Kinetic Study of the Photoisomerization of a Protonated Schiff Base of 11-*cis*-Retinal over the Picosecond-to-Second Time Regimes

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Abstract: The kinetics of the photoisomerization of the protonated *n*-butylamine Schiff base of 11-*cis*-retinal dissolved in methanol was studied. Laser-flash and steady-state experiments were performed to monitor spectral changes after excitation in the picosecond, nanosecond, microsecond, and second time regimes. Substantiation of the 11-cis to all-trans isomerization was made. From the microsecond experiments, no triplet transients or comparably long-lived transient species were observed. In the nanosecond experiments, cis-to-trans isomerization was seen to occur in less than 1 ns after laser excitation. Results from the picosecond experiments showed that the observed lifetime of isomerization was less than 8 ps. This rapid observed isomerization rate for the protonated 11-cis Schiff base in solution is comparable to the rate seen in rhodopsin.

The primary event in the visual sequence has been shown to be the conversion of the membrane-bound pigment rhodopsin into bathorhodopsin upon the absorption of light. It has been widely accepted that this primary event involves the cis-to-trans isomerization of the retinyl chromophore about the 11–12 double bond.[1] Initial picosecond laser-flash experiments on cattle rhodopsin (solubilized in LDAO) suggested that the formation of bathorhodopsin occurs within 6 ps after irradiation at room temperature.[2] Recent picosecond absorption and fluorescence experiments have been done on bovine rhodopsin with the retinyl chromophore locked into the 11-cis geometry.[3] The results of these experiments and others have verified that the 11-cis to all-trans isomerization of the chromophore is the crucial primary event in the photolysis of rhodopsin and also established that the isomerization occurs on the picosecond time scale (or faster[4]).

Laser-flash spectroscopy has also been used to study the photoisomerization process in retinal Schiff bases and protonated Schiff bases.[5-8] In early experiments, the 1-amino-2-propanol protonated Schiff base of 11-*cis*-retinal had been studied by using nanosecond laser-flash spectroscopy.[5] It was concluded from that work that the isomerization occurred in less than 10 ns in methylcyclohexane, although the photoproducts were not identified.

In subsequent nanosecond and picosecond experiments, both the 1-amino-2-propanol and *n*-butylamine protonated 11-*cis*-retinal Schiff bases were studied in methanol.[6] The picosecond experiments in both cases revealed an initial bleaching followed by a partial recovery to still negative optical density readings over the first 4.1 ns (λ_{mon} = 485 nm). From nanosecond studies on the *n*-butylamine-protonated Schiff base, the lifetime for the formation of the all-trans isomer was reported to be 11 ± 3 ns.

The reported lifetimes for the isomerization of the unbound chromophore and rhodopsin differ by as much as 4 orders of magnitude. This suggests that there could be a great difference in the dynamics of the photoisomerization of the model-protonated Schiff base in solution and when the chromophore is bound to opsin. This has led most investigators to believe that the protein–chromophore interactions are largely responsible for the increased isomerization rate, and, therefore, research emphasis has shifted away from the unbound chromophore in solution.[9,10]

We have recently completed a comprehensive study on the photoisomerization of both the protonated and unprotonated *n*-butylamine Schiff bases of 11-*cis*-retinal in solution.[8] From laser-flash and steady-state experiments, we observed a marked dependence of the photoisomerization quantum yield (ϕ) on the solvent polarity for the unprotonated Schiff base. In addition, the competitive formation of a nonexcited state transient was also noted after excitation of the unprotonated Schiff base. This long-lived transient was seen to thermally decay back to the 11-cis isomer. However, in the case of the protonated Schiff base, neither a solvent dependence nor a competitive formation of a long-lived transient was seen. The only detectable photochemical pathway was the 11-cis to all-trans isomerization. A detailed mechanism of the first step in the visual process was proposed.

In order to clarify the photoisomerization kinetics for the free chromophore, we have done further laser-flash experiments on solutions of the protonated *n*-butylamine Schiff base of 11-*cis*-retinal (henceforth called H^+-11-*cis*-SB) in methanol. Laser-flash experiments were done in the microsecond (0.5–400), μs, nanosecond (1–100), ns, and picosecond (8–100), ps, regimes all at an exciting wavelength of 532 or 530 nm. Steady-state irradiation experiments (second regime) were also employed to study the nature of the isomerization process.

Experimental Section

The 11-*cis*-retinal was obtained as a gift from Hoffmann-La Roche. TLC analysis of the retinal revealed the presence of no isomers other than 11-cis. The *n*-butylamine Schiff base was prepared as before [11] and protonated with trichloroacetic acid. Spectrograde methanol was dried over 3-Å molecular sieves and used without further purification.

The microsecond laser-flash experiments were carried out by using the 532-nm second harmonic from a Nd:YAG Q-switched laser. The laser-flash system has been described previously.[12] Laser pulses of 2–6 mJ and duration of 11 ns were employed. Power dependence studies showed that these energies fell into the linear region when optical density changes were compared with laser power. The H^+-11-*cis*-SB solutions used in the microsecond experiments had concentrations of 2×10^{-5} M. Optical density changes were monitored over a range of wavelengths in 10-nm steps, and differences (ΔOD spectra) were constructed.

The nanosecond experiments used the second harmonic light generated from a mode-locked Nd:YAG laser (200-ps fwhm, ~24-mJ full output). The intensity of the laser radiation on the sample was controlled by the use of wire-mesh screens. The kinetic absorption spectrometer (for de-

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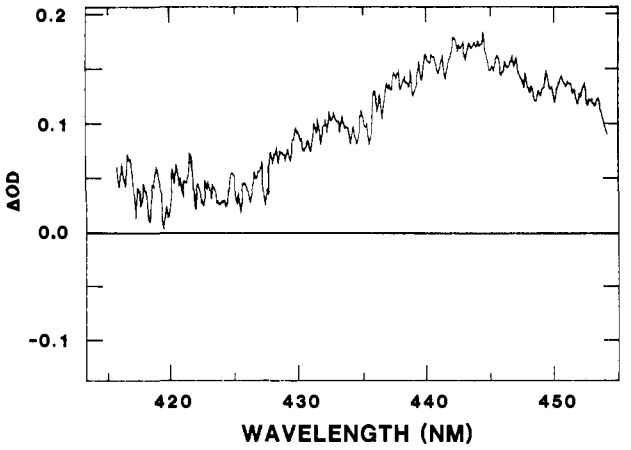

Figure 1. Optical density changes recorded at a delay time of 0 ps for the H^+-11-*cis*-SB in methanol.

tection on the nanosecond time scale) consisted of a continuously operating 150-W xenon arc lamp (monitoring source), two electromechanical shutters, quartz lenses, target cell holder, a Spex Minimate monochromator, and a photomultiplier tube (Hamamatsu R928). Detector output wave forms were processed by a Tektronix R 7912 digitizer and computer combination (PDP 11/70). The rise time of the detection system (photomultiplier tube and digitizer) was approximately 0.8 ns. Schiff base concentrations comparable to those used in the microsecond experiments were employed. To avoid photodegradation of the sample, the solution in the cuvette was changed after every two shots.

The picosecond experiments employed the second-harmonic 530-nm light generated from a mode-locked neodymium phosphate glass laser (8-ps pulse width). Absorbance changes produced by excitation were recorded over a 42-nm bandwidth at certain fixed delay times between excitation and probe pulses. The laser, optical arrangement, detection system, and data acquisition system are described elsewhere.[13] The H^+-11-*cis*-SB solutions used in the picosecond experiments had concentrations of 2.5×10^{-4} M. The total energy of the excitation pulses was in the range of 0.3 ± 0.1 mJ.

Steady-state irradiation experiments were employed to measure absorption changes after irradiation of the solutions for periods of up to 30 s. The excitation source consisted of an Optics Technology interference filter (No. 533) or a Jarell-Ash monochromator (6.5 nm/mm dispersion). Absorption changes before and after irradiation were recorded on a Cary 15 spectrophotometer.

Analysis of the primary photoproducts after laser excitation was accomplished by using TLC. The components remaining in solution after laser pulsing (530 nm, 8 ps) were first neutralized with *n*-butylamine and then separated on silica gel plates when using diethyl ether as the eluting solvent (R_f values for the Schiff bases were reported earlier).[14] Analysis of the photoproducts after steady-state irradiation was also done by using HPLC. After irradiation, the protonated Schiff bases were neutralized with triethylamine and then hydrolyzed back to their corresponding retinals and separated on a μ-Porasil column using 6% ether in hexane as the eluting solvent. Previously, high-performance TLC was also employed to verify 11-cis to all-trans isomerization; see the section on results.[8]

Results

Picosecond, nanosecond, and microsecond laser-flash experiments and steady-state experiments were employed to monitor spectral changes over a wide range of time scales. In the picosecond experiments, optical density changes were measured over the range of 416–456 nm. Accurate monitoring of OD changes at longer wavelengths was not possible due to Stokes and anti-Stokes lines in the continuum in the wavelength vicinity of 530 nm (the continuum was generated with a portion of the 530-nm pulse). For the H^+-11-*cis*-SB in methanol, an all-positive ΔOD spectrum was observed at a delay time of 0 ps; see Figure 1. This absorption band showed a maximum at 440–450 nm. This spectrum was not seen to change its wavelength maximum or

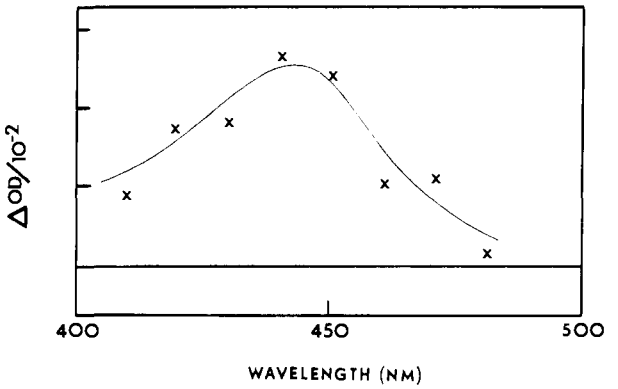

Figure 2. Difference (ΔOD) spectrum obtained for the H^+-11-*cis*-SB in methanol recorded as early as 1 ns after 532-nm laser excitation.

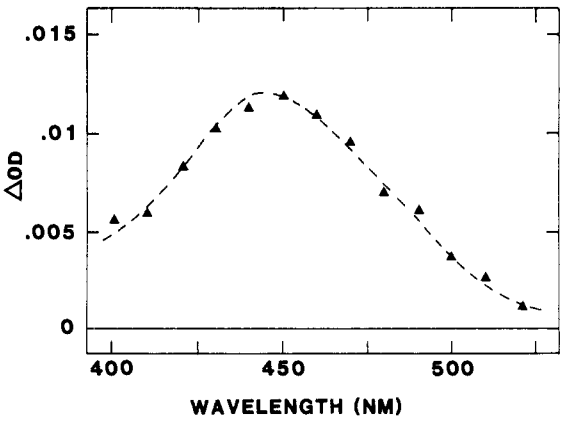

Figure 3. Difference (ΔOD) spectrum obtained for the H^+-11-*cis*-SB in methanol recorded up to 400 μs after the 532-nm laser flash.

change in magnitude at later delay times of 20 and 100 ps.

This rapid change in optical density was confirmed by results from nanosecond experiments on nitrogen-degassed solutions. An immediate rise in the optical density was seen in less than 1 ns after laser excitation, when monitored at 450 nm. After this initial rise, no further absorption changes were noted up to 100 ns. An all-positive ΔOD spectrum was recorded from 410 to 480 nm (Figure 2) with a maximum at ~445 nm. In addition, no detectable changes (positive or negative) in optical density were seen when monitoring from 550 to 700 nm. (Cutoff filters used to eliminate scattered laser light from entering the detection system prevented our monitoring in the range of 490–550 nm).

Net positive ΔOD's were also seen in the microsecond experiments for the H^+-11-*cis*-SB in methanol with a maximum occurring at ~445 nm. The rise time for this spectrum was also limited by the instrumental rise time (<0.5 μs). Optical density changes were recorded from 400 to 550 nm. Spectra constructed from optical density changes recorded at 10-nm intervals were not seen to change up to 400 μs. No triplet transients or other comparably long-lived species were seen. Figure 3 shows a typical ΔOD spectrum for the H^+-11-*cis*-SB in methanol. These spectra were not seen to change when nitrogen or oxygen were bubbled through the solution. These results also appeared to be insensitive to the presence of small amounts of water in solution.

Steady-state experiments revealed positive optical density changes in the wavelength region of 360–600 nm immediately (seconds) after irradiation of solutions of the H^+-11-*cis*-SB, with an absorption maximum at 445 nm. An isobestic point at ~350 nm characteristic of a cis-to-trans isomerization was also observed.

The optical density changes noted in each laser-flash and the steady-state experiments were indicative of an 11-cis to all-trans isomerization after excitation of the H^+-11-*cis*-SB. The ΔOD spectra for the H^+-11-*cis*-SB in methanol obtained in each of the different time regimes (pico-, nano-, and microseconds and seconds) all demonstrated positive optical density changes and the same absorption maximum (λ_{max} = 440–450 nm). This same

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absorption maximum is seen in the $\Delta\epsilon_{ct}$ spectrum for an 11-cis to all-trans isomerization ($\Delta\epsilon_{ct} = \epsilon_t - \epsilon_c$, where ϵ_c and ϵ_t are the extinction coefficients for the 11-cis and all-trans isomers, respectively).

Further substantiation of cis-to-trans isomerization was made by TLC. Separation of components in solution after laser pulsing revealed spots corresponding only to the 11-cis and all-trans isomers. Previously, we employed high-performance TLC (HPTLC) to identify the photoproducts after irradiation (λ_{exc} = 355 nm) of the H$^+$-11-*cis*-SB,[8] where the protonated Schiff bases were hydrolyzed back to their corresponding retinals and separated (by the HPTLC method). We found that the all-trans isomer was the only detectable photoproduct formed after irradiation. It should be noted that we also found that the quantum yield of isomerization of the H$^+$-11-*cis*-SB was essentially independent of excitation wavelength.[8] In addition, HPLC experiments also confirmed that the H$^+$-*all-trans*-SB was the only photoproduct.

In dichloromehtane, an observed lifetime of 300 ps for isomerization of H$^+$-11-*cis*-SB to the all-trans isomer has been found from preliminary laser-flash and steady-state experiments.

The present results confirm that 11-cis to all-trans isomerization is the only significant photochemical process seen after excitation of the protonated 11-cis Schiff base. This is in contrast to the unprotonated 11-cis Schiff base where the competitive formation of a nonexcited-state transient is seen in microsecond laser-flash experiments and also steady-state experiments after excitation, in addition to cis-to-trans isomerization.

Discussion

The results on photoisomerization over a wide range of time domains has led us to the conclusion that the H$^+$-11-*cis*-SB isomerizes with a lifetime much shorter than previously reported. From our picosecond experiments on the H$^+$-11-*cis*-SB in methanol, we observed a spectrum consistent with an 11-cis to all-trans isomerization at a delay time of 0 ps. Also, in the nanosecond, microsecond, and steady-state experiments, all-positive ΔOD spectra with the same wavelength maximum were observed. These results indicate that the absorption seen in the picosecond experiments could not be due to a short-lived transient species because no shifting in the absorption maximum as a function of time was seen nor was there decay of this spectrum over the picosecond-to-second time regimes. Therefore, we believe that the spectrum observed at the delay time of 0 ps was indicative of the formation of the all-trans ground state in a time less than 8 ps, the width of the excitation pulse.

The discrepancy between our results for the kinetics of isomerization and the kinetic results reported in the previous[6] pico- and nanosecond studies of the H$^+$-11-*cis*-SB may be the result of a couple of factors: (1) differences in sample preparation and (2) potentially large differences in laser power.[15]

The H$^+$-11-*cis*-SB is inherently a very difficult system to study by laser-flash spectroscopy. The relatively high absorption of the Schiff base at its maximum coupled with its low absorption at 532 nm (when methanol is the solvent) make it particularly troublesome to detect absorption changes when using low laser powers. The total energy of the excitation pulses in our picosecond experiments was in the range of 0.2–0.4 mJ. This energy range was the same one used by two of the present authors[13] for model hemoglobin systems and was found to be low enough to avoid nonlinear effects. It should be noted that these systems had molar extinction coefficients as much as 3 times greater (at 530 nm) than the H$^+$-11-*cis*-SB. This one fact suggests that the H$^+$-11-*cis*-SB should be even less susceptible to significant ground-state depletion at these energies and also to any significant nonlinear effects caused by multiphoton absorption. On the basis of preliminary data, a room-temperature fluorescence lifetime for the H$^+$-11-*cis*-SB in methanol of <10 ps[16] would make it appear that the excited state is very short-lived. This short lifetime would also decrease the chances of ground-state depletion. We have independently measured the lifetime of fluorescence to be <30 ps (instrument limited).

We have now observed an isomerization rate for the H$^+$-11-*cis*-SB which is comparable to that of rhodopsin. This rapid isomerization rate, together with the observation that the presence of oxygen in solution does not affect the isomerization process, suggests that isomerization occurs out of the singlet manifold after excitation. With observations of very low quantum yields of fluorescence[17] and triplet formation,[7] the only major pathways out of the excited singlet state of the H$^+$-11-*cis*-SB appear to be isomerization and radiationless return to the 11-cis ground state. These competing pathways can be summarized in the following mechanism:[18]

$$S_0\ (11\text{-cis}) \xrightarrow{h\nu} S^*\ (11\text{-cis})$$

$$S^*\ (11\text{-cis}) \xrightarrow{k_c} S_0\ (11\text{-cis})$$

$$S^*\ (11\text{-cis}) \xrightarrow{k_t} S_0\ (\text{all-trans})$$

Results from molecular orbital and molecular dynamics studies on an isolated ("gas phase") model similar to the H$^+$-11-*cis*-SB have predicted that cis-to-trans isomerization can occur in ~2 ps.[10] In light of the previously reported isomerization lifetime of 11 ns for the H$^+$-11-*cis*-SB in methanol,[6] it was concluded that a solution environment (particularly a polar one) significantly alters the excited-state potential surfaces and increases the energy barrier for isomerization and that a hydrophobic environment provided by the opsin protein was necessary for efficient isomerization.[10] It is clear from our data for the H$^+$-11-*cis*-SB in methanol that the lifetime for isomerization is very short and is comparable to the lifetime of rhodopsin in the absence of any hydrophobic protein environment.

Our results should further change the view of the role of the opsin protein in the primary steps of the visual sequence. The measured isomerization rate, together with the relatively high quantum yield of isomerization (ϕ = 0.26 in methanol),[8] indicates that rapid and efficient isomerization is intrinsic to the protonated chromophore in solution independent of the protein. As described in our recent work,[8] protonation of the 11-cis Schiff base provides for the necessary mixing of the "$^1A_g{}^{*-}$" and "$^1B_1{}^{*+}$" states and for the sufficient lowering of the activation energy for isomerization. This is not to say that the protein–chromophore interaction does not play any role in the isomerization process, but the protein very probably plays a more important role in the remaining steps of the energy transduction process.

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Registry No. H$^+$-11-*cis*-SB, 56053-76-0; *all-trans*-SB, 36076-04-7.

(15) In the previous picosecond study of the H$^+$-11-*cis*-SB (ref 5), no mention is made of the energy of the excitation pulse. From data given in that reference, we surmise that the energy of their excitation pulse could have been as great as 5 mJ per pulse.

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(18) The two pathways from the excited state, S* (11-cis), may actually exist as a series of steps leading to the ground state. Taking each of these steps as a first-order process, we can assume that each overall pathway is also first order and can be expressed by one rate constant.