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Photoisomerization Kinetics of 11-*cis*-Retinal, Its Schiff Base, and Its Protonated Schiff Base

Eva L. Menger and David S. Kliger*

Contribution from the Division of Natural Sciences, University of California, Santa Cruz, California 95064. *Received July 18, 1975*

Abstract: Kinetics of the photoisomerization of retinal (RET), a retinylidene Schiff base (SB), and a protonated Schiff base (PSB) have been studied using nanosecond laser photolysis techniques. Two paths of isomerization were observed, one occurring over 10's of nanoseconds and one occurring instantaneously in the time frame of the experiment. Data are presented identifying the former process as being mediated through the triplet state. Isomerization is the net result of a delicate balance between competing processes, and the relative amounts of isomerization via the two paths vary with the compound and with the solvent. A model is presented which allows an adequate and consistent interpretation of the experimental results. The significance of our results to visual pigments is discussed. The protonated Schiff base, which is the closest chemical analogue to rhodopsin we investigated, isomerizes through a singlet mechanism. The extremely rapid rate we observe for this isomerization is consistent with prelumirhodopsin being photogenerated from rhodopsin in picoseconds.

When rhodopsin is exposed to light it undergoes a series of spectral changes corresponding to the presence of various transient photoproducts. The first observable spectral change at 77 K or above involves absorption of a photon by rhodopsin to yield prelumirhodopsin. This photoproduct has been isolated and studied by cooling the rhodopsin solution to liquid nitrogen temperature.[1-3] Subsequent warming of the solution results in the formation of successive transient species. Only the first reaction in the series requires light for its initiation. The subsequent reactions, the so-called "dark reactions", proceed spontaneously at physiological temperatures in the absence of light.

The chromophore in rhodopsin is the 11-cis isomer of retinal[4] bound to the protein, opsin, through a protonated Schiff base linkage.[5-7] The final products of photolysis of bovine rhodopsin are opsin and retinal. This solution appears yellow compared to the red initial solution so it has become customary to refer to the photolysis process as "bleaching". When bleached with light the chromophore is released from the protein in the form of *all-trans*-retinal.[4] When "bleached" with heat, however, the opsin is denatured and releases the chromophore as 11-*cis*-retinal.[8] Thus, one deduces that the interaction of light with rhodopsin causes the cis → trans isomerization of the chromophore.[9] Since only the transition from rhodopsin to prelumirhodopsin requires light, one might suspect that this step involves isomerization of the retinylidene chromophore about the 11-12 double bond.

Recently several workers have investigated the kinetics of the photolysis of rhodopsin to produce prelumirhodopsin at physiological temperatures. Using a pulsed nitrogen laser to photolyze rhodopsin, Rosenfeld et al. found that the buildup of prelumirhodopsin occurred on a time scale faster than 10 ns.[10] Using a mode locked Nd^{3+} glass laser, Busch et al. established that the upper limit for the buildup of prelumirhodopsin is less than 6 ps.[11] Thus, there is strong evidence documenting the rapid conversion of rhodopsin to prelumirhodopsin and linking this reaction to a cis → trans isomerization.

Much work has been done in trying to determine the mechanism for isomerization of visual pigments. Most of this work has involved studies of the isomerization of retinal as a model system for the chromophoric moiety. Unfortunately, much of this work, both theoretical and experimental, is inconclusive or contradictory.

Some theoretical studies indicate that there is a high barrier to rotation for the isomerization of 11-*cis*- to *all-trans*-retinal out of the first $\pi\pi^*$ singlet state but a low barrier from the first $\pi\pi^*$ triplet state.[12,13] However, Becker et al. find low barriers to rotation in both the singlet and triplet states[14] and Pullman et al. find high barriers to rotation in both states.[15]

Experimental results tend to support the hypothesis that 11-*cis*-retinal isomerizes efficiently out of the first triplet state but the degree and nature of the triplet involvement are not clear. Raubach and Guzzo report that the isomerization quantum yield, ϕ_{isom}, is about 0.75 out of the triplet state, as determined by triplet sensitization studies.[16] Taking the intersystem crossing quantum yield to be $\phi_T = 0.11$[17] and the isomerization yield to be $\phi_{isom} = 0.2$[18] following direct irradiation, they determined that about half the isomerization of

11-*cis*-retinal is mediated through the first triplet state and half through the first excited singlet state. This result is incompatible with the more recent determinations of intersystem crossing quantum yields. These vary from 0.6 ± 0.1,[19] to 0.7 ± 0.1 for *all-trans*-retinal and 0.6 ± 0.1 for 11-*cis*-retinal,[20] to 0.50 ± 0.05.[21]

Rosenfeld et al.[20] interpret their results to give a different picture from that of Raubach and Guzzo. They state that isomerization takes place from a thermally excited level of the first triplet state. This state, they suggest, is reached by intersystem crossing from the singlet state to the second triplet state followed by an internal conversion to the thermally excited first triplet.

Rosenfeld et al.[22] also looked at the isomerization of retinal Schiff bases. They found that $\phi_{isom} = 0.45 \pm 0.1$ when the 11-*cis*-retinal is excited by triplet sensitizers but ϕ_{isom} was only 0.004 ± 0.001 when the retinal was irradiated directly. Although Busch et al. state that the extremely fast buildup of prelumirhodopsin is not inconsistent with known rapid radiationless processes such as some intersystem crossing rates, Rosenfeld et al. point out that one would have to postulate an unusually fast deactivation process out of the triplet state were it a precursor to prelumirhodopsin.[22] A triplet mechanism is also made questionable since ϕ_T for a protonated Schiff base has been found to be less than 0.001.[21]

The picture presented above gives a rather fragmentary view of the isomerization process of the chromophore in visual pigments. We sought to clarify the issue through a direct measurement of the kinetics of the isomerization of 11-*cis*-retinal (RET), 11-*cis*-retinylidene Schiff base (SB), and 11-cis-protonated Schiff base (PSB). This was done by taking advantage of the differences in the spectra of these species when they are in their 11-cis or all-trans conformations. In all cases the extinction coefficient of the low-energy or α peak increases and that of the higher energy or β peak decreases when the molecule goes from the 11-cis to the all-trans conformation.[23,24] Thus by photolyzing the species with a short pulse of light and observing the increase of the absorbance as a function of time at the α-peak maximum wavelength, a direct measure of the isomerization kinetics can be made.

Experimental Section

Material and Methods. 11-*cis*-Retinal was obtained as a gift from Hoffmann-La Roche Corp. TLC analysis carried out in the dark revealed no other retinal isomers present. Schiff base of the retinal and 1-amino-2-propanol was made by the method described by Erickson.[25] All reactions were carried out under dim red light. Samples were stored under nitrogen at −20 °C. Protonation of the Schiff base was accomplished by acidifying the Schiff base solutions (~5 × 10^{-5} M) with reagent grade trichloroacetic acid (TCA, ~10^{-2} M). Solvents in all cases were Spectrograde and used without additional purification. Degassing of the solvents was accomplished by the freeze-pump-thaw method. Isomeric purity of the RET, SB, and PSB solutions was checked by taking their spectra on a Cary 14 spectrophotometer before and after irradiation of the samples.

The equipment used to perform these experiments utilized a nitrogen laser as a photolyzing source and a xenon flashlamp as an analyzing source. The xenon lamp produced a light pulse with energies of approximately 8 J lasting approximately 3 μs. The duration of the lamp was lengthened to 10 μs for degassed samples where transients had longer lifetimes. The nitrogen laser is a Molectron UV-1000 which produced 1 mW pulses with durations of 10 ns. The two beams intersected in a 1-cm pathlength cell. The detection system monitoring the flash intensity as a function of time consisted of a Bausch and Lomb 0.25-monochromator and a 1P28 photomultiplier whose output was measured on a Tektronix 475 oscilloscope. The detection system had a 7-nm or better spectral resolution and a risetime faster than 2 ns. Care was taken to ensure that photolysis of the sample by the flashlamp was negligible. Maximum overlap of the flashlamp and laser beams was accomplished by monitoring the triplet–triplet absorption of retinal at 448 nm and adjusting the position of the beams to maximize the signal. The viewing wavelength was then changed to the maximum of the α peak of each molecule studied in order to monitor changes in the sample optical density due to isomerization.

Results and Discussion

The first isomerization process we studied was that of 11-*cis*-retinal in methylcyclohexane (MCH). The retinal α peak appears at 373 nm in this solvent and has an extinction coefficient of about 25 400 for 11-*cis*-retinal and 48 000 for *all-trans*-retinal.[21,23] The triplet quantum yield for *all-trans*-retinal is about 0.5 and the triplet–triplet absorption shows a maximum at 448 nm with an extinction coefficient at 76 000.[21] Figures 1a and 1b show the changes in the flashlamp intensity, seen when the retinal is excited by the laser, when monitoring at 373 and 448 nm, respectively. The sample in this case is aerated and the triplet–triplet absorption shows strictly exponential behavior over three lifetimes. This is shown in Figure 2. The lifetime of the triplet in the aerated MCH solution is 90 ± 2 ns, i.e., the rate constant for triplet disappearance is 1.11 × 10^7 s^{-1}.

In order to analyze the isomerization data we assumed, following the observation of Ottolenghi,[26] that the excited states created by the laser excitation do not absorb light at 380 nm. We allowed for the possibility that 11-*cis*-retinal could isomerize directly to any other isomer. However, we expect the vast majority of isomerized 11-cis molecules to be in the all-trans conformation. The absorbance of the solution as a function of time is

$$A(t) = \log [I_0(t)/I_L(t)] = l[\epsilon_{11}C_{11}(t) + \epsilon_{AT}C_{AT}(t) + \epsilon_9C_9(t) + \epsilon_{13}C_{13}(t) + \epsilon_{DC}C_{DC}(t)] \quad (1)$$

Here, $I_0(t)$ is the incident intensity of the xenon flashlamp at time t. $I_L(t)$ is the intensity of light transmitted through the laser-excited sample at a time t. The various ϵ's and $C(t)$'s are extinction coefficients and concentrations at time t, respectively, of the various isomers denoted by the subscripts (11-cis, all-trans, 9-cis, 13-cis, and various di-cis isomers). Finally, l is the sample pathlength.

The kinetic scheme we assumed was

$$\text{11-}cis\text{-retinal} \xrightarrow{h\nu} C^*$$

$$C^* \begin{cases} \xrightarrow{k_{11}} \text{11-}cis\text{-retinal} \\ \xrightarrow{k_{AT}} \textit{all-trans}\text{-retinal} \\ \xrightarrow{k_9} \text{9-}cis\text{-retinal} \\ \xrightarrow{k_{13}} \text{13-}cis\text{-retinal} \\ \xrightarrow{k_{DC}} \text{di-}cis\text{-retinal isomers} \end{cases}$$

Here C^* represents excited state species formed by the laser excitation whose decay to stable products we observed. The nature of these species will be discussed later.

With this mechanism, the concentration of excited species is given by

$$\frac{dC^*(t)}{dt} = -(k_{11} + k_{AT} + k_9 + k_{13} + k_{DC})C^*(t) \quad (2)$$

$$= -kC^*(t)$$

where $k \equiv k_{11} + k_{AT} + k_9 + k_{13} + k_{DC}$. For any of the stable product species one may write

$$C_n(t) = C_n(0) + \frac{k_n}{k} C^*(0)\,[1 - e^{-kt}] \quad (3)$$

Substituting (3) into (1) yields

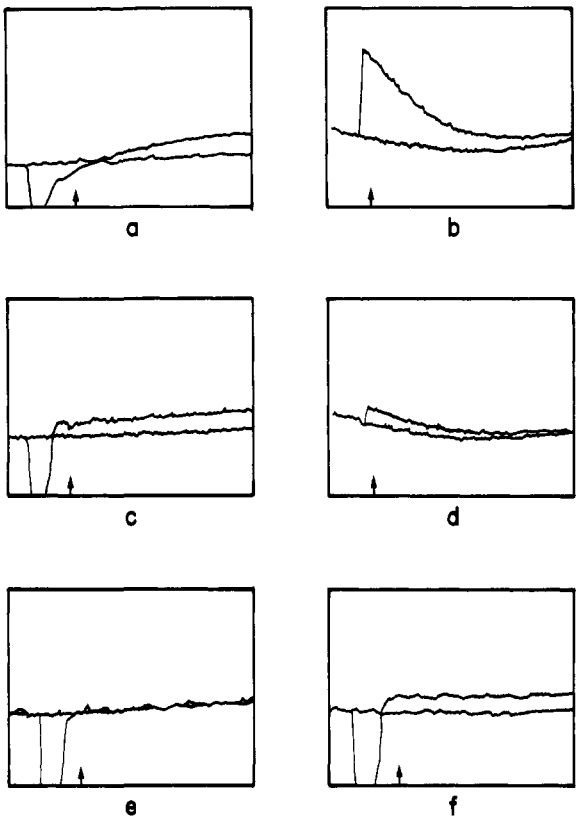

Figure 1. Changes in flashlamp intensity upon laser excitation when observing (a) isomerization of RET in MCH at 373 nm, (b) triplet–triplet absorption of RET in MCH at 448 nm, (c) isomerization of RET in MeOH at 380 nm, (d) triplet–triplet absorption of RET in MeOH at 460 nm, (e) isomerization of SB in MCH at 360 nm, and (f) isomerization of PSB in MCH at 463 nm. The isomerization data cover time spans of 200 ns and the triplet–triplet absorption data cover time spans of 500 ns. Flashlamp intensities are shown in all cases both in the presence and absence of laser excitation. The large increases in intensity to the left of the arrows in a, c, e, and f are due to scattered laser radiation. The arrows indicate the times corresponding to the end of the laser pulse.

$$\log \frac{I_0(t)}{I_L(t)} = a[1 - e^{-kt}] + b' \tag{4}$$

where a and b' are given by

$$a = \frac{lC^*(0)}{k} \sum_j \epsilon_j k_j$$

$$b' = l \sum_j \epsilon_j C_j(0)$$

where the summations are over all product species in the kinetic scheme.

Equation 4 can be expressed in a more convenient form for experimental analysis if we consider what the absorbance is in the absence of laser excitation. In that case the absorbance is simply proportional to the amount of 11-*cis*-retinal present initially, since we start with a pure sample of 11-*cis*-retinal. Calling this initial amount of 11-*cis*-retinal $C_{11}{}^0$, we have

$$\log [I_0(t)/I_x(t)] = l\epsilon_{11}C_{11}{}^0$$

Here $I_x(t)$ is the light transmitted through the sample in the absence of laser excitation. Subtracting this initial absorbance from total sample absorbance of eq 4 gives

$$\log \frac{I_0(t)}{I_L(t)} - \log \frac{I_0(t)}{I_x(t)} = \log \frac{I_x(t)}{I_L(t)}$$

$$\log \frac{I_x(t)}{I_L(t)} = a[1 - e^{-kt}] + b \tag{5}$$

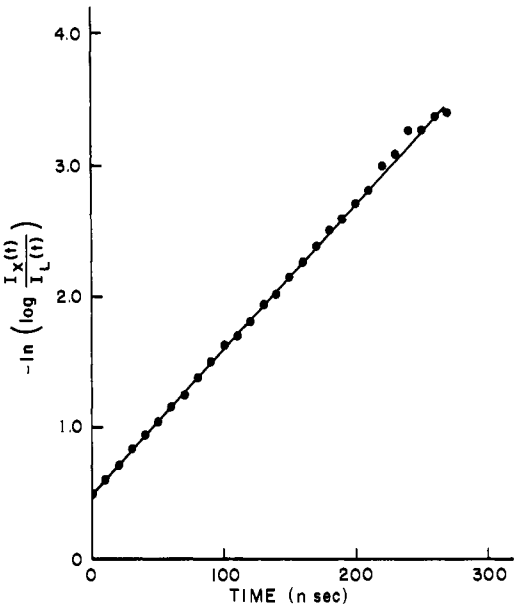

Figure 2. Decay of triplet–triplet absorption in aerated solution of RET in MCH.

where $b = b' - l\epsilon_{11}C_{11}{}^0$.

Figure 1a shows a typical signal obtained when observing the isomerization of 11-*cis*-retinal in MCH. Note that immediately after laser excitation there is a decrease in the absorbance followed by a relatively slow (that is, lasting some tens of nanoseconds) increase in absorbance. The decrease in absorbance, after laser scatter becomes negligibly small, is a result of ground-state depletion. Since ground-state RET molecules absorb strongly at 373 nm and the excited states do not, the absorbance decreases as the ground-state concentration decreases and nonabsorbing excited states are produced.

To make sure that the increase in absorbance we observed was due to isomerization, we measured the absorbance at 300 nm, the isosbestic point for the various RET isomers,[18] before and after photolysis. The absorbance did not change, confirming that isomerization is the only photoreaction.

The relatively slow buildup of the absorbance suggested the possibility that the triplet state is involved in this isomerization process. This possibility was subject to direct test since the rate constant, k, of eq 5 would be simply the triplet lifetime if isomerization were coming from the triplet state. This rate constant we have already measured as $k = 1.11 \times 10^7\ s^{-1}$ from our triplet–triplet absorption measurement shown in Figure 1b. Figure 3 shows the isomerization data plotted according to eq 5 with $k = 1.11 \times 10^7\ s^{-1}$. The agreement shows that the photoisomerization of 11-*cis*-retinal in MCH is consistent with the process emanating from a state with a lifetime equal to that of the triplet state.

Degassing MCH solutions of 11-*cis*-RET lengthened both the triplet lifetime and the buildup time of isomerized species as expected. The triplet quantum yield was essentially unchanged upon degassing but the triplet decay no longer shows strictly exponential behavior. It exhibits a bimolecular component in agreement with observations of Fischer and Weiss.[21]

As a further check of triplet state involvement in RET isomerization, we looked at the isomerization of 11-*cis*-RET in methanol (MeOH). While ϕ_T for RET in MCH is 0.5, it is reduced to 0.08 in MeOH.[21] We expected, then, that when we looked at the triplet–triplet absorption (λ_{max} 460 nm) and the isomerization signal (at λ 380 nm) of RET in MeOH, the signals would be similar in character to, but with magnitude reduced to about 16% of, those seen for RET in MCH. The signals we saw are shown in Figures 1c and 1d. The triplet–triplet absorption signal was 15% of that seen in MCH as ex-

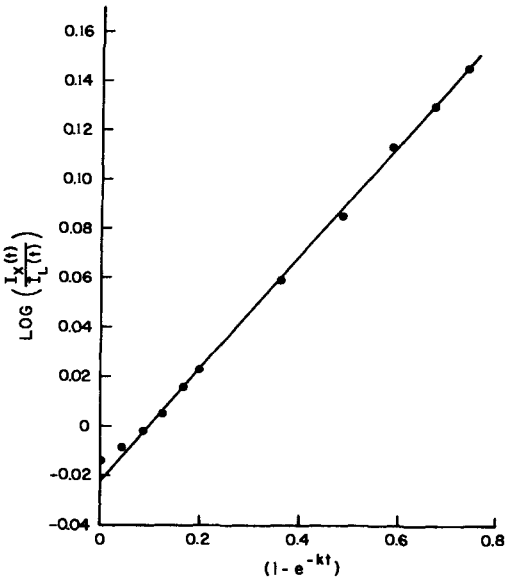

Figure 3. Fit of the isomerization data of RET in MCH to eq 5. The value of k is $1.1 \times 10^7\ s^{-1}$, the rate constant for the decay of the triplet state in this system.

pected. The lifetime of the triplet in aerated MeOH solution increases to about 207 ns. The isomerization signal, however, showed a much different kinetic pattern from that seen in MCH.

The isomerization signal for RET in MeOH, Figure 1c, does not have a ground-state depletion component followed by a slow buildup of isomerized species. Instead, there is a very fast increase in the sample's absorbance followed by an additional slower increase. The slow component of the absorbance change is consistent with eq 5 for a value of $k = 4.83 \times 10^6\ s^{-1}$. This k corresponds to our observed triplet lifetime of retinal in MeOH of 207 ns. The low triplet quantum yields in this solvent make the triplet lifetime and extent of triplet involvement in the isomerization uncertain to 10%. The fast component shows a buildup on a time scale too fast for us to measure and must take place on a time scale less than 10 ns.

After studying the isomerization process of retinal, we began looking at the process in MCH solutions of retinylidene-1-amino-2-propanol (SB). We also studied the protonated Schiff base (PSB) which we made by acidifying the SB solution with trichloroacetic acid (TCA).

The α peak of the SB lies at 360 nm and the triplet-triplet absorption peaks lie at 434 nm.[21] We were unable to detect any triplet absorption in this system. This is not surprising since the intersystem crossing quantum yield in the *all-trans*-SB is only $\phi_T = 0.008$.[21] As can be seen in Figure 1e, we also observed no isomerization with a single laser pulse. The isomerization quantum yield in this system is only $\phi_{isom} = 0.004$.[22]

The PSB in MCH has a triplet-triplet absorption peak at 570 nm and an α peak at 463 nm.[21] The intersystem crossing yield in this system is very small, $\phi_T < 0.001$,[21] so we saw no triplet absorption again in this system. We did, however, see a reasonable signal from the PSB isomerization. This is shown in Figure 1f. The isomerization in the PSB occurs very quickly. As in the case of RET in MeOH, it has a component too fast for us to measure. We can only say the isomerization takes place in a time faster than 10 ns. However, unlike RET in MeOH there is no detectable triplet component to the isomerization process in the PSB.

Conclusions

Let us now briefly summarize the experimental results. In the case of RET, we observed two paths for isomerization, greatly differing in rates. There was a fast reaction which appeared to be instantaneous with our temporal resolution of a few nanoseconds. In addition, there was a slower isomerization which we showed to originate from the triplet state. This identification we made by showing that the rate of the slow isomerization was identical with the rate of triplet decay, as measured by triplet-triplet absorption. The relative amounts of the fast and slow isomerization in RET depend strongly on solvent. For RET in MCH, the slow isomerization path predominated, while in MeOH, the fast reaction predominated. This is consistent with the observation that the intersystem crossing yields for *all-trans*-RET in MCH and MeOH are 0.5 and 0.08, respectively.[21] Although we did not measure absolute intersystem crossing yields, the relative yields for the 11-*cis*-RET were in this same ratio for the two solvents.

In the SB system, we could observe no isomerization or triplet-triplet absorption. The PSB exhibited the fast isomerization, but not the slow.

Let us see if these observations are consistent with calculations of the energy levels of the RET, SB, and PSB systems. If we follow the common practice and label these as if the molecules had the C_{2h} symmetry of linear polyenes, the ground state would have A_g symmetry and the lowest $\pi\pi^*$ excited states would have A_g^+, A_g^-, and B_u symmetries. There is also a low-lying $n\pi^*$ state in the RET and SB systems. There has been some controversy over the past years as to the ordering of these states but recent calculations by Birge et al.[27] and experiments by Birge et al.[28] give very strong evidence for a solvent-dependent ordering of RET states. The lowest energy excited singlet state was shown to be a ^1B_u in hydrocarbon solvents with the next state, only slightly higher in energy, being an $^1A_g^-$. In polar solvents, however, these states reverse order and the lower energy state is the $^1A_g^-$ followed by the ^1B_u. Above these states lies an $^1n\pi^*$ state and above that is the $^1A_g^+$ state. The ordering of triplet states is not known.

The slow isomerization we have shown as arising from a triplet state, presumably the lowest $\pi\pi^*$ triplet state. The energies of the triplet $\pi\pi^*$ and $n\pi^*$ states have not been calculated. One would expect, however, that a $^3n\pi^*$ in the vicinity of the lowest excited singlet state would greatly enhance the rate of intersystem crossing.[29,30] Thus, we speculate that in MCH a $^3n\pi^*$ state lies below the $^1A_g^-$ and ^1B_u states so there is a high intersystem crossing quantum yield and we see a large amount of triplet-mediated isomerization. As we go to the more polar MeOH environment this $^3n\pi^*$ state should blue shift relative to the $\pi\pi^*$ states.[31] The $^3n\pi$ state might then lie above the $^1A_g^-$ state (the lowest excited singlet in polar media[27]) and internal conversion from the ^1B_u state would compete kinetically with intersystem crossing. This could account for the decreased intersystem crossing yield and for the observed singlet-mediated isomerization.

In the SB system little experimental or theoretical data are available to enable us to interpret our results with a high degree of confidence. The extremely low intersystem crossing quantum yields lead us to believe that in this system the $^3n\pi^*$ state lies above both the lowest excited $^1\pi\pi^*$ states. Recent calculations on the SB system which include singly and doubly excited CI indicate that there are two states with highly mixed ($^1A_g^-$ and ^1B_u) character.[32] It is not known how the energies of these states vary with C_{11}-C_{12} bond twisting. Our results imply a large increase in energy upon twisting and thus a large barrier to isomerization. Calculations to check this hypothesis are clearly in order.

In the PSB system there is no low-lying $^3n\pi^*$ state and the intersystem crossing quantum yield is very small. The lowest lying $^1\pi\pi^*$ state is calculated to be a ^1B_u state and the $^1A_g^-$ state lies at much higher (~0.85 eV) energy.[32] Suzuki et al.[33] have calculated adiabatic potentials for cis-trans isomerization

of the PSB system and find, in agreement with our results, that isomerization should take place efficiently out of the lowest $^1\pi\pi^*$ state.

On the basis of a lack of any O_2 effect on the isomerization yield of retinal, Ottolenghi et al.[20] ruled out a thermalized triplet state as a precursor to the isomerized species. Our mechanism implicates the thermalized triplet in isomerization and we explain Ottolenghi's observation by the alternative assumption that O_2 quenches the thermalized triplet without substantially effecting its branching ratio into the various ground-state isomers. Our explanation would apply whether the triplet existed in one common, presumably twisted, conformation or as a mixture of all isomers. Ottolenghi further observed that triplet-sensitized isomerization occurred from the 11-cis isomer but not from *all-trans*-retinal. This interesting result he took as further evidence that the thermalized triplet is not involved in isomerization. He explained the observation by assuming that isomerization from a nonrelaxed triplet can efficiently compete with thermalization in the case of 11-*cis*-retinal but not of all-trans. We propose that in the sensitization studies, insufficient energy is available for the all-trans triplet to overcome the potential barrier and reach the twisted triplet configuration.

Finally, we would like to point out that our observation of a single isomerization lifetime faster than 10 ns is consistent with the observation of Busch et al. that prelumirhodopsin is formed from rhodopsin in a matter of picoseconds.[11] This gives us more confidence that a protonated retinylidene Schiff base is a good model system for rhodopsin. The vastly different behaviors of retinal and its Schiff base point out the danger in using these compounds as rhodopsin model systems.

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Structural Implications of the Electronic Spectra of Quinacrine–Deoxyribonucleic Acid Complexes in the Ultraviolet Region (250–300 nm)[1]

M. Nastasi,[2] J. M. Morris,[2] D. M. Rayner,[2] V. L. Seligy, A. G. Szabo,* D. F. Williams, R. E. Williams, and R. W. Yip

Contribution from The Division of Biological Sciences and the Division of Chemistry, National Research Council of Canada, Ottawa, Ontario, Canada K1A 0R6.
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Abstract: Ultraviolet difference absorption spectra have been measured for quinacrine–polynucleotide complexes. The difference absorption spectra of quinacrine–native calf thymus DNA and quinacrine–poly(dA-dT)·poly(dA-dT) complexes include two bands at 270 and 290 nm, whereas in the spectrum of quinacrine itself only one band at 280 nm is observed. In the difference spectrum of quinacrine–poly(dG)·poly(dC) complexes, a broadened band is observed in the 280-nm region. The effect of the phosphate/dye (P/D) ratio on the observed absorption band splitting indicates that the splitting resulted from highly localized nearest neighbor interactions of the dye and nucleic acid bases. It does not originate from dye–dye interactions. Observation of an isosbestic point in these studies indicates that the splitting was associated with only one type of binding, the strong "primary" binding process. The absorption band splitting is attributed to the interaction between exciton states of the complex and requires the close proximity between the dye and nucleic acid bases. We conclude that quinacrine is bound closer (intercalated) to A-T base pairs than to G-C base pairs in the polynucleotides. Fluorescence quenching experiments with iodide ions confirm these conclusions.

Recently several workers[3–8] have studied the complexes formed between the antimalarial drug and chromosomal staining agent quinacrine (QAC) and nucleic acids. This interest has been stimulated by the potential use of complexes formed between cationic acridine dyes and nucleic acids as regioselective labels in the staining of chromosomal DNA in