Rho pigments are shown in Figure 1. The 11-*cis*-9-dm-Ret chromophore regenerates well with wild-type opsin to form an artificial recombinant pigment with a λ_{max} of 465 nm. The λ_{max} value agrees with that obtained by Ganter et al. (*12*) and represents a blue shift of ∼35 nm (∼1505 cm^{-1}) from that of native rhodopsin. Since the absorption maxima of 11-*cis*-9-dm-Ret (372 nm) and 11-*cis*-retinal (379 nm) differ by only 7 nm (495 cm^{-1}) in ethanol, the large blue shift in 9-dm-Rho compared to Rho must involve changes in retinal−opsin interactions. The spectral ratio (A_{280}/A_{465}) of 9-dm-Rho (2.5) is larger than that of rhodopsin (A_{280}/A_{500}) regenerated and purified in parallel (1.8). Since 9-dm-Rho has an extinction coefficient, ϵ, similar to that of Rho (*30*), the increase in spectral ratio is not due to a difference in ϵ and may reflect

[2] According to suggested formal retinoid nomenclature, the carbon of the methyl group on the C_9 position is numbered C_{19}, and 9-demethylretinal would be denoted 19-norretinal. For clarity and consistency with much of the existing biochemical literature, we use the common name 9-demethylretinal in this report to denote the retinal analogue in which the C_{19} methyl group is replaced by H (see Figure 1).

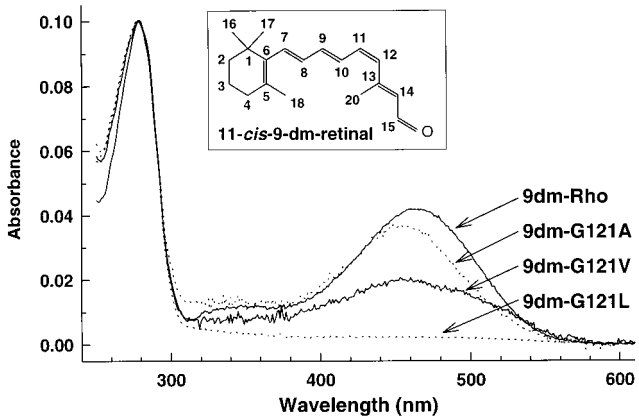

FIGURE 1: UV−visible absorption spectra of the artificial recombinant pigments 9-dm-Rho, 9-dm-G121A, 9-dm-G121V, and 9-dm-G121L. No detectable visible absorption is noted for 9-dm-G121L. In addition, 9-dm-G121I and 9-dm-G121W pigments could not be purified in DM (not shown). The structure of 11-*cis*-9-demethylretinal is shown in the inset.

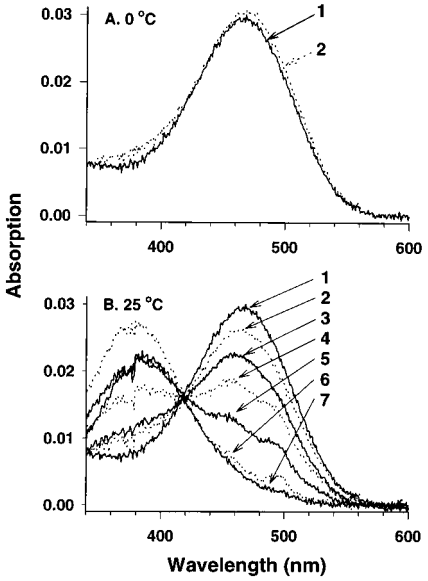

FIGURE 2: Photobleaching properties of 9-dm-Rho. (**A**) 9-dm-Rho was illuminated at 0 °C. *Trace 1*, spectrum obtained in the dark; *trace 2*, spectrum obtained after a 2-s flash. No further spectral change was observed after the first 2-s flash. (**B**) 9-dm-Rho illuminated at 25 °C. Several representative stages of the bleaching process are displayed. The spectral conversion exhibits an isosbestic point at 419 nm. *Trace 1*, spectrum obtained in the dark; *trace 2*, spectrum obtained after a 5-s flash; *trace 3*, spectrum 2 followed by incubation at 25 °C in the dark for 90 min; *trace 4*, spectrum 3 followed by an additional 5-s flash; *trace 5*, spectrum 4 followed by incubation at 25 °C in the dark for 20 min and a subsequent 5-s flash; *trace 6*, spectrum 5 followed by incubation at 25 °C in the dark for 14 min and a subsequent 20-s flash; *trace 7*, spectrum 6 followed by two successive 10-s flashes and the addition of HCl.

lowered affinity of 11-*cis*-9-dm-Ret for opsin and/or lowered stability of the 9-dm-Rho pigment in DM.

Photoproducts of 9-dm-Rho were also characterized by UV−visible spectroscopy. Photolysis was first carried out at 0 °C in order to decrease the rate of thermal decay of the resulting photoproducts. The initial spectrum after illumination (Figure 2A, trace 2) is very similar to the dark spectrum (Figure 2A, trace 1), and exhibits a slightly red-shifted λ_{max} (467 nm), a hyperchromic change in ϵ, and a slightly broader bandwidth. These results are in general agreement with those reported by Ganter et al. (*12*). The 467-nm λ_{max} of the 9-dm-Rho photoproduct implies that the Schiff base is still protonated. Only a small amount of a 380-nm species is noted after illumination. In contrast, under the same conditions wild-type Rho forms MII (λ_{max} = 380 nm) with a deprotonated Schiff base (not shown). The Schiff base deprotonation to form MII is crucial for the formation of the active receptor (R*) (*6*). A steady state is reached within the first 2-s illumination of 9-dm-Rho. Additional photolysis with 2-s flashes did not alter the spectrum.

At 25 °C, a complicated bleaching process is observed (see Figure 2B). A 5-s flash, which was sufficient to establish a steady state at 0 °C, resulted in a spectrum with a decreased absorbance at 465 nm and a noticeable increase in absorbance at 380 nm (Figure 2B, trace 2). This is in contrast to the bleaching behavior at 0 °C, where a hyperchromic shift was observed in the visible region (Figure 2A, trace 2). After this first 5-s flash, the sample was kept in the dark and absorption spectra were recorded at 2-min intervals. A gradual decay of absorption at 465 nm was observed within the first 10 min. Further incubation for 80 min did not result in an additional change in the spectrum (Figure 2B, trace 3). In Figure 2B, trace 3, the λ_{max} is blue-shifted to 460 nm and the total absorption decrease in the visible region is ∼22% of the original absorbance. This observation implies that the protonated Schiff base of the photoproduct is not stable and undergoes deprotonation and/or hydrolysis. The application of another 5-s flash decreased the remaining 465-nm absorption by about an additional 12% (Figure 2B, trace 3). Further incubation at 25 °C for 20 min did not result in additional change in the spectrum. When additional flashes were applied, a decrease in absorbance in the visible region and a concomitant increase in 380-nm absorption were observed (Figure 2B, traces 4 and 5). The spectral conversion displayed an isosbestic point at 419 nm, implying that as few as two species were involved. No further change in the spectrum occurred after a total of 35 s of illumination. The sample was then acidified to denature the protein while trapping any Schiff base linkage to give a 440-nm species. The spectrum of the acidified sample gave a λ_{max} of 382 nm, essentially the same as that of the bleached product without acidification (Figure 2B, traces 6 and 7). This indicates that the majority of the pigment after the bleaching and incubation described above was converted to opsin and free retinal. Interestingly, a small shoulder absorbing at a λ_{max} of 493 nm was observed in traces 3−6 in Figure 2B, which disappeared upon acidification (trace 7). This shoulder was also observed in the other pigments studied but its identity has not yet been investigated.

Hydroxylamine reactivity has been commonly employed to probe the Schiff base environment of visual pigments and mutant pigments. The Schiff base bond in rhodopsin in the dark does not react with hydroxylamine (*23*). Despite the blue-shifted λ_{max} of 9-dm-Rho, indicating a possible change of the protonated Schiff base environment, the pigment was fairly stable to hydroxylamine in the dark. It decayed slowly with a $t_{1/2}$ of 169 min (Table 1). In contrast, the photoproduct of 9-dm-Rho decayed rapidly in the presence of hydroxylamine at 25 °C (not shown). This is also the case with rhodopsin, which reacts readily with hydroxylamine upon illumination.

Table 1: Properties of 9-dm-Rho and 9-dm-Gly^{121} Mutant Pigments[a]

	λ_{max} (nm)	blue shift[b] (cm^{-1})	relative spectral ratio[c]	$t_{1/2}$ in hydroxylamine (min)
9-dm-Rho	465 ± 0.5 (3)	1505	1.38 ± 0.06 (2)	169 ± 11
9-dm-G121A	456 ± 0.3 (3)	1850	1.4 ± 0.2 (2)	51 ± 2
9-dm-G121V	458 ± 0.7 (5)	870	3.2 ± 0.6 (2)	10.2 ± 0.7
9-dm-F261A	466 ± 1.0 (4)	1459	1.26 ± 0.04 (2)	111 ± 2
9-dm-G121L/F261A	451 ± 0.6 (3)	1377	1.1 (1)	4.8 ± 0.2

[a] 9-dm-G121L, 9-dm-G121I, and 9-dm-G121W do not display detectable visible pigment under the same purification conditions in DM (Figure 1). Data are presented as the mean ± SE (*n*). [b] Blue shift is defined as the difference in λ_{max} between the 9-dm pigment and the corresponding native pigment, in wavenumbers. The λ_{max} values of the Gly^{121} mutant pigments are from refs *23* and *31*. [c] Relative spectral ratio is defined as the spectral ratio of the 11-*cis*-9-dm-Ret artificial pigment ($\boldsymbol{A}_{280}/\boldsymbol{A}_{\lambda max}$) divided by that of the corresponding 11-*cis*-retinal pigment.

UV−Visible Spectroscopy of 9-Demethylrhodopsin Mutants. Five Gly^{121} mutants (G121A, G121V, G121L, G121I, and G121W) were regenerated with 11-*cis*-9-dm-Ret. The resulting artificial pigments were purified in DM, and UV−visible spectra were recorded (Figure 1, Table 1). There is a gradual decrease in pigment yield as the size of the amino acid at position 121 increases. This trend is slightly more pronounced than the same general trend found for mutant opsins regenerated with 11-*cis*-retinal (*23*). The pigments 9-dm-G121A and 9-dm-G121V displayed significantly higher spectral ratios than the corresponding mutant pigments regenerated with 11-*cis*-retinal, as demonstrated by the relative spectral ratio, defined as the spectral ratio of the 9-dm pigment normalized to that of the corresponding 11-*cis*-retinal pigment (Table 1). No detectable 9-dm-G121L, 9-dm-G121I, or 9-dm-G121W pigments could be purified in DM. The λ_{max} values of 9-dm-G121A (456 nm) and 9-dm-G121V (458 nm) were significantly blue-shifted compared to pigments G121A (498 nm) and G121V (477 nm). The blue shifts (in wavenumbers) of the 9-dm pigments compared to their respective 11-*cis*-retinal pigments are listed in Table 1.

The hydroxylamine reactivities of the 9-dm mutant pigments were measured (Table 1). The half-life of pigment decay in the presence of hydroxylamine, $t_{1/2}$, was derived as described previously (*23*). The mutant opsin pigments reacted with hydroxylamine more rapidly than 9-dm-Rho under identical conditions: 9-dm-Rho ($t_{1/2}$ = 169 min), 9-dm-G121A ($t_{1/2}$ = 51 min), and 9-dm-G121V ($t_{1/2}$ = 10.2 min). The 9-dm-G121A pigment reacted more rapidly than the corresponding 11-*cis*-retinal G121A pigment ($t_{1/2}$ = 175 min) (*23*). However, the 9-dm-G121V pigment reacted more slowly than the corresponding 11-*cis*-retinal G121V pigment ($t_{1/2}$ = 2.6 min), implying that the Schiff base in the 9-dm-G121V pigment is less reactive than that in the G121V pigment (*23*).

We have shown in a previous report that a second-site mutation, F261A, on TM helix 6 can rescue the defects caused by the bulky substitutions of Gly^{121} on TM helix 3 (*31*). For example, G121L has very poor pigment yield, while the double mutant G121L/F261A displays a pigment yield close to that of wild-type rhodopsin. We concluded on the basis of these and other observations that Gly^{121} on TM helix 3 and Phe^{261} on TM helix 6 are key residues defining the retinal-binding site and are located on opposite sides of the retinal. It was therefore of interest to test whether or not the F261A mutation could rescue the lack of pigment formation in 9-dm-G121L. As shown in Figure 3, 9-dm-G121L/F261A displays a pigment yield similar to that of

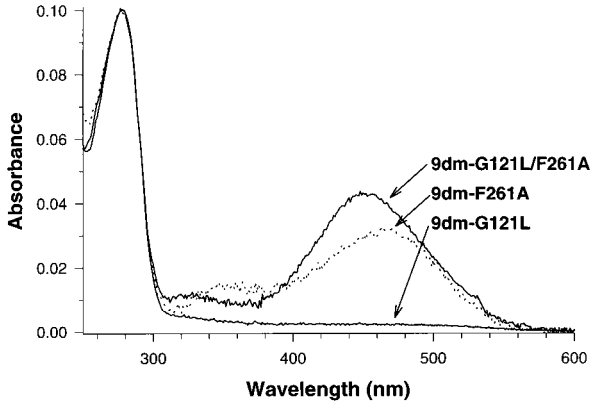

FIGURE 3: UV−visible absorption spectra of 9-dm-G121L, 9-dm-F261A, and 9-dm-G121L/F261A. The ability of the second-site F261A mutation to rescue the defect of G121L opsin in incorporating 11-*cis*-9-dm-retinal is demonstrated.

9-dm-Rho. In contrast, 9-dm-G121L is entirely incapable of forming a stable pigment in DM. Therefore, the rescue effect is very similar to that described earlier with the same mutant opsin regenerated with the 11-*cis*-retinal (*31*). This observation suggests that the 11-*cis*-9-dm-Ret analogue may dock roughly into the same pocket as 11-*cis*-retinal between residues Gly^{121} and Phe^{261}. The λ_{max} of 9-dm-G121L/F261A (451 nm) (Table 1) is significantly blue-shifted compared to that of G121L/F261A (480 nm) (*31*). The hydroxylamine reactivity ($t_{1/2}$ = 4.8 min) of 9-dm-G121L/F261A is higher than that of G121L/F261A ($t_{1/2}$ = 11 min). Interestingly, 9-dm-G121L/F261A yields more pigment than 9-dm-F261A (Figure 3), although both 9-dm-pigments still have lowered yields than the same respective opsins regenerated with 11-*cis*-retinal (relative spectral ratio greater than 1). This is in contrast to the previous case where F261A displayed higher visible absorption than G121L/F261A (*31*).

Transducin Activation by 9-Demethyl Pigments in DM. Light absorption by 9-dm-Rho in DM leads to a decreased level of transducin activation (43% ± 4% when compared to rhodopsin) under our standard assay conditions (Table 2). In contrast, Ganter et al. (*12*) reported transducin activity of only 8%. The major difference between the two assays employed is the concentration of DM detergent (0.6 mM or 0.03% in ref 12 and 0.2 mM or 0.01% in this study). To assess the dependence of DM concentration, the ability of 9-dm-Rho to activate transducin was measured at different DM concentrations ranging from 0.01% to 0.1% for both Rho and 9-dm-Rho (Figure 4). A drastic decrease in transducin activity was observed with increasing DM concentration for both pigments, indicating that the assay is extremely sensitive to the amount of DM present. When

Table 2: Transducin Activation by Mutant Opsin in the Presence of 11-*cis*-9-dm-Retinal[a]

pigment	in DM[b] (%)	light activity in membranes[c] (%)	dark activity in membranes[c] (%)	opsin activity[d] (%)
9-dm-Rho	43 ± 4 (5)	16.1 ± 3.0	0.4 ± 0.4	0.9 ± 0.2
9-dm-G121A	49 ± 14	10.5 ± 2.0	0.2 ± 0.1	1.6 ± 0.3
9-dm-G121V	13 ± 4	5.6 ± 0.1	0.2 ± 0.2	0.4 ± 0.1
9-dm-G121L	no pigment	17.8 ± 1.6	1.8 ± 0.4	1.9 ± 0.3
9-dm-G121I	no pigment	18.6 ± 0.9	0.7 ± 0.1	1.0 ± 0.4
9-dm-G121W	no pigment	25.6 ± 3.8	1.9 ± 0.6	2.3 ± 0.4

[a] Data are listed as the mean ± SE (n); $n = 3$ unless otherwise specified. [b] Transducin activation in DM is presented as the percent of the transducin activity of the 9-dm pigment normalized to the corresponding mutant pigment with native retinal (*e.g.*, 9-dm-G121A activity is normalized to G121A activity). Pigment concentration is determined from the visible spectrum assuming that the 9-dm mutant pigment has the same ϵ as the respective opsin containing native retinal. [c] Transducin activities of the 9-dm-opsin complex in COS cell membranes in the dark (dark activity) or under continuous illumination (light activity) are presented as the percent of the transducin activities normalized to the light activity of the same opsin sample incubated with 11-*cis*-retinal and measured in parallel under illumination. [d] From ref *23*.

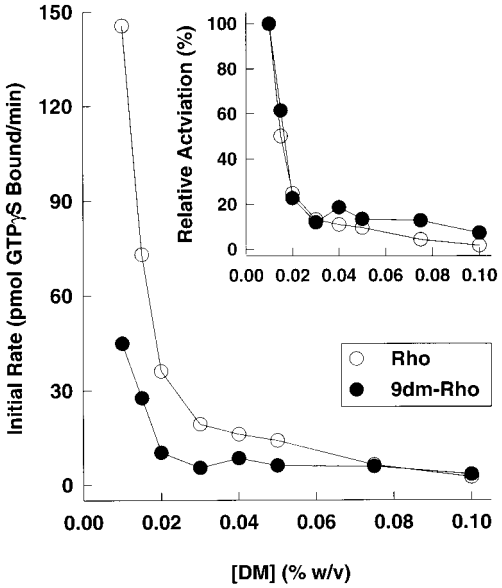

FIGURE 4: Transducin activation by Rho (○) and 9-dm-Rho (●) as a function of DM concentration under continuous illumination. The relative activity as a function of DM concentration is shown as an inset. Each data point is normalized to the activity measured at 0.01% DM for either Rho or 9-dm-Rho. The relative activity demonstrates the similarity in DM concentration dependence of transducin activation of the two pigments.

the DM concentration was greater than 0.075%, the ability of Rho to activate transducin was essentially undetectable.

The influence of DM concentration on 9-dm-Rho *compared to* wild-type Rho is more clearly seen in Figure 4 where the transducin activity for both pigments is normalized to the transducin activity observed at 0.01% DM. This normalization clearly shows the similarity in DM concentration dependence between Rho and 9-dm-Rho. This observation implies that high concentrations of DM are likely to affect the ability of transducin to bind R* and/or to exchange GDP for GTP. We also measured the ability of 9-dm-Rho to activate transducin relative to Rho under buffer conditions similar to those used by Ganter et al. (*12*) (110 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, 0.6 mM DM, 0.1 mM EDTA,

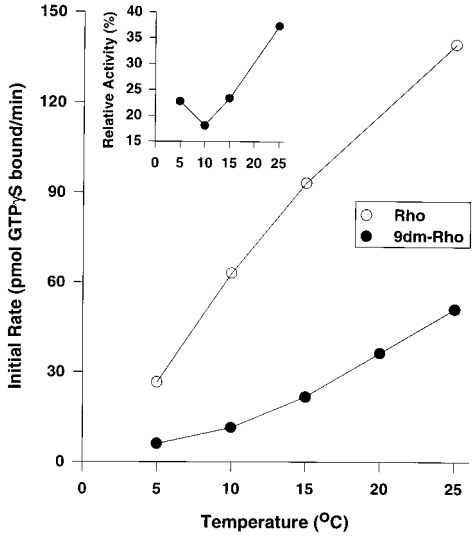

FIGURE 5: Transducin activation by Rho (○) and 9-dm-Rho (●) as a function of temperature. Relative activation of 9-dm-Rho normalized to that of Rho at a given temperature is shown in the inset.

and 20 mM Tris-HCl, pH 7.4). The relative activation level measured, 42.3% ± 0.8% ($n = 2$), is essentially the same as observed under our standard assay condition (100 mM NaCl, 4 mM $MgCl_2$, 1 mM DTT, 0.2 mM DM, and 50 mM Tris-HCl, pH 7.2). The difference in transducin activity measured in the two studies is likely due to differences in the pigment purification procedures.

Transducin activation by both Rho and 9-dm-Rho is also sensitive to temperature as shown in Figure 5. However, in contrast to the dependence on DM concentration, the temperature dependence differs for the two pigments. The relative transducin activity for 9-dm-Rho ranges from 18% at 10 °C to ~40% at 25 °C (Figure 5, inset).

Transducin activation by the 9-dm mutant pigments relative to their respective opsin mutants regenerated with 11-*cis*-retinal was also measured (Table 2). The relative activity of 9-dm-G121A versus G121A was 49%, very similar to the value of 43% for 9-dm-Rho versus Rho described above. This indicates that 11-*cis*-9-dm-Ret affects transducin activation by mutant 9-dm-G121A to a similar degree as in 9-dm-Rho. The value for 9-dm-G121V versus G121V was 13%, significantly lower than that of either 9-dm-Rho versus Rho or 9-dm-G121A versus G121A. The light-dependent transducin activation by 9-dm-G121L/F261A was 56.8% of that of G121L/F261A (Table 2).

Transducin Activation by 9-Demethyl Mutants in COS Cell Membranes. The five Gly^{121} mutant opsins and wild-type opsin were combined with 11-*cis*-9-dm-Ret in COS cell membranes to form six opsin−9-dm-retinal complexes. Transducin activation by these complexes was measured either in the dark (dark activity) or under continuous illumination (light activity). Both dark and light activities of the opsin−9-dm-retinal complexes were normalized relative to the light activity of the corresponding opsin incubated with 11-*cis*-retinal (Table 2). Basal activities of Gly^{121} mutant opsins alone (opsin activity) are also listed for comparison. The dark activities of Gly^{121} mutant opsins in the presence of 11-*cis*-9-dm-Ret are invariably equal to or lower than their respective opsin activities, in contrast to the high dark activity observed for these mutant opsins in

the presence of 11-*cis*-retinal (*32*). The partial agonism of 11-*cis*-retinal in Gly^{121} mutants was interpreted as an increased steric interaction between the amino acid at position 121 and the retinal ligand that partially mimicked the R* conformation. This result demonstrates that the presence of the C_9 methyl group of retinal is crucial for the partial agonist activity of 11-*cis*-retinal observed in Gly^{121} mutant opsins.

In COS cell membranes, 9-dm-Rho exhibited 16% of the light activity of Rho (Table 2). The low transducin activity is primarily due to an intrinsic inability to activate transducin rather than an inability of opsin to bind 11-*cis*-9-dm-Ret, because it has been shown that 11-*cis*-9-dm-Ret regenerates well with opsin to form a stable pigment (Figure 1, Table 1). The transducin activation level observed in COS cell membranes is markedly lower than that observed in DM (43%). However, it is closer to the 25% level of phosphorylation of 9-dm-Rho in rod outer segments (ROS) (*15*) and the 17% integrated quantal response measured in salamander rods (*13*). The difference in the activation levels observed in detergent and membranes implies that the ability of 9-dm-Rho to form R* is dependent on the environment of the receptor.

The regeneration levels of 11-*cis*-9-dm-Ret with mutant opsins are not well characterized except for G121A, which is shown to regenerate with 11-*cis*-9-dm-Ret nearly as well as wild-type opsin (Figure 1, Table 1). The low yield of purified pigment for G121V, and the absence of pigment for G121L, G121I, and G121W, when incubated with 11-*cis*-9-dm-Ret may reflect the combined effects of low chromophore incorporation and low pigment stability in DM. If chromophore incorporation is low even in the COS cell membrane preparations, the light activities observed in these 11-*cis*-9-dm-retinal− Gly^{121} mutant complexes (Table 2) may reflect both lower intrinsic transducin activation and lower ligand incorporation. As the size of the residue at position 121 increases, light activation of the opsin mutants G121V, G121L, G121I, and G121W in the presence of 11-*cis*-9-dm-Ret shows an increase in activity (Table 2). As discussed above, it is unlikely that the increased light activity with increasing residue size is due to an increased level of ligand incorporation. Therefore, when the volume of the residue at position 121 is large enough, it is able to counteract the detrimental effect on transducin activation caused by the absence of the crucial C_9 methyl group.

DISCUSSION

The C_9 methyl group of retinal is crucial for rhodopsin photoactivation (*12*, *16*). Recently, we demonstrated that Gly^{121} on TM helix 3 of rhodopsin is one of the key residues that define the retinal-binding site (*23*, *31*). We also showed that Gly^{121} regulates properties of early rhodopsin photoproducts (*33*) and proposed that Gly^{121} might interact with the retinal C_9 methyl group via specific van der Waals interactions (*24*, *31*). In this study, we combined the synthetic retinal analogue 11-*cis*-9-dm-Ret with opsin mutants containing amino acid replacements at position 121 in order to better understand the role of the retinal C_9 methyl group in receptor activation.

As a necessary control, we studied the properties of the recombinant artificial pigment 9-dm-Rho, which was made by regenerating recombinant wild-type opsin with 11-*cis*-9-dm-Ret. The 9-dm-Rho obtained from opsin expressed in COS cells exhibits the same λ_{max} (465 nm) as the 9-dm-Rho previously prepared from ROS opsin (*12*). However, the bleaching properties of solubilized recombinant 9-dm-Rho in our hands were somewhat different from those reported earlier (*12*). We observed more 380-nm absorbing photoproduct at 25 °C (Figure 2B) and a higher rate of transducin activation in DM detergent (Table 2). These discrepancies are due in part to differences in the purification procedures used in the two studies.

Misdocking of 9-dm-Ret May Be Responsible for the Blue Shift in λ_{max}. The large blue shift in the λ_{max} of 9-dm-Rho compared to that of Rho has been an intriguing observation since it was first noticed by Kropf and co-workers (*30*). The origin of the change in electronic structure of the bound retinal in 9-dm-Rho is likely to be associated with other properties of the analogue pigment, such as (1) the lack of chromophore distortions in bathorhodopsin (*16*), (2) a red-shifted photoproduct (*12*), (3) lowered transducin activity (*12*), (4) a lowered quantal response (*13*), and (5) reduced light-dependent phosphorylation (*15*). The increase of the residue size at position 121 of opsin and the removal of the C_9 methyl group of retinal both lead to blue shifts in the λ_{max}. We propose two possible reasons for the large blue shift of 9-dm-Rho. First, the retinal C_9 methyl group provides specific hydrophobic packing interactions with opsin that may function as a crucial anchor for correctly docking the ligand in its binding site. Lacking the crucial methyl group, 11-*cis*-9-dm-Ret does not bind in the "correct" orientation. Even small changes in the rotational orientation or tilt of the 11-*cis*-9-dm-retinal could cause the large blue shift in λ_{max} observed. Second, the lack of the C_9 methyl group in 9-dm-Rho may create a cavity where water can bind and, if oriented properly, induce a blue shift. The importance of a single methyl group in ligand recognition has been previously demonstrated in a number of cases. In one study of antiviral compounds, a single methyl group has been shown to dictate the mode of binding of drug to human rhinovirus 14. The loss of the methyl group in one compound completely altered the stereochemical specificity with its target virus and also caused translation of another compound in the binding pocket (*34*).

Role of the C_9 *Methyl Group in the Steric Component of Receptor Activation*. We reported earlier that 11-*cis*-retinal can activate Gly^{121} mutant opsins in the dark without chromophore isomerization (*32*). In these mutants, the native 11-*cis*-retinal chromophore displays partial agonist activity rather than inverse agonist activity as it does in the wild-type receptor. We interpreted the partial agonism of 11-*cis*-retinal in the Gly^{121} mutants as resulting from increased steric interaction between the larger residue at position 121 and the retinal ligand such that the R* conformation was partially mimicked. Replacement of Gly^{121} by a larger residue was suggested to result in an outward movement of TM helix 3 from the seven-TM helix bundle. Rigid-body movement of TM helices 3 and 6 relative to each other was reported to be a major feature in reaching the active state of the receptor (*8*).

Among the five 9-dm-Gly^{121} mutant pigments, only two (9-dm-G121A and 9-dm-G121V) formed pigments with reasonable yield and stability in DM. The G121L, G121I,

and G121W mutant opsins did not combine with 11-*cis*-9-dm-Ret to form stable pigments in DM (Figure 1). Interestingly, a second-site mutation, F261A, can rescue the lack of pigment formation of 9-dm-G121L (Figure 3), reminiscent of the rescuing effect observed for 11-*cis*-retinal with the same mutants (*23*). However, in COS cell membranes none of the Gly^{121} mutants showed dark activity in the presence of 9-dm-Ret. This negative result demonstrates that the presence of the C_9 methyl group is crucial for the partial agonist activity of 11-*cis*-retinal observed in Gly^{121} mutant opsins in the dark.

In contrast, the light activity of Gly^{121} mutants in the presence of 11-*cis*-9-dm-Ret exhibited a biphasic trend. When the volume of the residue at position 121 increases going from Rho to G121A to G121V, there is a decrease in transducin activation. When it increases going from G121V to G121L to G121I to G121W, there is a recovery in transducin activation. Among the mutants tested, G121W in the presence of 11-*cis*-9-dm-Ret displays the highest transducin activation. A higher activation level can result either from a higher level of ligand incorporation, so that more active photoproduct is formed, or from formation of an R* with higher intrinsic activity. It is unlikely that 11-*cis*-9-dm-Ret incorporates to higher levels as the size of residue on 121 increases, judging by the decrease of pigment yield in DM as a function of residue size at position 121. Moreover, 9-dm-G121L, 9-dm-G121I, and 9-dm-G121W did not form stable pigments at all in DM, yet the G121L, G121I, and G121W opsins in COS cell membranes display equal or higher levels of transducin activation compared to wild-type opsin in the presence of 11-*cis*-9-dm-Ret. Therefore, when the volume of the residue at position 121 is large enough, it seems to be able to partially compensate for the detrimental effect caused by the absence of the crucial C_9 methyl group in R* formation.

Role of the C_9 Methyl Group in the Electrostatic Component of Receptor Activation. In native rhodopsin, the Rho → MI transition is driven by steric interactions, while electrostatic interactions are essential for converting MI to MII and forming the activated receptor (*24*, *35*). This transition is characterized by deprotonation of the Schiff base and protonation of its counterion, Glu^{113}. Jäger et al. (*7*) assigned the 1712-cm^{-1} band in the Fourier transform infrared difference spectrum of MII to the C═O stretching vibration of protonated Glu^{113}. A peak at nearly this frequency, 1710 cm^{-1}, is also present in the 467-nm photoproduct of 9-dm-Rho. It displays a similar D_2O effect as the corresponding peak in Rho (*12*). This observation suggests that Glu^{113} is unprotonated in 9-dm-Rho in the dark and becomes protonated in the red-shifted photoproduct of 9-dm-Rho. The proton for Glu^{113} protonation in native Rho is provided by the deprotonation of the protonated Schiff base. Because the visible spectrum of the 9-dm-Rho photoproduct clearly reveals a protonated Schiff base (*12*, this study), the identity of the proton donor in the 9-dm-Rho photoproduct is unclear but is currently under study. This raises the interesting possibility that Glu^{113} becomes at least partially protonated following retinal isomerization while the Schiff base remains protonated, implying that the protonation of Glu^{113} can be decoupled from the deprotonation of the Schiff base. Presumably, an anion would need to be recruited from the solvent to function as the counterion to the positively charged protonated Schiff base in the photoproduct of 9-dm-Rho. A neutral residue at position 113 is one of the most important elements in defining the active state of the receptor (*35*). Our observation that an increase in the 380-nm photoproduct of 9-dm-Rho correlates with an increase in transducin activity suggests that the series of steps leading to R* is reversed in 9-dm-Rho from that in wild-type Rho. Glu^{113} protonation precedes, or is coincident with, the structural rearrangement normally associated with the Rho → MI transition. On the basis of the amide I vibrations, Ganter et al. (*12*) conclude that in the red-shifted photoproduct of 9-dm-Rho the protein still has a lumirhodopsin-like structure, but the retinal has relaxed to a structure characteristic of MI.

The conclusion from this study that the C_9 methyl group of retinal interacts with Gly^{121} is also supported by the results of a related study in which retinal analogues with ethyl or propyl groups at the C_9 position were reconstituted with opsin mutants (*36*).

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