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A New Spectral Window on Retinal Protein Photochemistry

Boris Loevsky,[†] Amir Wand,[†] Oshrat Bismuth,[†] Noga Friedman,[‡] Mordechai Sheves,[‡] and Sanford Ruhman*[,†]

[†]Institute of Chemistry and the Farkas Center for Light-Induced Processes, The Hebrew University, Jerusalem 91904, Israel
[‡]Department of Organic Chemistry, The Weizmann Institute of Science, Rehovot 76100, Israel

ABSTRACT: A VIS pump/hyperspectral NIR probe study of all-*trans*-retinal protonated Schiff base (RPSB) in ethanol is presented. Upon irradiation, a short-lived absorption band covers the recorded range of $\lambda = 1-2\,\mu$m. It decays to reveal the tail of S_1 emission at $\lambda < 1.3\,\mu$m, along with a residual absorption at longer wavelengths, both of which decay with the known kinetics of internal conversion to S_0. The existence of this hitherto unrecorded excited-state absorption deep in the NIR will require a revision of current models for RPSB electronic structure. The phenomenological similarity of these observations with ultrafast NIR studies of carotenoids raises the question of whether three, and not two, electronic states participate in RPSB photochemistry as well. The relevance of these observations to retinal protein photochemistry is discussed.

Retinal protonated Schiff base (RPSB) energizes activity in all bacterial retinal proteins (RPs), and in most visual pigments, by photoisomerizing rapidly about a specific retinal C=C double bond. Binding to the protein has far-reaching effects on this photochemistry, increasing isomerization quantum yields from 20 to ~60%,[1] and conferring the said bond specificity.[2] It also tunes RPSB's absorption peak by thousands of cm^{-1} to optimize a specific RP's biological activity.[3] Investigating the crucial step of photoisomerization is a prerequisite for understanding the function of these important photoreceptors. RPSB photochemistry in solution and within RPs has therefore been probed with ultrafast time resolution from the UV to the far IR.[4] These studies show that protein-dependent internal conversion (IC) ranges in duration from <1 to > 10 ps and exhibits nonexponential dynamics in both RPs and their chromophores alike.[5] Most of these studies are rationalized in terms of two electronic surfaces which meet at conical intersections when properly deformed, directing the chemical reactivity. This is, however, not unanimously accepted, with numerous suggestions that more states may be involved, in the case of both free RPSB[4b] and various RPs.[6]

In the related carotenoids (CARs), as reviewed extensively in ref 7, involvement of at least two excited states is a cornerstone of photoreactivity, due to the optical "darkness" of the lowest excited singlet. This aspect of CAR photochemistry, whose manifestation was first observed in weak fluorescence signals from excited CARs,[8] has recently been corroborated and clarified by ultrafast spectral probing in the near IR (NIR). In this spectral range, absorption between low-lying excited states is directly accessible, providing the lifetimes and relative potential energies of the reactive electronic surfaces.[9]

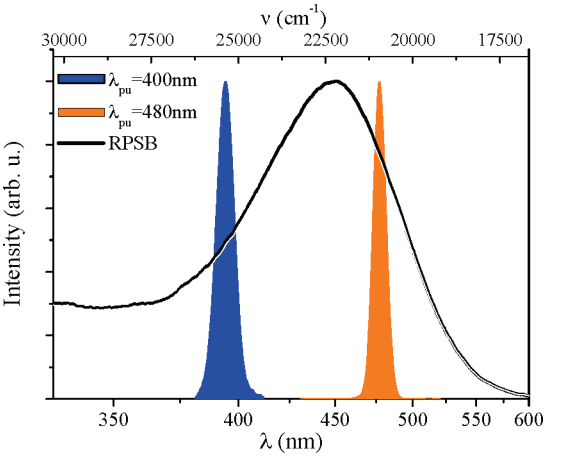

Figure 1. Normalized absorption spectra of all-*trans*-RPSB in ethanol together with normalized intensity spectra of the excitation pulses employed in experiments.

In particular, the subpicosecond shift from intense photoinduced absorption assigned to $S_2 \rightarrow S_N$ transitions, to weaker $S_1 \rightarrow S_2$ activity, is the NIR hallmark of consecutive stages of internal conversion in excited CARs. Oddly, despite the seminal insights afforded by NIR probing of excited CARs, this range has been overlooked in the spectral coverage of ultrafast photochemistry in RPs and of RPSB. Here, we report the first femtosecond visible-pump/1−2 μm−NIR-probe study of internal conversion in RPSB. Results reveal an excited-state absorption band deep in the NIR, which evolves rapidly in the first 100 fs of delay. Comparison of the results with those from similar experiments in CARs suggests the possible involvement of multiple excited states in RPSB photochemistry, which may carry over to the photochemistry of RPs as well.

Pump pulses of ~30 fs time duration were obtained at 400 nm by doubling the Ti:Sapph multipass amplifier output (~25 fs, 0.5 mJ/pulse), or at 480 nm using an optical parametric amplifier (TOPAS, light conversion) pumped by the same system. Multifilament white light continuum probe pulses were derived by focusing 800 nm fundamental in 3 mm of sapphire, and refocusing with reflective optics into the sample through a visible cutoff filter. The transmitted probe was fed into an InGaAs array spectrograph covering the range from 1 to 2 μm. Cross correlations of ~60 fs across the full spectral range, and the probe group delay dispersion (GDD) were obtained by pump−probe scans in 0.3 mm of silicon. All-*trans*-RPSB in

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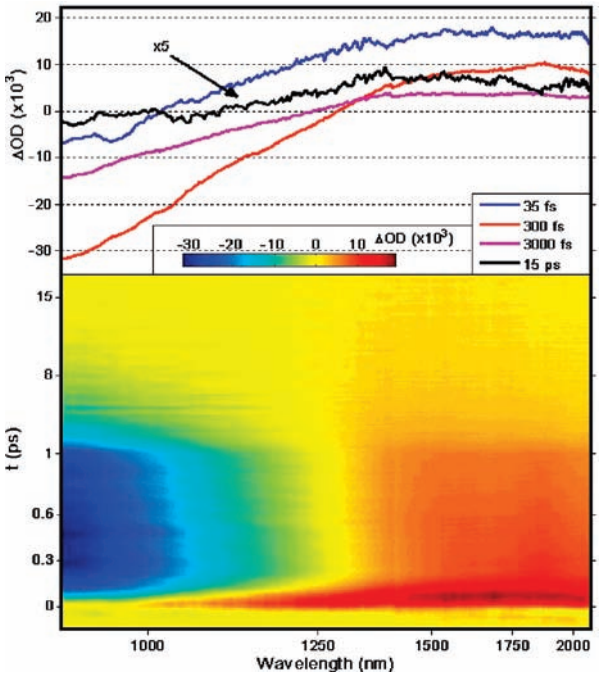

Figure 2. Hyperspectral transient NIR absorption data for RPSB in ethanol for excitation at λ_{pump} = 400 nm. Bottom: full mapping of $\Delta OD(\lambda,t)$ as a color-coded contour map according to the ruler inset above. Top: cuts in the data at selected delays depicted in the legend. The difference absorption spectrum at 15 ps is multiplied by 5.

ethanol solution was prepared and treated as previously described[10a] and pumped through a 300 μm cell, with a concentration that afforded a peak optical density of ~0.8 at 470 nm. Sample absorption, and laser excitation spectra are depicted in Figure 1.

The lower panel of Figure 2 displays a color-coded mapping of $\Delta OD(\lambda,t)$ for RPSB in ethanol after irradiation at t = 0 with 400 nm pulses, time corrected for probe GDD. Upon irradiation, a steep rise and rapid decay of absorption is apparent across most of the probed range. This short-lived absorptive signal rapidly shifts to stimulated emission for λ_{pr} < 1.3 μm, in agreement with previous studies.[4,10] Instead of this emission tapering to zero with increasing λ, a transition to absorption is apparent above λ_{pr} = 1.3 μm. To further clarify these points, the upper panel of Figure 2 presents cuts in the data at a series of probe delays. As in previous visible pump−probe studies of RPSB, both panels show the existence of a weak residual difference spectrum extending beyond 20 ps—long after completion of IC, possibly due to photoisomers, or triplet formation.[10a] Designating this product as "P", the data were globally fit according to the following scheme:

$$P \xleftarrow{1/\tau_5} A^* \xrightarrow{1/\tau_1} B^* \xrightarrow{1/\tau_2} C^* \xrightarrow{1/\tau_3} D^* \xrightarrow{1/\tau_4} A$$

A being the ground-state reactant. The main purpose of this fitting procedure was to rule out the possibility that these NIR signatures reflect species other than the same reactive RPSB excited state reported in the literature.[5,10] The upper panel of Figure 3 presents the five resulting evolution associated difference spectra (EADS) which characterize A*, B*, ... etc. The fit afforded by the model is shown on the bottom, as solid lines accompanying the data at selected wavelengths.

Parallel generation of P and B* schematically generates the long-lived product described above. While mechanistically questionable (P could equally have been a product of later intermediates), the low ΔOD of P makes changing or distributing this branching point of little spectral consequence to the extracted EADS. The same can be said about the use of sequential kinetics to describe stages of internal conversion which should not be norm conserving. Since the ground state does not contribute to absorption in this range, the only consequence of this simplification is the reduction in amplitude of the D* EADS.

As shown in Figure 4, all these trends, albeit with small changes in the resulting EADS, were also obtained for experiments pumping at 480 nm. Notice that this data set was obtained with an extended InGaAs array spectrograph only, and covers a narrower spectral range than that shown in Figures 2 and 3. Again, two initial subpicosecond EADS are obtained, but their appearance is somewhat different in the data extracted with the two pump wavelengths. This is most likely the result of slight inaccuracies in the measured probe GDD giving rise to imperfect separation of these phases. The data show no significant differences in the appearance of this feature as observed in the lower panels of Figures 3 and 4. The inclusion of a nondecaying EADS in the case of 480 nm excitation is marginal, indicating that whatever the nature of "P", its yield is much lower when exciting to the red of RPSB's absorption peak. This is also in accord with previous RPSB experiments with tunable excitation pulses.[10a] Thus, in this case the last product EADS is superfluous and discarded. As before, C* and D* decay with time constants that match those obtained from visible probe experiments within error, likewise assigning them to the reactive excited state (S_1).

A 100−200 fs evolution component has been observed in all high time resolution visible probe studies of RPSB within RPs or in solution, in full agreement with the present B*. In the case of RPSB in solution, slower spectral decay phases well fit with decay times of ~2 and ~7 ps, respectively, were detected as well.[5] Thus, all of the latter four temporal components (including a long-lived photoproduct) agree with studies based upon probing in the visible within error. Along with the detection of emission at the blue edge of the NIR, this close match in decay times demonstrates that the newly detected NIR spectral features emanate from the same excited state followed in the visible and assigned to the reactive singlet state, often coined S_1.

The existence of an even earlier A* "species" is not obvious from vis probing experiments. In line with the measured temporal resolution and considerable group velocity mismatch (GVM), the optimal Gaussian instrument response function (IRF) was ~90 fs (fwhm), convoluting the two subpicosecond components, and making it difficult to differentiate a short-lived intermediate from an instantaneous wave-mixing artifact. First, this feature could not be the result of a nonresonant cross-phase modulation, since its spectrum was insensitive to large variations in that of the probe. Moreover, pump−probe scans in pure solvent differed both in spectrum and temporal evolution from this initial feature, and were of negligible intensity. Second, the assignment of this feature to an intermediate of finite lifetime consistently led to superior fits relative to models which represent it as a coherent artifact.

To demonstrate this, Figure 5 presents a short-term 1.3 μm cut in the 400 nm data along with two optimal fits. The first is a Gaussian function, while the latter is an exponential decay convolved with a Gaussian IRF. Not only does the latter reduce the sum of square deviation by an order of magnitude, it does so with a best-fit IRF of 95 fs duration, very close to that determined experimentally. In contrast, the former requires an unphysical IRF of 135 fs. Accordingly, the

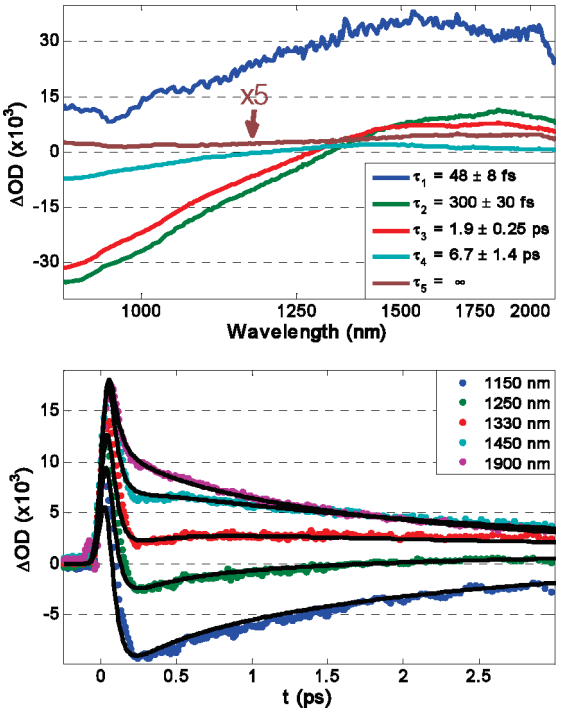

Figure 3. Global fitting results for excitation at λ_{pump} = 400 nm. Top: the EADS which provide the best fit to the entire data set, using the proposed scheme (see text) and the decay times designated in the figure. Bottom: fit provided by these spectra and decay times as continuous lines along with the data points for comparison.

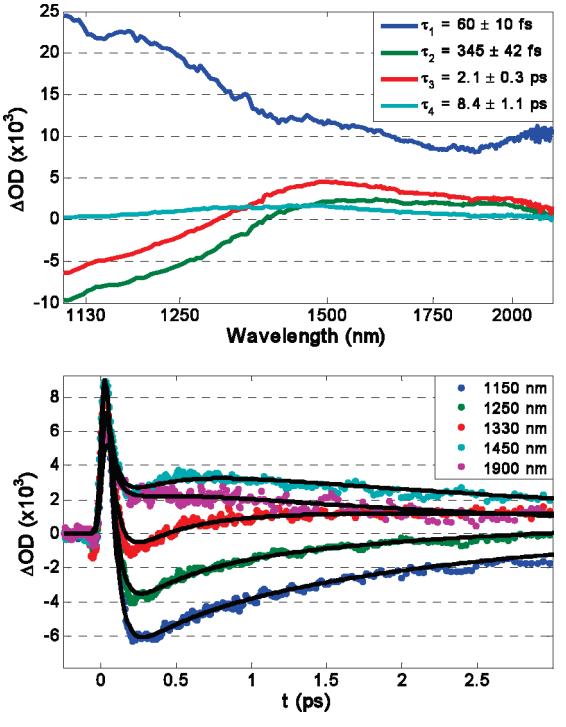

Figure 4. Same as Figure 3 except for excitation at λ_{pump} = 480 nm. The global fitting procedure for this data set does not require a residual difference spectrum at long times and involves one less EADS. See text for details.

initial absorption is assigned to a separate state. It is noteworthy that, even if this feature were partly or wholly due to a coherent artifact, it

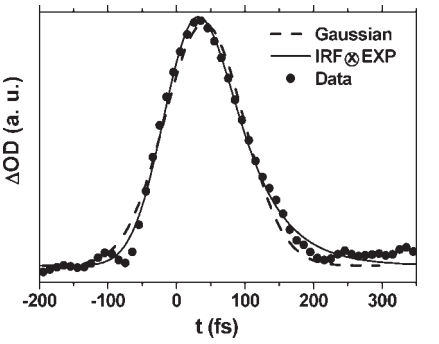

Figure 5. Showing 1.3 μm cut in the 400 nm data along with best-fits to a Gaussian IRF and an exponential decay convoluted with Gaussian IRF.

would still demonstrate the existence of an absorptive resonance deep in the NIR. As one can show with a simple diagrammatic approach to perturbative generation of a resonantly enhanced coherent artifact, a dominant emissive resonance would lead to an increase in probe transmission when it overlaps with the pump and not to absorption as detected here.[11]

The salient findings of this study are, first, that the picosecond excited-state spectral signatures reported in the literature, and whose evolution has served to follow the dynamics of IC in this chromophore, are accompanied by an absorption feature extending from $\tilde{\nu} \leq 7600$ cm^{-1} to below 4700 wavenumbers. The other is the observation of a strong initial absorptive feature, which precedes the others mentioned above. The former observation poses the question of the identity of the electronic states giving rise to this absorption. To demonstrate the relative intensity and extent of this feature in context, Figure 6 presents a vis−NIR transient absorption spectrum for the 400 nm photoexcitation of RPSB in ethanol at a delay of 250 fs, compiled from the data described above, combined with spectra presented in ref 10a. The involvement of multiple higher electronic excited states in the transient spectroscopy of excited RPSB is now well-documented. Aside from a strong ∼500 nm excited-state absorption, another bordering the NIR at ∼750 which overlaps with stimulated emission, gives rise to the "dimple" observed in the middle of the emission band (see Figure 6). Similar signatures have also been reported in transient absorption studies of excited *salinarum* and *pharaonis* halorhodopsins,[12] and in bacteriorhodopsin (BR).[6a] Since absorption bands "tail" to the blue, it is unlikely that this newly discovered feature and the overlapping emission involve transitions to the same electronic potential. If so, the transient absorption feature would have to extend over 10,000 cm^{-1} and exhibit multiple absorption peaks. In any case, the existence of an electronic state to which optical transitions are allowed, in such energetic proximity to S_1 throughout the course of IC, whether separate from those absorbing at higher frequencies, must now be worked into any theoretical description of RPSB electronic structure and photochemistry. The possibility that an excited population in the vicinity of a conical intersection can exhibit such broad spectral features extending all the way to zero frequencies has recently been presented for IC in bovine visual pigments.[13]

The more tentative observation of a short-lived and strongly absorbing state preceding the latter stages of spectral evolution is noteworthy as well. A provocative and phenomenological comparison with early spectral features observed on similar time scales in photoexcited carotenoids reveals a strong resemblance. An immediate rise of intense NIR absorption followed by a weaker residual net reduction in transmission which decays with later stages of IC characterizes those symmetric polyenes as well. The similarity of this

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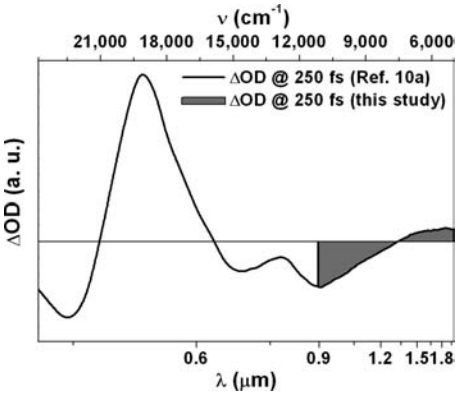

Figure 6. Vis−NIR transient spectrum after excitation at λ_{pump} = 400 nm at a delay of 250 fs (shaded portion is this study).

scenario with ultrafast photochemistry in CARs makes one wonder whether, as in CARs, this early stage of relaxation is due to an ultrafast IC process as well. Time-resolved emission studies of this same reaction have been recently interpreted as indicating that, at the wavelengths employed, excitation can take place to more than one excited singlet state, identified there as S_1 and S_2 of the RPSB chromophore.[4b] However, no clear stage of IC was suggested to take place on such short time scales in that report. It is important to point out that the scenario of a very rapid initial electronic decay explaining the ultrafast spectral shifts referred to above is not equivalent to some of the envisioned three-state models for RP photochemistry, which often ponder the later stages of the photochemical reaction.[6,14] One way or another, all would require multiple crossings between at least three states to describe the reactivity.

Whether in relation to emission dynamics in RPSB or through possible analogy with CARs, all of the above findings demonstrate that multiple closely spaced excited electronic states are essential for understanding the spectroscopy of excited RPSB, and most likely even to understand its photochemistry. These findings also raise the question of whether at least three electronic states are required to explain photochemical dynamics in RPs as well. The extrapolation from the case of RPSB in solution to the process of IC in RPs must, however, be taken with care, since theoretical treatments have suggested significant changes in electronic structure of the protein-attached RPSB chromophore, which could change this aspect of RP photochemistry.[15] Nonetheless, preliminary experiments on BR suspensions suggest that, contrary to these theoretical predictions, the experimental observations in RPSB carry over to RPs as well. The existence of a sub-100 fs spectral evolution in the visible, following excitation of BR with ∼10 fs pulses, may be related to the NIR feature described in this study.[16]

AUTHOR INFORMATION

Corresponding Author
sandy@fh.huji.ac.il

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