Quantitative Aspects of the Photochemistry of Isomeric Retinals and Visual Pigments

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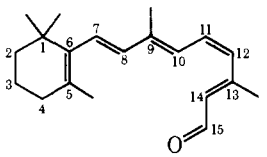
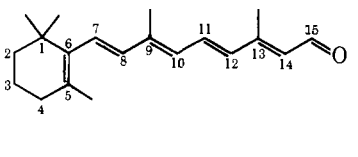
Abstract: The photoisomerization quantum efficiencies of *all-trans*-, 9-*cis*-, 11-*cis*-, 13-*cis*-, and 9-*cis*,13-*cis*-retinal were determined upon direct excitation in polar and nonpolar solvents and biacetyl triplet sensitization in a polar solvent. The four *cis*-retinals had quantum yields of 20% in nonpolar solvents and 4–5% in polar solvents. The biacetyl triplet sensitized quantum yields of the cis isomers were also ca. 20%, while *all-trans*-retinal underwent no detectable triplet isomerization. High-pressure liquid chromatography was used to analyze the photoproducts. It was found that only two types of carbon double bonds isomerized, the cis bonds and the terminal carbon double bond. Photobleaching of rhodopsin, isorhodopsin, and 9,13-isorhodopsin (isorhodopsin II) gave stereospecifically *all-trans*-retinal. When rhodopsin was treated with the unique triplet sensitizer, trimethyl-1,2-dioxetane, the protein was denatured and the chromophore isomerized. Based on product studies and quantum yield measurements, it is concluded that in the *cis*-retinals the photoisomerization occurred from the singlet and triplet states. In visual pigments it is not possible to assign the state from which isomerization occurred.

Introduction

The visual pigment rhodopsin was found by Wald and Hubbard[3] to consist of a chromophore, 11-*cis*-retinal (**1**), bound covalently to the apoprotein opsin. Recently, it has been shown that **1** is bound via a protonated Schiff base[4] to the terminal amino group of a lysine.[5-7] The primary photochemical event results when rhodopsin absorbs a photon of light (λ_{max} 498 nm, ϵ_{max} 40 600 M^{-1} cm^{-1}) and the 11-cis chromophore is isomerized to the all-trans form. This photoisomerization process, which occurs with an efficiency of 67%,[8] initiates a series of thermally induced chemical reactions[9] which alter the conformation of the protein and the chromophore. *all-trans*-Retinal (**2**) and opsin are the final products of this bleaching process.



1, 11-*cis*-retinal



2, *all-trans*-retinal

Both 9-*cis*-[3] and 9-*cis*,13-*cis*-retinal[10] bind with opsin to afford stable pigments, isorhodopsin (I) and 9,13-isorhodopsin (isorhodopsin II), respectively. Upon irradiation, these pigments also exclusively yield *all-trans*-retinal and opsin, with an efficiency of approximately 33%.[10]

The solution photochemistry of the retinal isomers has been previously studied, and the quantum yields of photoisomerization by direct photolysis[11,12] and triplet sensitization[12,13] have been measured. In addition, a number of theoretical studies concerning the photoisomerization of isomeric retinals have been conducted.[14] In spite of this widespread interest in the retinals, there are several interesting photochemical aspects of retinals that remain to be explained. For example, 9-*cis*-retinal is reported to have a higher photoisomerization efficiency than 11-*cis*-retinal,[11] while the reverse is true for their pigments;[10] reports of the triplet sensitized photoisomerization efficiencies of the retinals vary widely,[12,13] and it has not been established whether or not the triplet state is involved in the photoisomerization process in retinals and rhodopsin; the retinal photoisomerization products have not been specifically identified for each isomer, and although a multiple bond isomerization occurs in 9,13-isorhodopsin,[10] the photochemistry of 9-*cis*,13-*cis*-retinal itself is not known. In an attempt to clarify these points, we have made a quantitative study of the solution photoisomerization of the isomeric retinals and have examined the chromophores in the protein system. Our study includes the use of high-pressure liquid chromatography as an analytical tool in the redetermination of the photoisomerization quantum yields of retinals in solution upon direct excitation and triplet sensitization, the triplet sensitized isomerization of rhodopsin using a 1,2-dioxetane sensitizer, and an analysis of the primary photoisomerization products of retinals, rhodopsin, isorhodopsin, and 9,13-isorhodopsin.

Results

Retinal photoisomerization products were determined by high-pressure liquid chromatography (HPLC), while product studies on rhodopsin and other pigments were carried out by the denaturation–extraction process with methylene chloride[15] and subsequent HPLC analysis. Quantum yields for the direct photoisomerization (ϕ_{PI}) of *all-trans*-, 9-*cis*-, 11-*cis*-, 13-*cis*-, and 9-*cis*,13-*cis*-retinal in 3-methylpentane and methanol solutions at room temperature are summarized in Table I. Within experimental error we find that the cis isomers, 9-*cis*-, 11-*cis*-, 13-*cis*-, and 9-*cis*,13-*cis*-retinal, have similar photoisomerization yields in either 3-methylpentane or in methanol with the quantum yields being four–five times lower in the polar solvent. In addition to this polarity effect, ϕ_{PI} for 11-*cis*-retinal decreases with increasing viscosity, namely, ϕ_{PI} = 0.24, 0.15, and 0.12 in *n*-hexane, *n*-dodecane, and *n*-hexadecane, respectively.

Flash photolysis of *all-trans*-retinal in 3-methylpentane yielded only 13-*cis*-retinal as its primary photoproduct and 13-*cis*-retinal gave only *all-trans*-retinal, Table I. On the other hand, 9-*cis*- and 11-*cis*-retinal yielded products that reflect isomerizations about the cis double bond and the terminal carbon double bond, i.e., all-trans and di-cis products.[16] Direct excitation of 9-*cis*,13-*cis*-retinal afforded 9-*cis*- and 13-*cis*-retinal with a slight preference for the 13-cis product; *all-trans*-retinal was observed as a secondary photoproduct upon continued irradiation.[17]

In addition to the photoisomerization quantum yields being four–five times lower in methanol compared with 3-methylpentane, there are two notable changes in the product ratios. In methanol, photolysis of 9-*cis*-retinal yielded both 9-cis,13-cis and all-trans products, but the formation of *all-trans*-retinal is higher than in nonpolar solvent. Irradiation of 9-*cis*,13-*cis*-retinal in methanol produced some *all-trans*-retinal directly; however, the percentage conversion was ca. 25% and it is possible that *all-trans*-retinal is a secondary photoproduct.

Table I. Photoisomerization Quantum Yields for Isomeric Retinals by Direct Excitation[a]

	3-Methylpentane		Methanol		Hexane[c]
Isomer	ϕ_{PI}	Products[b]	ϕ_{PI}	Products	ϕ_{PI}
all-trans	0.04	13-*cis*	0.006	13-*cis*	0.06–0.2
9-*cis*	0.18	*trans*/9-*cis*,13-*cis* (2:1)	0.04	*trans*/9-*cis*,13-*cis* (4:1)	0.5
11-*cis*	0.24	*trans*/11-*cis*,13-*cis*[d] (5:1)	0.04	*trans*	0.2
13-*cis*	0.21	*trans*	0.05	*trans*	0.4
9-*cis*,13-*cis*	0.20	9-*cis*/13-*cis*[e] (1:1)	0.04	*trans*/9-*cis*/13-*cis* (1:8:10)	

[a] λ_e = 350 nm, 10-nm band-pass, ca. 10^{-4} M, aerated solutions, room temperature, ferrioxalate actinometer, <10% conversions, $\phi_{PI} \pm 15\%$. [b] Xenon flash lamp, $t_{1/2}$ = 50 μs, HPLC analysis. [c] A. Kropf and R. Hubbard, *Photochem. Photobiol.*, **12**, 249 (1970). [d] 11-*cis*,13-*cis*-Retinal has not been fully characterized. The tentative assignment is based on HPLC retention time and photoisomerization trends of the 9-cis isomer. The ratio of 5:1 was determined by assuming ϵ_{350} = 25 000 for 11-*cis*,13-*cis*-retinal. A trace of 11-*cis*,13-*cis*-retinal may be formed in methanol. [e] A small percentage (<~2%) may be formed directly; however, the percent conversions of this isomer is ~25%.

Table II. Biacetyl Triplet Sensitized Photoisomerization Quantum Yields of Isomeric Retinals[a]

Isomer	$^3\phi_{PI}$[b]	Products	ϕ_{ET}[c]
all-trans	<0.003	13-*cis*	0.66
9-*cis*	0.20	*trans*/9-*cis*,13-*cis* (5:1)	0.70
11-*cis*	0.17	*trans*	0.78
13-*cis*	0.15	*trans*	0.75
9-*cis*,13-*cis*	0.20	*trans*/9-*cis*/13-*cis* (2:3:5)	0.77

[a] CH_3CN solutions, deaerated by N_2 bubbling (180 s, −20 °C), λ_e = 450 nm, 10-nm band-pass, <20% conversions, [biacetyl] = 0.6 M. [b] $^3\phi_{PI}$ corrected for ϕ_{ISC} of biacetyl; $^3\phi_{PI} \pm 20\%$. [c] $\phi_{ET} = k_{ET}[\text{retinal}]/(k_D + k_{ET}[\text{retinal}])$ where $k_D^{-1} = \tau_p = 3.4 \times 10^{-4}$ s (photon counting techniques) and $k_{ET} = 2.5 \times 10^9\ M^{-1}\ s^{-1}$ (Stern–Volmer analysis of biacetyl phosphorescence quenching).

The triplet sensitized photoisomerization efficiencies ($^3\phi_{PI}$) of isomeric retinals were remeasured in acetonitrile solutions at room temperature using 2,3-butanedione (biacetyl) as a sensitizer (Table II). Biacetyl was employed since it could be removed easily by evaporation prior to HPLC analysis, because it could be selectively excited in the presence of ~5×10^{-5} M retinal and because a comparison could be made with previous studies on $^3\phi_{PI}$ of retinals[12,13] which also used biacetyl. Acetonitrile was used in order to observe the room temperature phosphorescence of biacetyl for the Stern–Volmer analysis. Previous studies were made in CCl_4 and hexane; however, at 0.6 M biacetyl our solutions would be quite polar in any solvent. The $^3\phi_{PI}$ of the *cis*-retinals were of comparable magnitudes, ca. 15–20% (Table II), whereas *all-trans*-retinal underwent no detectable triplet isomerization. We estimate the upper limit to be 0.003.[18] Upon direct excitation, 11-*cis*-retinal formed *all-trans*-retinal with a quantum efficiency of 0.12 in acetonitrile.

The product ratios resulting from the triplet sensitized photoisomerizations in acetonitrile are similar to those observed in methanol by direct excitation (Tables I and II). The notable exception is that triplet sensitization of 9-*cis*,13-*cis*-retinal produced *all-trans*-retinal directly in ca. 20% yields. Liu and Butt[19] report that for 2,6-dimethyl-2,4,6-octatriene, multiple bond isomerizations occur via the triplet state.

Using tetramethyl-1,2-dioxetane[20] as a thermally generating triplet sensitizer, *all-trans*- and 13-*cis*-retinal were isomerized to a steady-state mixture of 13:5:2 *all-trans*-, 13-*cis*-, and 9-*cis*-retinal in *n*-hexane and 13:3:4 in acetonitrile, respectively.

Irradiation of rhodopsin, isorhodopsin, and 9,13-isorhodopsin with a single 300-ns laser pulse (460 nm) in Ammonyx-LO yielded *all-trans*-retinal predominantly (Table III). The formation of *all-trans*-retinal from 9,13-isorhodopsin is a one-photon/two-bond photoisomerization. In spite of the lifetime of excited rhodopsin[21,22] being shorter than our laser pulse, we believe that it is a one-photon process for several reasons: only ~40% of the pigment was bleached, the number of photons and molecules was approximately equal, and kinetic studies[10] conclude that it is a one-photon process. It has been shown that varying amounts of *cis*-retinals are formed as a result of a one-photon/two-bond isomerization when rhodopsin is bleached in different detergents.[23]

In order to determine whether or not the chromophore in rhodopsin can be isomerized and detached from the protein by a triplet state reaction, rhodopsin was sensitized with the unique triplet sensitizer, trimethyl-1,2-dioxetane, which thermally decomposes to triplet excited states at temperatures where rhodopsin is thermally stable, ca. 0 °C. The dioxetane completely bleached rhodopsin in the dark at 0 °C (uv/visible absorption spectrum). The pigment was denatured, and the chromophore was extracted and analyzed by HPLC to yield a mixture of *cis*-,[24] 9-*cis*-, and *all-trans*-retinals. The ratio of the products is unimportant since multiple isomerizations could have occurred. Acetone, decomposed trimethyl-1,2-dioxetane, and tetramethyl-1,2-dioxetane[25] (which gives triplet acetone at higher temperatures) neither denatured rhodopsin (absorption spectrum) nor isomerized the chromophore (HPLC analysis) at 0 °C. Since trimethyl-1,2-dioxetane forms excited state acetone with an efficiency of ca. 10%[26] and has a triplet/singlet ratio of >300,[20] it is evident that rhodopsin undergoes a triplet sensitized decomposition to retinal and opsin similar to that observed for direct excitation.

Discussion

The quantum yields of photoisomerization of *all-trans*- (lower limit) and 11-*cis*-retinal upon direct excitation are similar to those reported by Kropf and Hubbard,[11] but those for 9-*cis*- and 13-*cis*-retinal differ from this report and are similar to ϕ_{PI} for 11-*cis*-retinal (Table I). The triplet sensitized photoisomerization quantum yields of *all-trans*- and 11-*cis*-retinal in acetonitrile are essentially identical with the values determined by Rosenfeld, Alchalal, and Ottolenghi[12] in hexane when biacetyl or biphenyl are used as triplet sensitizers. Also, as reported previously,[12] the addition of oxygen had no measurable effect upon ϕ_{PI} of 11-*cis*-retinal.

Direct excitation of the isomeric retinals in 3MP gave primary photoproducts corresponding to one carbon–carbon double bond isomerizations, including 9-*cis*,13-*cis*-retinal

Table III. Pulsed Laser Excitation of Visual Pigments in Ammonyx-LO[a]

Pigment	ϕ_{PI}[b]	Products[c]
Rhodopsin[d]	0.67	*all-trans*-Retinal, 2% 9-*cis*-retinal
Isorhodopsin	0.33	*all-trans*-Retinal, 5% 13-*cis*-retinal
9,13-Isorhodopsin	0.33	*all-trans*-Retinal, 7% 11-*cis*-retinal

[a] λ_e = 460 nm pulsed laser excitation, $t_{1/2}$ = 300 ns, ~40–50% conversions. [b] See ref 8, 10. [c] 11-*cis*-Retinal was an impurity in isorhodopsin and could not be analyzed; 9-*cis*-retinal was an impurity in 9,13-isorhodopsin and could not be analyzed. The amount of cis isomers is ±33%. [d] Laser photolysis of rhodopsin in different detergents yields varying amounts of *cis*-retinals, ref 23.

(*all-trans*-retinal was a secondary photoproduct[17]). Zechmeister's[27] assumption, that in polyenes only one double bond isomerizes per photon thus applies to this class of polyenals. One interesting result is that in spite of the observed solvent effects on ϕ_{PI} and the product ratios in methanol and 3-methylpentane, only the terminal and/or the cis double bond is photoisomerized. Wald, Hubbard, and co-workers[3,28,29] determined that photolysis of *all-trans*-retinal in ethanol at wavelengths greater than 410 nm yielded steady-state mixtures containing ca. 20% 11-*cis*-retinal.[28] In our experiments, irradiation of the trans isomer in methanol at 350 nm yielded only 13-*cis*-retinal. Because of the presence of two low-lying π,π^* states and an n,π^* state in retinals,[30,31] it is possible that a wavelength-dependent photochemistry exists.[11] At 77 K in 3MP or a mixed polar solvent, EPA, a wavelength-dependent fluorescence is observed for *all-trans*-, 9-*cis*-, and 13-*cis*-retinal.[30,32]

Retinals have a high intersystem crossing efficiency (ϕ_{ISC} ca. 0.5),[12,33-35] and the question of the involvement of the triplet state in the photoisomerization of retinals has received considerable attention. Because of the lack of isomerization upon triplet sensitization, Ottolenghi et al.[12] concluded that the photoisomerization of *all-trans*-retinal occurred through the singlet state, a result that we confirm. In addition, they concluded that 11-*cis*-retinal must either isomerize from the singlet state or from a vibrationally excited triplet state since addition of oxygen had no effect upon ϕ_{PI}. Our observations lead us to the conclusion that photoisomerizations of the *cis*-retinals occurs via the singlet as well as the triplet state.

In the product studies, 9-*cis*,13-*cis*-retinal gave ca. 5% *all-trans*-retinal upon direct excitation in methanol, whereas upon triplet sensitization a 20% yield is obtained in acetonitrile. If the triplet alone were to be involved in the photoisomerization process upon direct excitation we would expect to observe more *all-trans*-retinal in methanol. Quantum yields are another important factor. Becker and co-workers[32,36] report that no fluorescence is observed from the retinals at room temperature in solution $\phi_F < 10^{-3}$. Bensasson et al.,[33,34] Ottolenghi et al.,[12] and Fisher and Weiss[35] report that in *all-trans*- and 11-*cis*-retinal (nonpolar solutions, room temperature), intersystem crossing accounts for ca. 50% of the excitation energy. Since the total isomerization observed upon direct excitation is equal to the isomerization in the singlet and in the triplet states (eq 1), and for 11-*cis*-retinal, ϕ_{PI} = 0.24 and $^3\phi_{PI} \simeq$ 0.15–0.17 in nonpolar solvents,[12]

$$\phi_{PI} = {}^1\phi_{PI} + ({}^3\phi_{PI})(\phi_{ISC}) \quad (1)$$

we conclude that the triplet state alone cannot account for the observed photoisomerization in 11-*cis*-retinal; however, approximately one-third can be accounted for if this state were to be involved in the photoisomerization process. A similar argument may be proposed for the other *cis*-retinals if ϕ_{ISC} and $^3\phi_{PI}$ (nonpolar solvents) were of comparable magnitude as for the 11-cis isomer.

The question of the involvement of the triplet state in the photoisomerization process of rhodopsin and other pigments is a more important question since it relates directly to the mechanism of visual excitation. The triplet state of rhodopsin has never been observed by spectroscopic methods;[22,37] however, a rhodopsin model, the protonated 11-*cis*-retinal Schiff base, has $^3\phi_{PI} \simeq 1.0$.[38] The dioxetane results indicate that the triplet state can sensitize the photoisomerization of the chromophore in rhodopsin.[39] In addition, 9,13-isorhodopsin yields *all-trans*-retinal (a two-bond photoisomerization) in high yields upon direct excitation, a result which contrasts the solution photochemistry of the chromophore. While these results are suggestive of a triplet reaction, we must keep in mind that the protein can maintain the chromophore in a particular environment (twisting the chromophore, or nonbonded interactions) that may reorder the energies of the transition states of photoproducts to favor the formation of *all-trans*-retinal.[23] It is currently not possible to assign the state involved in the photoisomerization of visual pigments.

In conclusion, the photoisomerization quantum yields of 9-*cis*-, 11-*cis*-, 13-*cis*-, and 9-*cis*,13-*cis*-retinal, either by direct excitation or triplet sensitization, are identical within experimental error. Upon direct irradiation in nonpolar solvents, only one double bond is isomerized per unit photon of light, the isomerization occurring via the singlet as well as the triplet states. Regardless of multiplicity of the state and the nature of the solvent, only the cis and/or terminal carbon–carbon double bond undergoes photoisomerization. Pulsed laser excitation of rhodopsin, isorhodopsin, and 9,13-isorhodopsin in the detergent A-LO lead to *all-trans*-retinal as the major primary product but other *cis*-retinals are also formed as result of a one-photon/two-bond isomerization. It has been shown with trimethyl-1,2-dioxetane that visual pigments can undergo triplet isomerizations. However, it is not clear whether the triplet state is involved in the photoisomerization process upon direct excitation of visual pigments.

Experimental Section

Materials. *all-trans*-, 9-*cis*-, and 13-*cis*-retinal were purchased from Sigma Chemical Co. 11-*cis*-Retinal and 9-*cis*,13-*cis*-retinoic acid were a generous gift of the Hoffmann-La Roche Chemical Co. 9-*cis*,13-*cis*-Retinal was prepared from the acid.[10] Retinals were purified to 99+% by recrystallization, TLC, or HPLC. Rhodopsin was obtained from bovine retinae (Hormel-Austin Co) by the sucrose flotation method.[40] The synthetic pigments, isorhodopsin (I) and 9,13-isorhodopsin (isorhodopsin II), were prepared from the bovine opsin[10] and purified by chromatography on hydroxyapatite (1.5% Ammonyx LO).[41] 3-Methylpentane, 3MP (Matheson Coleman and Bell), *n*-hexane, *n*-dodecane, and *n*-hexadecane (K & K Labs) were purified by passing through an 18-in. column of silver nitrate–alumina.[42] Trimethyl- and tetramethyl-1,2-dioxetane were prepared according to literature methods.[43] 2,3-Butanedione (Aldrich) was distilled three times prior to usage. All work was carried out under a dim red light.

Quantum Yields. Quantum yields of photoisomerization by direct excitation were determined by irradiating a volume (0.5–2 ml) of retinal (concentration ~10^{-4}–10^{-5} M) for several minutes (3–5) at room temperature using a 150-W Xenon lamp with ¼ M monochromator from a Hitachi Perkin-Elmer MPF-3L spectrophotometer, λ_{ex} = 350 nm, 10 nm band-pass. The lamp flux was calibrated with potassium ferrioxalate actinometry.[44,45] The extent of photoisomerization was determined by high-pressure liquid chromatographic (HPLC) analysis using a 1 ft × ¼ in. μ-Porasil column, 1000 psi, ~2% ether/hexane, and 350 nm uv detection. The elution sequence was 13-*cis*-, 9-*cis*,13-*cis*-, 11-*cis*-, 9-*cis*-, and *all-trans*-retinal. The integrated areas were corrected for isomer response by dividing the area by ϵ_{350} nm for each isomer and corresponded to 5–10% products based upon a nonirradiated sample. The percentage absorption was calculated from the optical density, adjusted for the photoproduct absorption. Quantum yields were calculated from

$$\phi_{PI} = \frac{N_A VC\Delta}{tFA} \quad (2)$$

where N_A = Avogadro's number, V = volume in liters, C = concentration M, Δ = percentage conversion, t = time in seconds, F = the lamp flux in seconds, and A = percentage absorption at the excitation wavelength. Quantum yields determined in this manner were reproducible to ±15%.

Triplet Sensitized Quantum Yields. Triplet sensitized quantum yields were determined using 2,3-butanedione (biacetyl) as a sensitizer and the following equation:

$$^3\phi_{PI} = \frac{N_A VC\Delta}{tFA\phi_{ET}} \quad (3)$$

where $\phi_{ET} = k_{ET}[\text{retinal}]/(k_D + k_{ET}[\text{retinal}])$, F = the photon flux (corrected for ϕ_{ISC} biacetyl), and A = the percentage absorption of the sensitizer. Samples were prepared, irradiated, evaporated to dryness (by blowing nitrogen gas over the surface to remove the biacetyl), and HPLC analyzed. k_{ET} was determined by a standard Stern–Volmer analysis,[46] and k_D was calculated from $k_D = 1/\tau_p$ where τ_p was measured directly by photon counting techniques on an apparatus described elsewhere.[43] All solutions were deaerated by bubbling nitrogen gas directly into the solution for 180 s at −20 °C.

Product Studies. The products from the direct excitation and triplet sensitized isomerizations of retinals were determined by excitation with a Phase-R DL2100B tunable coaxial flash lamp pumped dye laser, half bandwidth of 300 ns, or a 50 μs xenon flash lamp followed by HPLC analysis.

Pigments were irradiated in 2% Ammonyx-LO detergent solutions using laser excitation λ_{ex} = 460 nm. The chromophores were extracted with cold methylene chloride[10] at 0 °C, centrifuged (12 000 rpm, 10 min), dried and analyzed by HPLC.

Thermolysis of Retinals with Tetramethyl-1,2-dioxetane. Approximately 10^{-4} M solutions of *all-trans*- and 13-*cis*-retinal in *n*-hexane or acetonitrile containing an ~100-fold excess of tetramethyl-1,2-dioxetane (TMD)[20] were incubated in degassed, sealed ampules at 37 °C for ~50 h. HPLC analysis of all solutions indicated that steady-state mixtures were reached. No isomerizations were observed for identical solutions without TMD. Attempts to obtain quantitative data, by varying TMD concentrations, were unsuccessful since large excesses of TMD were required.

Triplet Sensitization of Rhodopsin. Four aliquots of rhodopsin (1–2 mg each) in Triton X-100 were prepared at 0 °C under a dim red light. Trimethyl-1,2-dioxetane (50 mg) was added to the first aliquot, 50 mg of decomposed (by prior heating for 30 min at ~75 °C) trimethyl-1,2-dioxetane to the second, 50 mg of acetone to the third, and nothing to the fourth. After ~2 h, the long-wavelength absorption band characteristic of rhodopsin remained for solutions 2, 3, and 4; however, solution 1 was denatured. Extraction of the four solutions at 0 °C with anhydrous diethyl ether revealed that only for solution 1 was chromophore present (uv spectrum), while subsequent extraction of solutions 2, 3, and 4 with cold methylene chloride yielded 11-*cis*-retinal (HPLC analysis). 70% *trans*-, 20% 9-*cis*-, and 10% *cis*-retinal[24] was found for solution 1 (HPLC). Repeats of these experiments yielded similar results, i.e., rhodopsin was denatured by trimethyl-1,2-dioxetane. Similar treatment of rhodopsin with tetramethyl-1,2-dioxetane yielded no chromophore at 0 °C; however, at room temperature some denaturing of the protein (uv) was observed.[25] When 11-*cis*-retinal was treated with excess trimethyl-1,2-dioxetane at 0 °C in hexane, isomerization occurred (HPLC) as it did when treated at 37 °C with tetramethyl-1,2-dioxetane.

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(16) 11-*cis*,13-*cis*-Retinal has not been fully characterized. This tentative assignment is based upon HPLC retention time and the photoisomerization trends of 9-*cis*-retinal.
(17) *all-trans*-Retinal may be present as a primary, but minor, photoproduct, < ~2%.
(18) Although some singlet sensitization can occur since ϕ_{ISC} of biacetyl is 90%, we should point out that Es(biacetyl) < Es(retinal), ϕ_{ISC} for retinals is ~50% (ref 33–35), hence only 5% retinal singlets could be populated, and our reproducibility (±20%) is greater than the error introduced by possible singlet sensitization.
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