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Steric Hindrance between Chromophore Substituents as the Driving Force of Rhodopsin Photoisomerization: 10-Methyl-13-Demethyl Retinal Containing Rhodopsin

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ABSTRACT

A visual chromophore analogue, 10-methyl-13-demethyl (dm) retinal, was synthesized and reconstituted with bleached bovine rhodopsin to form a visual pigment derivative with absorbance maximum at 505 nm. The investigations with this new compound were stimulated from recent results using 13-dm retinal as a chromophore that revealed a remarkable loss in quantum efficiency (ϕ of 13-dm retinal-containing rhodopsin: 0.30, Ternieden and Gärtner, *J. Photochem. Photobiol. B Biol.* 33, 83–86, 1996). The quantum efficiency of the new pigment was determined as 0.59 by quantitative bleaching using reconstituted rhodopsin as a reference. The very similar quantum efficiencies of rhodopsin and the new pigment give experimental support for the recently presented hypothesis that a steric hindrance between the substituents at positions 10 and 13 in 11-*cis*-retinal is elevated during the photoisomerization and thus facilitates the rapid photoisomerization of the visual chromophore (Peteanu *et al.*, *Proc. Natl. Acad. Sci. USA* 90, 11762–11766, 1993). Such steric hindrance is removed from the molecule by the elimination of the methyl group from position 13 and can be re-established *via* a rearrangement of the substitution pattern by introducing a methyl group at position 10 of 13-dm retinal.

INTRODUCTION

The visual pigment rhodopsin constitutes the first component of the visual transduction cascade that converts the energy of incident light into a neuronal response (1). The absorption of light by rhodopsin is accomplished by the 11-*cis*-retinal chromophore that is covalently bound to a lysine residue of the protein *via* a protonated Schiff base. The mechanisms that underlie the most efficient (ϕ = 0.67) and extremely fast kinetics of the photoisomerization of the retinal chromophore (τ: *ca* 0.2 ps (2)) are still not understood in full detail. In particular the observation that the kinetics for the 11-*cis* → all-*trans* photoisomerization of rhodopsin is even faster than the all-*trans*-derived photochemistry of other retinal containing chromoproteins (*e.g.* bacteriorhodopsin, halorhodopsin) has evoked strong experimental efforts. A chromophore-inherent steric hindrance due to its 11-*cis* geometry (between the hydrogen atom at C-10 and the methyl group at C-13) has been suggested to facilitate the double-bond rotation during the photoisomerization (2,3). Whereas in solution the retinal molecule can escape such steric interaction by a distortion of adjacent single bonds, it was proposed that the protein binding site may enforce a steric stress in the polyene and prevent relaxation of the chromophore.

Besides site-directed mutagenesis of various amino acids, a number of retinal analogues have been synthesized, particularly in order to alter the geometric constraints that the retinal molecule experiences from the surrounding protein (4–6). In most cases, either an increase of steric hindrance has resulted (by the synthesis of 10-substituted retinals (7)) or a more relaxed conformation was achieved by the withdrawal of the methyl group from position 13, 13-demethyl (dm)†-retinal (8,9). In accordance with the above-discussed steric hindrance, strong effects on the extent and velocity of pigment generation, the quantum efficiency and the process of photobleaching have been observed for these retinal derivatives. In particular for the 10-substituted retinals, a decrease in the quantum efficiency was reported, suggestively caused by an enhanced interaction with the protein that was most prominent for 10-fluororetinal (10). If the steric hindrance is reduced by removing the methyl group from position 13, 13-dm-retinal, again a remarkable decrease of the quantum yield to less than half of its original value had been determined ($\phi \sim 0.30$ (9)), probably because this chromophore can adopt a more relaxed conformation in the protein binding pocket. With the aim of maintaining the absence of the 13-dm group, we synthesized 11-*cis*-10-methyl-13-dm-retinal as a retinal derivative that by a rearrangement of substituents should re-establish the bleaching capacity of the reconstituted new pigment to the high efficiency of the native pigment (Scheme 1).

MATERIALS AND METHODS

Chemical procedures. All chemicals used were of highest available quality.

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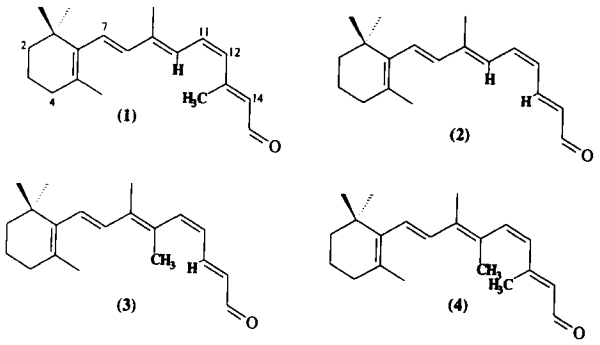
†*Abbreviations*: DIBAH, diisobutyl aluminum hydride; dm, demethyl; NH_2OH, hydroxylamine; VP, visual pigment.



Scheme 1. Structural formula of retinal (**1**) and retinal derivatives 13-dm (**2**), 10-methyl-13-dm (**3**) and 10-methyl retinal (**4**). All compounds are shown as 11-*cis* isomers. The substituents suggested to sterically interact are given in bold.

Synthesis of 10-methyl-13-dm-retinal (3) (Scheme 2). Compound **3** was synthesized following literature protocols. In brief, 2-β-ionylidene propionitrile (**7**) was generated by condensing β-ionone (**6**) with α-dimethylphenylsilyl propionitrile (**5**) (11,12). Compound **7** was converted into the corresponding aldehyde (**8**) by diisobutyl aluminum hydride (DIBAH) reduction. Condensation of **8** with 4-diethylphosphonato-crotonitrile (**9**) afforded 10-methyl-13-dm-retine-nitrile (**10**) that was converted into 10-methyl-13-dm-retinal (**3**) by DIBAH reduction. Because the reaction product was finally purified by preparative HPLC, no special care was given toward photoisomerizations. All reaction products showed spectral properties (UV/visible, infrared, ^{1}H-NMR and mass spectra) as expected.

HPLC. The HPLC system consisted of an LC-10AS pump (Shimadzu) for analytical chromatography or an LC-8A pump for preparative separation and a diode array detection unit (Shimadzu, SPD-M10AV). Separation was performed with silica columns, 5 μm, using *n*-hexane/diethylether (96:4, vol/vol) at a flow of 1 (analytical) or 7 mL/min (preparative separation) as eluent.

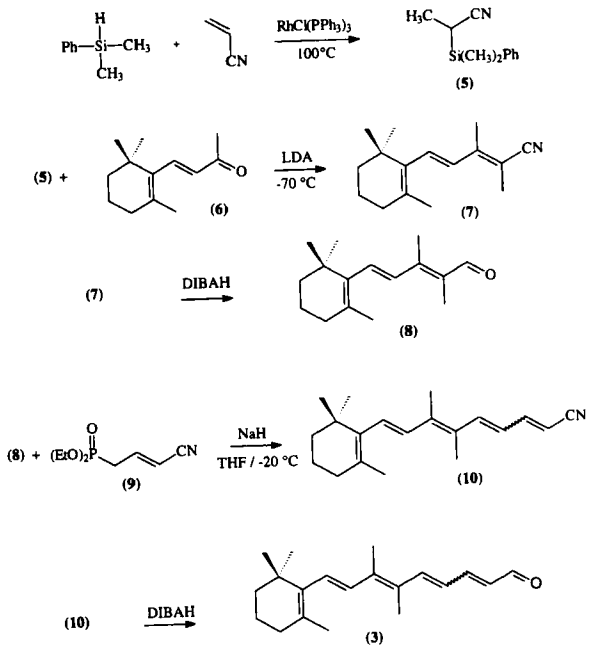
Photoisomerization. A sample of isomerically pure all-*trans* **3** in 2-propanol or hexane (1 m*M*) was irradiated with light from a projector bulb at a distance of *ca* 15 cm. Aliquots were withdrawn after several minutes (see data points in Fig. 1) and injected into the HPLC system.

Oxime formation. Oxime formation from all-*trans* **3** was performed under red light by the addition of a 100-fold molar excess of hydroxylamine to a 20–30 μ*M* solution of the aldehyde in ethanol. The reaction was followed spectroscopically and by thin-layer chromatography.

Biochemical procedures

Preparation of rhodopsin. Rhodopsin preparation from bovine eyes and bleaching of opsin followed literature protocols (13). The extent of regeneration of opsin using 11-*cis*-retinal was routinely between 60 and 70% of the original absorbance. Samples for quantitative bleaching were prepared by incubating opsin in Ringer's suspension (10 n*M* in a volume of 2.5 mL) in parallel with the 11-*cis* isomers of 10-methyl-13-dm-retinal (*ca* 40 n*M*) and retinal (*ca* 40 n*M*) at ambient temperature. Pigment formation was followed by absorption spectroscopy (Perkin-Elmer 356 spectrophotometer).

Quantitative bleaching. Fully reconstituted pigment samples (A_{max} was between *ca* 0.05 and 0.3 in a volume of 2.5 mL for the various preparations) were adjusted to 2.0 m*M* hydroxylamine (NH_2OH) and incubated in the dark until no further change of the absorbances was observed (usually less than 5% of the pigment decomposed). Before start of the bleaching experiments, an absorption spectrum was recorded that served as reference spectrum for the generation of difference spectra. Bleaching was performed with the light from a projector bulb (250 W). The irradiation beam passed through a neutral density filter (attenuation to 50%) and then through an interference filter (λ = 516 ± 11 nm). Irradiation of the samples in parallel (artificial pigment and regenerated rhodopsin) was performed for intervals of 5 s, after which time the samples were allowed to react in the dark for at least 15 min. After that time, a new



Scheme 2. Reaction scheme for the synthesis of 10-methyl-13-dm-retinal (**3**).

absorption spectrum was recorded. After exposure for a total of 1 min, the neutral density filter was removed and the samples were completely bleached by irradiation for 15 min, followed by recording of a final spectrum. The manipulation of the spectral changes that yields the photosensitivity curves was performed according to Dartnall (14) and as recently described (9,15).

RESULTS

Synthesis of 10-methyl-13-dm-retinal (3)

The yet unknown retinal derivative 10-methyl-13-dm-retinal (**3**, numbering of atoms follows the retinal nomenclature) has been synthesized starting from β-ionone (**6**) in an overall yield of *ca* 5%, see Scheme 2. Generation of the tetra-substituted double bond (C_9–C_{10}) turned out to be the most critical step in this reaction sequence. Formerly described re-

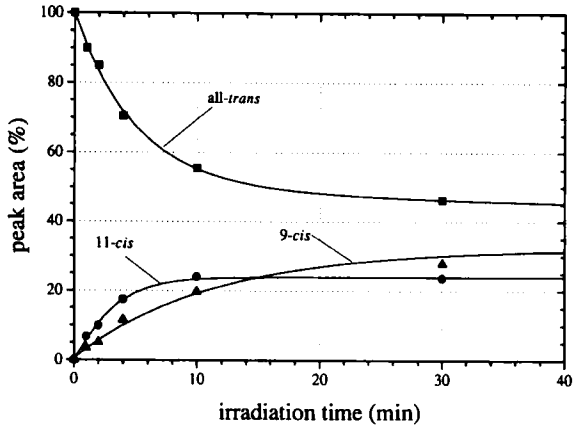


Figure 1. Time course of light-induced 9- and 11-*cis* isomer formation from 100% of all-*trans* (**3**). A solution of pure all-*trans* (**3**) in 2-propanol was irradiated (see Materials and Methods). At the given times, aliquots were withdrawn and subjected to HPLC analysis. The detection wavelength was set at 370 nm.

Table 1. The ^{1}H-NMR signals ($CDCl_3$) for the polyene side-chain hydrogen atoms of *E*- and various *Z*-isomers of 10-methyl-13-dm-retinal **3** and of 10-methyl-C_{15}-aldehyde **8** (coupling constants in Hz are given in brackets; except for the hydrogens at aldehyde and C_{14} only couplings along double bonds are given; couplings along single bonds were always on the order of 12 Hz)

Compound	H–C_7	H–C_8	CH_3–C_9	CH_3–C_{10}	H–C_{11}	H–C_{12}	H–C_{13}	H–C_{14}	CHO
All-*trans* **3***	6.66 (16.0)	6.41	2.06	2.02	7.3 (14.9)	6.53	7.24 (15.1)	6.17	9.55 (8.0)
13-*cis* **3**	6.66 (16.0)	6.40	2.05	1.99	7.24‡ (14.9)	7.22‡	7.05 (10.9)	5.82	10.21 (7.7)
11-*cis* **3**	6.55 (16.1)	6.28	2.0	1.85	6.63 (11.2)	6.25	7.27 (15.1)	6.15	9.55 (8.1)
9-*cis* **3**	6.72 (15.9)	6.29	2.02	1.96	7.38 (14.8)	6.46	7.22 (15.0)	6.16	9.54 (8.1)
9,13-di*cis*†	6.7 (15.9)	6.28	2.05	2.01	7.25‡ (14.8)	7.22‡	7.03 (10.8)	5.81	10.19 (7.8)
10-me-C_{15}-aldehyde **8**	6.95 (15.9)	6.45	2.08	1.85	—	—	—	—	10.26

*Positions of cyclohexenyl ring hydrogens for all isomers of **3**: CH_3–C_1: 1.02; H–C_2: 1.45–1.49; H–C_3: 1.57–1.63; H–C_4: 2.0–2.02; CH_3–C_5: 1.72.

†The 9,13-di*cis* isomer was contaminated by another compound that coeluted in the HPLC. This compound exhibited a singlet for the aldehyde proton and two signals in the range of the olefinic hydrogens (6.7 and 7.25 ppm).

‡Positions of H–C_{11} and H–C_{12} are partially obscured by the $CDCl_3$ signal and may possibly need to be interchanged.

actions using isopropylidene cyclohexylamine in a directed aldol condensation (16) were reported difficult to repeat by other groups (7). An alternative route based on the preparation of dimethyl-phenyl-silyl-2-propionitrile (**5**) and its condensation with β-ionone (**6**) (11,12) was found advantageous and yielded 2-β-ionylidene propionitrile (**7**) and after reduction with DIBAH the corresponding aldehyde (**8**). Further polyene chain extension followed the general reaction route by condensation of 10-methyl-C_{15}-aldehyde with 4-phosphonato-crotonitrile (**9**) in a Wittig–Horner-type reaction. The resulting C_{20}-nitrile (**10**) was converted into the target compound, 10-methyl-13-dm-retinal (**3**) by DIBAH reduction. Although stereoselective synthesis for polyenes has been reported (16–18), no special care was taken for light exposure and avoidance of photoisomerization. The resulting E,Z-isomeric mixture was finally irradiated into a photoequilibrium and isomerically purified by preparative HPLC. The isolated isomers were thermally stable in the dark at least for several weeks when kept under argon at −60°C.

Table 2. Absorption and bleaching parameters of 10-methyl-13-dm-retinal, its oximes and reconstituted VP

Compound	λ_{max} (nm, EtOH)	ϵ_{max} (M^{-1} cm^{-1})	*m**	ϕ
VP (**3**)†	505	44–58 000	0.02	0.59–0.46
Rhodopsin‡	500	40 000	0.02	0.67
11-*cis* **3**	378	24 900	—	—
All-*trans* **3**	384	44 200	—	—
All-*trans* **3** oxime	361	59 600	—	—
All-*trans* **1**‡	383	44 000	—	—
All-*trans* **1** oxime‡	357	54 300	—	—

*The parameter *m* defines the slope of the photosensitivity curves (insets in Fig. 3). The value given derives from an average of four individual experiments.

†See text for explanation of ranges.

‡Values for retinal, retinal oxime and rhodopsin were included as reference and were taken from the literature.

Photoisomerization

Photoisomerization of all-*trans* **3** in 2-propanol yielded three major photoisomers that after preparative HPLC and ^{1}H-NMR analysis (see Table 1) were found to be ≥95% pure. They were identified as 11-*cis*, 9-*cis* and all-*trans* **3** in the order of elution with λ_{max} 360, 364 and 370 nm (values given for the HPLC solvent and determined during HPLC separation with a diode array detector; values given in Table 2 refer to measurements performed in ethanol). In particular the 11-*cis* isomer exhibited a remarkably large "*cis*" peak at 250 nm, indicative of a strong inherent steric hindrance. The ratio between both absorption peaks is *ca* 1:1.35 (A_{360}:A_{250}), which is nearly identical to the ratio reported for 11-*cis*-10-methyl retinal (7). Though not a quantitative measure, the intensity of a "*cis*" peak often indicates a steric hindrance within the chromophore. This has been reported in the cited work (7) but is also found in the spectra of sterically hindered isomers of retinal in solution (17,19). Because besides the substitution pattern, the solvent used during the photoisomerization is also critical for the production of particular isomers (20–23), irradiation was performed in hexane, which as a solvent of low polarity has been found to facilitate the formation of 13-*cis* isomers of retinal derivatives void of the 13-methyl group (21). Irradiation in hexane again produced the same elution pattern of three major products in only slightly altered composition plus the formation of two additional isomers in low yield (between 2 and 3% of the total). Detailed ^{1}H-NMR analysis of these new HPLC peaks revealed NMR signals indicative of a 13-*cis* geometry (see Table 1). Besides the mono-13-*cis* isomer, the NMR signals of the second new peak gave evidence also for the 9–10 double bond being in the *cis* configuration. Thus, 13-*cis* and also 9,13-di*cis* (**3**) were formed upon irradiation in hexane. Apparently, the low probability for

13-*cis* isomer formation, already reported for 13-dm-retinal (20,21), is even further diminished for this retinal derivative.

The time course of the photoisomerization of all-*trans*-10-methyl-13-dm-retinal is shown in Fig. 1. Preferentially the 11-*cis* isomer is formed and reaches a plateau of *ca* 25% after 10 min (percentages are given as HPLC peak areas at 370 nm and do not directly reflect the molar ratio). Formation of the 9-*cis* isomer is a much slower process and is nearly complete (reaching *ca* 34%) after 2 h of continuous irradiation. Incubation of this isomeric mixture in the dark for 44 h did not cause a change of isomer composition.

Pigment formation with 11-*cis*-10-methyl-13-dm-retinal

The 11-*cis* isomer of 10-methyl-13-dm-retinal formed a visual pigment (VP) (λ_{max}: 505 nm) with remarkably slow regeneration kinetics. Complete pigment formation (no further increase at λ_{max}) required several hours. Furthermore, in cases of about equal stoichiometry of 11-*cis* **3** and opsin, no complete reconstitution of the bleached rhodopsin was observed as was evident from a relatively low absorbance increase around 500 nm and a remainder at 370 nm. Similarly unexpected, we observed a high sensitivity of the reconstituted pigment toward NH_2OH in the dark. Whereas 10-substituted retinals in rhodopsin were resistant to decomposition by NH_2OH concentrations of up to 40 m*M* in the dark (7), and 13-dm rhodopsin was found to be stable at least up to 8 m*M* NH_2OH (9), the 10-methyl-13-dm pigments underwent remarkable decomposition at such NH_2OH concentrations. Bleaching had thus to be performed at low NH_2OH concentration of *ca* 2 m*M*, under which conditions less than 5% of the reconstituted pigments decomposed in the dark. This resulted in slow kinetics and the transient accumulation of bleaching intermediates, and afforded long dark-incubation times after irradiation. It was also found from the exposure of reconstituted samples to NH_2OH in the dark that a remarkable amount of the 10-methyl-13-dm chromophore had been trapped in a reconstitution intermediate (λ_{max} around 440 nm) and did not form the pigment with 500 nm absorption (Fig. 2). This intermediate species was much more susceptible toward decomposition in the dark. Bleaching was started only after complete disappearence of the absorbance around 440 nm, in order to avoid interference of two different processes.

Quantitative bleaching of 10-methyl-13-dm rhodopsin

The quantum efficiency of a VP can be determined in a comparative manner when its photochemical reactions are investigated using (reconstituted) rhodopsin as reference (15,24,25). A series of bleaching experiments was performed with samples of rhodopsin and 10-methyl-13-dm rhodopsin, both regenerated under identical conditions from the same batch of opsin. Simultaneous irradiation of both samples was performed in the presence of 2 m*M* NH_2OH for intervals of 5 s each, and the photoisomerized molecules were allowed to react to completion for at least 15 min, after which time an absorption spectrum was recorded with the spectrum of the reconstituted pigment taken at time zero as reference (see Materials and Methods section). The changes in absorbance were read at 525 nm in order to minimize contributions from still present bleaching intermediates. When the absorption

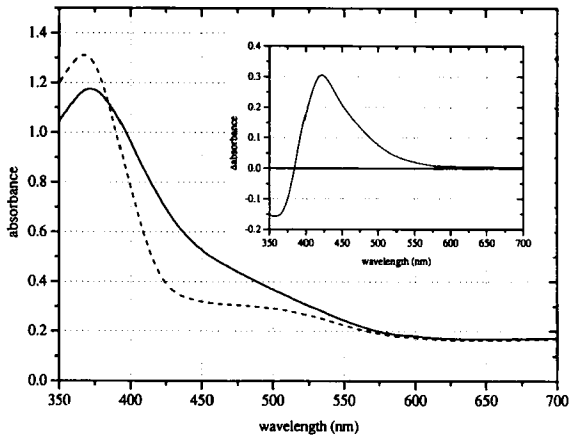


Figure 2. Absorption spectrum of reconstituted, 10-methyl-13-dm-retinal containing VP (solid line). The pigment was prepared by incubating 10 n*M* of opsin with 40 n*M* of 11-*cis* (**3**) in the dark at ambient temperature in a volume of 2.5 mL. The pH of the buffered solution was set to 7.2. No further increase around 500 nm was observed after 24 h. Dotted line: Resulting spectrum after incubation of the pigment with 2 m*M* NH_2OH in the dark for 2 h.

changes were recorded after a shorter dark-incubation time, a transient absorbance increase around 440 nm was observed, indicating changes in the bleaching kinetics and relatively long-lived bleaching intermediates. A typical bleaching experiment is shown in Fig. 3.

Due to the low NH_2OH concentrations that were tolerated by the artificial pigment, special care was taken to (1) the completeness of the thermal reactions after irradiation and to (2) suspected back reactions from bleaching intermediates or the released chromophore before its conversion into the oxime. The former case was accounted for by an extended dark incubation time (>15 min) before reading the absorbance changes. The latter situation was probed by incubating a *ca* 50% bleached sample in the dark for 30 min. No absorbance increase around 500 nm was detected when an absorption spectrum recorded 3 min after the irradiation was compared with a spectrum taken after a further incubation time of 30 min, indicating that no back reactions occurred.

Following the theoretical approach, a plot of $-\ln (10^{A_t - A_f} - 1)$ *vs* time of irradiation should yield straight lines for a bleaching experiment (A_t, A_f: absorbance differences at time [t] of irradiation and at final [f] bleach). From the slope of such lines the product from the absorption coefficient and quantum efficiency of a VP is derived. Employing identically treated rhodopsin as reference, all experimental constants disappear and the ratio from both slopes directly reveals the quantum efficiency of the unknown pigment. Inspection of the plots showed that the criterion of linearity was fulfilled for a total irradiation time of *ca* 30 s after which time deviations were observed. Thus, only this very early period was used for determining the quantum efficiency (insets to Fig. 3).

Determination of the absorption coefficient of 10-methyl-13-dm VP

Determining the quantum efficiency demands knowledge of the absorption coefficient. Routinely, the absorption coefficient of a new VP is derived from the ratio of the absorbance

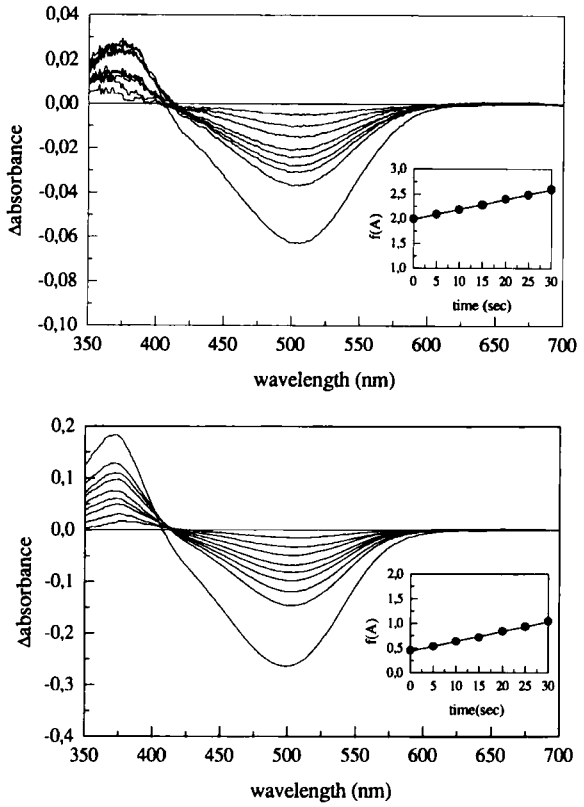


Figure 3. Examples for quantitative bleaching of (top) 10-methyl-13-dm-retinal-rhodopsin and of (bottom) reconstituted rhodopsin in the presence of 2 m*M* NH_2OH. The pH of the buffered solution was set to 7.2. The baselines (zero line) correspond to the spectrum of the fully reconstituted pigment recorded before the beginning of the experiment. Bleaching, *i.e.* reduction of the absorption band around 500 nm is shown as a difference spectrum (negative absorbance) and is accompanied by an absorbance increase around 360 nm, indicative of oxime formation. The difference spectra correspond to exposure to light of 516 ± 11 nm for 5, 10, 15, 20, 25, 30, 40, 55 s (curves 1–8). Due to deviation from linearity only the values for a total of 30 s were used for the generation of the photosensitivity curves. After these irradiation intervals, the samples were completely bleached after removal of the neutral density filter by an exposure to the light for 15 min (last difference spectrum). The insets show the plot of $-\ln(10^{A_t-A_f}-1)$ *vs* time of irradiance for each bleaching experiment.

changes at λ_{max} (bleached pigment) and at 360 nm (oxime formed) because the latter value can be determined independently. The absorption coefficient of all-*trans* 10-methyl-13-dm-retinal (ethanol) was determined as 44 200 M^{-1} cm^{-1}. Complete conversion into the oxime yielded the corresponding ϵ-value as 59 600 M^{-1} cm^{-1} (Table 2), which was reduced by *ca* 10% upon incubation with opsin.

The calculation of the absorbance ratio has to take into account the contribution of the pigment under investigation to the absorbance in the wavelength range of oxime formation (around 370 nm), which is not negligible. Because this pigment absorbance is concomitantly decreased upon bleaching, this process leads to too small apparent absorbance increases of the oxime band. This correction was recently determined (for rhodopsin) as *ca* 15% of the maximal VP absorption (15). In the particular case of 10-methyl-13-dm-retinal, however, the observed changes of absorbances at 360 and 500 nm showed a ratio of *ca* 0.9 (taken from spectrum before last), which would result in an absorption coefficient of about 58 000 M^{-1} cm^{-1} for the new pigment, a value too large and rather improbable for a visual pigment. Apparently, an additional process partially compensated for the rise of the oxime absorption. A possible explanation has been proposed for 10-methyl retinal-containing pigments for which a large "*cis*" peak was reported (7). Similarly, also the 10-methyl-13-dm pigment exhibits a "*cis*" peak of *ca* 25% of the maximal absorbance that upon bleaching interferes with the increase of oxime absorbance. Taking this additional contribution to the absorption changes around 360 nm into account one arrives at an ϵ_{max} of *ca* 44 800 M^{-1} cm^{-1} for 10-methyl-13-dm rhodopsin.

Quantum efficiency of 10-methyl-13-dm rhodopsin

The quantitative bleaching experiments performed with 10-methyl-13-dm rhodopsin and with regenerated rhodopsin yielded photosensitivity curves with nearly equal slopes *m* (Table 2). Employing the absorption coefficient for 10-methyl-13-dm rhodopsin and of the corresponding value for rhodopsin (ϵ_{max}: 40 000 M^{-1} cm^{-1}), one arrives at a quantum efficiency for the artificial pigment of 0.59, a value very similar to that of rhodopsin (0.67).

Formation of 10-methyl-13-dm isorhodopsin

Attempts to reconstitute an isorhodopsin derivative of 10-methyl-13-dm-retinal (9-*cis* isomer) and opsin were nearly unsuccessful and led to only a minimal absorbance increase around 505 nm. Attempts at quantitative bleaching turned out to be impossible due to low reconstitution yields for this pigment and a remarkably reduced bleaching rate.

DISCUSSION

Pursuing the investigation of the impact of chromophore structural changes on the photochemistry of rhodopsin, we have synthesized 10-methyl-13-dm-retinal, followed its photoisomerization kinetics and reconstituted its 11- and 9-*cis* isomer with bleached rhodopsin. This study was stimulated by the finding that the quantum efficiency of the VP containing 13-dm-retinal dropped remarkably to less than half (ϕ = 0.30 (9)) of the value of the native system (ϕ = 0.67). The alteration of the substitution pattern of 10-methyl-13-dm-retinal should further prove the hypothesis that a steric interaction in retinal facilitates the photoisomerization (2,26). The results reveal that with a different substitution pattern the steric interactions of the native chromophore can also be mimicked.

For a discussion of the changes of the quantum efficiencies for rhodopsins carrying sterically modified chromophores, recently reported reconstitution and bleaching experiments are of major importance. The 9-*cis* and 11-*cis* isomers of 10-methyl retinal had formerly been used for the reconstitution experiments of gecko and bovine visual pigments (27). Rhodopsins and isorhodopsins had been obtained in good yield (83 and 94%, respectively) from bovine opsin. However, low quantum efficiencies of *ca* 0.32 and 0.08 (10-methyl isorhodopsin) were reported. Interestingly, reconstitution experiments employing the gecko opsin, failed with 10-methyl retinal (27).

The chromophore structure investigated here apparently restores the high bleaching efficiency of rhodopsin. The slope for the photosensitivity, which was nearly identical to that of rhodopsin, already predetermines the range for the absorption coefficient. With the derived value of 44 800 M^{-1} cm^{-1} for the absorption coefficient of the VP, a quantum efficiency (0.59), very close to that of rhodopsin results. The absorption coefficient calculated here was confirmed by Mathies *et al.* who determined the absorption coefficient for this new VP by an alternative method (G. Kochendörfer, personal communication). Interestingly, if an absorption coefficient of *ca* 35 000 M^{-1} cm^{-1}, as was assumed for the 10-methyl retinal pigment (27), is adopted for this pigment, a quantum efficiency of *ca* 0.76 is calculated.

The data presented here further elucidate the effects of structural changes of the chromophore on the reconstitution and photochemistry of rhodopsin. With respect to reconstitution velocity and extent, the removal of the methyl group from position 13 slows down both parameters and also causes reduced stability toward NH_2OH in the dark. This effect is not compensated by the introduction of a methyl group at position 10 (10-methyl-13-dm-retinal, this study), whereas the bleaching capability can virtually be restored.

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