Photochemistry of a Retinal Protonated Schiff-Base Analogue Mimicking the Opsin Shift of Bacteriorhodopsin

Oshrat Bismuth,[†] Noga Friedman,[‡] Mordechai Sheves,[‡] and Sanford Ruhman*[,†]

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

Received: October 23, 2006; In Final Form: December 4, 2006

A retinal Schiff base analogue which artificially mimics the protein-induced red shifting of absorption in bacteriorhodopsin (BR) has been investigated with femtosecond multichannel pump probe spectroscopy. The objective is to determine if the catalysis of retinal internal conversion in the native protein BR, which absorbs at 570 nm, is directly correlated with the protein-induced Stokes shifting of this absorption band otherwise known as the "opsin shift". Results demonstrate that the red shift afforded in the model system does not hasten internal conversion relative to that taking place in a free retinal-protonated Schiff base (RPSB) in methanol solution, and stimulated emission takes place with biexponential kinetics and characteristic timescales of approximately 2 and 10.5 ps. This shows that interactions between the prosthetic group and the protein that lead to the opsin shift in BR are not directly involved in reducing the excited-state lifetime by nearly an order of magnitude. A sub-picosecond phase of spectral evolution, analogues of which are detected in photoexcited retinal proteins and RPSBs in solution, is observed after excitation anywhere within the intense visible absorption band. It consists of a large and discontinuous spectral shift in excited-state absorption and is assigned to electronic relaxation between excited states, a scenario which might also be relevant to those systems as well. Finally, a transient excess bleach component that tunes with the excitation wavelength is detected in the data and tentatively assigned to inhomogeneous broadening in the ground state absorption band. Possible sources of such inhomogeneity and its relevance to native RPSB photochemistry are discussed.

Introduction

Retinal proteins perform a diverse range of light driven biological activities, from differential sensing of solar radiation, to vectorial transport of ions across cellular membranes. They are indispensable building blocks in all three domains of living organisms,[1] with frequent discovery of new family members.[2] In all these proteins the biological activity is fueled by photon energy absorbed by a retinal chromophore covalently linked to the protein via a protonated Schiff base which consequently isomerizes around a specific C═C double bond. The broad natural use of this basic architecture stresses the importance of understanding how variations in the protein backbone serve to reapply this design to new cellular tasks. The protein effect can be best evaluated by comparing retinal excited-state energetics and dynamics in the proteins to that of a retinal protonated Schiff base (RPSB) in solution. The PSB and not the retinal itself provides this benchmark since protonation of the imine group significantly alters the electronic structure of the polyene, red shifting its intense absorption from the UV to peak at ∼440 nm in methanol solution or ∼610 nm in vacuum.[3] This protonation also modifies the ensuing photophysics presumably due to changing the state ordering of the lowest excited singlet states.

In the particular case of bacteriorhodopsin (BR), the light-absorbing retinal chromophore is initially all-trans once light adapted and functions as a proton pump embedded in the purple membrane of the brine resistant archea *Halobacterium salinarum*.[4] That the protein strongly influences the retinal prosthetic group is obvious from the red shift induced in the RPSB's $S_0 \rightarrow S_1$ absorption by more than 100 nm upon its incorporation into the protein pocket, giving the membrane its characteristic purple color. This protein-induced Stokes shifting of BR's absorption is coined the "opsin shift"[5−10] and has been assigned to dielectric and Coulombic interactions of protein residues with the polyene moiety including screening of the imine groups counterion[11−14] and increased π electron delocalizeation due to steric planarization of the β-ionone ring.

Experiments with synthetic chromophores have been conducted to assess the contribution each of these mechanisms makes to the opsin shift.[15] While the 570-nm absorption peak in BR is actually blue shifted with respect to the 610-nm band observed in isolated RPSB cations in the gas phase, recent calculations suggest that spectra obtained in solution are more appropriate yardsticks for assessing the protein effects on the absorption of the retinal chromophore.[16] The idea behind experimenting with altered chromophores and pigments was by properly tailoring the structure of the prosthetic group or the protein, to "switch on or off" specific protein effects one at a time, allowing their individual contributions to the opsin shift to be estimated.

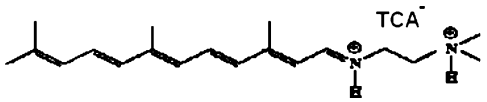
The early light-induced dynamic responses in BR events are believed to hold the key for understanding the mechanisms of storage and fixation of the absorbed photon energy. To assess the role played by the protein in controlling these important events the ultrafast spectroscopy of BR has been compared with

* To whom correspondence should be addressed. E-mail: sandy@fh.huji.ac.il.
[†] Hebrew University.
[‡] The Weizmann Institute of Science.

10.1021/jp0669308 CCC: $37.00
Published on Web 02/14/2007

that of RPSB in solution. Following femtosecond photoexcitation of solvated RPSB, the course of internal conversion (IC) has been probed by transient transmission experiments in the visible and NIR,[17,18] the MIR,[19] and also by time-resolved emission.[20] Such a comparison shows that incorporation of RPSB into the protein not only influences its spectroscopy but also has dramatic effects on its dynamics. While interpretation of these results has not led to full consensus concerning the underlying photoinduced dynamics, particularly concerning a sub-picosecond component of spectral evolution apparent in most, it is clear that IC of RPSB in alcohol solutions takes place nearly 10 times slower than in BR. It exhibits non-exponential kinetics which can be fit well to a biexponential decay function with ~2 and ~7 ps components. The source of this nonexponential internal conversion is still not fully understood. Isomerization of the C_{13}═C_{14} bond, which is the exclusive reactive outcome in BR, is not even the main RPSB photoproduct where isomerization takes place primarily about other double bonds. In addition, the total quantum efficiency for RPSB isomerization in solution around all double bonds is less than half that found in BR.[21,22]

The present study is aimed at exploring whether the dynamic changes described above are correlated with the protein-induced shifts in the chromophores absorption spectrum. Such a trend is plausible since the magnitude of the opsin shift has been shown to be correlated with photoinduced shifts in bond length alternation, which should in turn determine the weakening of retinal C═C bonds, and be reflected in IC dynamics.[5,10,11,13,23,24] It might also be expected if reaction dynamics are determined by intertwining low-lying excited states that tune differently with environmental changes, causing significant restructuring of the reactive potential surface topology.[21,25] While no such simple correlation was uncovered in a recent comparative study of IC dynamics in different archaeal rhodopsins, the simultaneous presence of steric, electrostatic, and dielectric effects of the protein make it difficult to separate the various factors influencing IC catalysis in BR.[26] In order to investigate the mechanisms by which the protein modifies the retinal's photochemical *dynamics* in a systematic fashion, a modified RPSB is investigated, hitherto referred to as compound I, which was previously used to assess the source of BR's opsin shift.[27]



Compound 1

A femtosecnd pump multichennel probe experiment was conducted to record its photoinduced dynamics in trifluoroethanol (TFE) solution. The absorption maximum of the retinal chromophore was shifted to 520 nm as shown in Figure 1 due to a combination of factors: (1) Eliminating a major part of the β-ionone ring removed steric hindrance and induced a planar s-trans configuration in the polyene which is more stable than the s-cis conformation. (2) Introducing a second protonated amine residue in the protonated Schiff base vicinity enhanced charge delocalization in the polyene and destabilized mainly the ground state due to positive charge repulsion. (3) Weakening the protonated Schiff base counterion interaction was achieved by using a solvent which preferentially stabilized the negative charged counterion via effective hydrogen bonding. Accumulating experimental and theoretical evidence stresses the importance of mechanisms 1 and 3 above in the red shifting of BR's visible absorption band, while 2 was implemented in order to boost the shift farther by accentuating the importance of charge migration relevant to point 3.

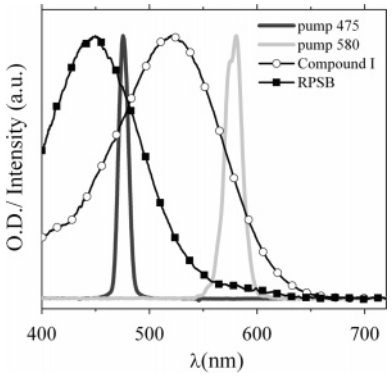


Figure 1. Normalized absorption spectra of compound I in TFE and RPSB in ethanol together with normalized intensity spectra of the excitation pulses used in our experiments.

Analysis of our results leads to several new insights. Despite the significant tuning of the state energies, rates of excited-state decay are only mildly modified: the ~2-ps component is unchanged, with later stages of IC decaying exponentially in 10.5 ps—about 40% slower than the measured for RPSB in ethanol. This demonstrates that even in controlled model systems no simple correlation exists between the S_0-S_1 energy gap and the IC dynamics. This result is significant in view of recent theoretical studies,[28] which claim that subtle potential features along the reaction channel are responsible for the multiexponential kinetics in this relaxation process. The robustness of this feature in spite of extensive tuning of the vertical S_0-S_1 gap must signify a more fundamental aspect of the photochemistry of this family of reactions. A strong sub-picosecond component of spectral evolution observed here, analogues of which have been reported for native PSB and photoexcited retinal proteins,[20,29,30] is suggested to reflect electronic relaxation between excited states. This is attested to by identical spectral evolution observed in the initial ~1 ps of delay, including a clear isosbestic point at ~520 nm, following excitation above and below the peak of ground state absorption. The decay associated spectra derived from the data also indicate that the bleach signals induced by pumping at 580 and 475 nm differ at all delays, suggesting long-lived inhomogeneity of the reactant absorption spectrum. Possible sources of inhomogeneity and implications of this finding are discussed.

Experimental Methods

The aldehyde derivative of compound I was prepared as previously described.[31] It was later dissolved in ethanol and then condensed with four equivalents of *N*,*N*-dimethylethylenediamine at 25 °C for 2 h. The solvent and excess amine were evaporated, and the residue was dissolved in trifluoroethanol followed by protonation with trichloroacetic acid to achieve full Schiff base protonation which is evident in its absorption spectrum (see Figure 1).

A room-temperature sample was pumped through a 0.25-mm path length flow cell, which was equipped with 0.2-mm quartz windows using a peristaltic pump, at a rate which ensured sample replenishment between shots, and a concentration which produced an optical density (OD) of 0.8 @ 520 nm. The extinction coefficient of the reactant was determined to be 23 000 M^{-1} cm^{-1}. In order to minimize oxidation of the sample during storage it was kept under nitrogen, in the dark, at a temperature of −32 °C, and its integrity was determined by measuring its absorption spectrum before and after each run. The presented data was shown to be reproducible with multiple samples and to be free of the effects of sample degradation due

to the accumulation of photoproducts or oxidation due to contact with air during the experimental runs.

The laser system and methods of measurement have been described in detail elsewhere.[32] Briefly, a homemade titanium sapphire oscillator producing 17-fs pulses, centered at 790 nm, are used to seed a 9-pass amplifier, which is a modified version of the design devised by Bakcus et al.[33] The amplifier is pumped with an intracavity doubled flash lamp pumped Nd:YLF laser (Quantronix) producing 150-ns pulses at 527 nm, with a pulse energy of ~7 mJ. The ultimate output consists of 30-fs pulses centered at 800 nm containing ~0.5 mJ of energy. 70% of this is used to pump a TOPAS (light conversion) and produce pump pulses at 475 and 580 nm (see Figure 1) by signal mixed with amplifier fundamental and frequency doubled signal in BBO respectively. Probing pulses were derived using the remaining amplifier fundamental by white-light generation in CaF_2 or sapphire.

Excitation was achieved at both 475 and 580 nm by focusing pulses of ~400 nJ to a spot of ~200 μm in diameter. The pump beam waist was determined by measuring the transmission through a 100-μM pinhole assuming a Gaussian intensity distribution. By use of this measure, the excited-state density per cm^2 is determined by measuring pump intensity before and after the cell, with an estimated error of ±15%. At both excitation wavelengths only a small fraction (~12%) of the molecules in the illuminated volume was excited with each laser pulse. Magic-angle transient spectra were recorded in a double spectrometer system which measures the transient pump induced variation in OD for consecutive pulse pairs with and without pump pulse irradiation and averages this for a predetermined number of such pairs.[34] The linearity of the signal with pump intensity was verified up to at least a factor of 2 higher than that used in analysis. The group delay of the probe pulses was determined as described in the literature[34] and found to cover a range of ~250 fs from 450 to 900 nm. Given the 20−30-fs pump pulses, this results in a pump−probe cross correlation ranging from 70 to 45 fs across this spectral range. This estimate agrees with the rise times observed in the data at pertinent wavelengths.

Results

Time corrected multichannel data obtained with 475 and 580 nm excitation pulses are depicted in Figures 2−4. Figure 4 displays pump-induced difference spectra on a split time axis from −0.2 to 25 ps in the form of color-graded contour plots. This map is the compilation of data sets with closely spaced transient spectra conducted over the initial 4 ps of pump−probe delay and more coarsely spaced data for coverage of the later stages of spectral evolution up to delays of 100 ps. Transient difference spectra pertaining to selected pump−probe delays are displayed in Figures 2 and 3. Notice the separate color scales for the different panels of Figure 4, despite the similar estimates for the excited-state concentrations obtained in both experiments. Both maps reflect dramatic spectral evolution consisting of an instantaneous buildup of excess transmission near the ground state absorption band, simultaneous rise of absorption above and below this feature, and the buildup of excess transmission in the red, which extends beyond the edge of our spectral coverage at 950 nm. The excess transmission observed near the ground-state absorption and that appearing in the red presumably reflect ground-state bleaching and excited-state fluorescence, respectively. During the initial ps of delay a rapid and discontinuous shift in the emergent absorption features surrounding the S_0 absorption bleach takes place in both sets of

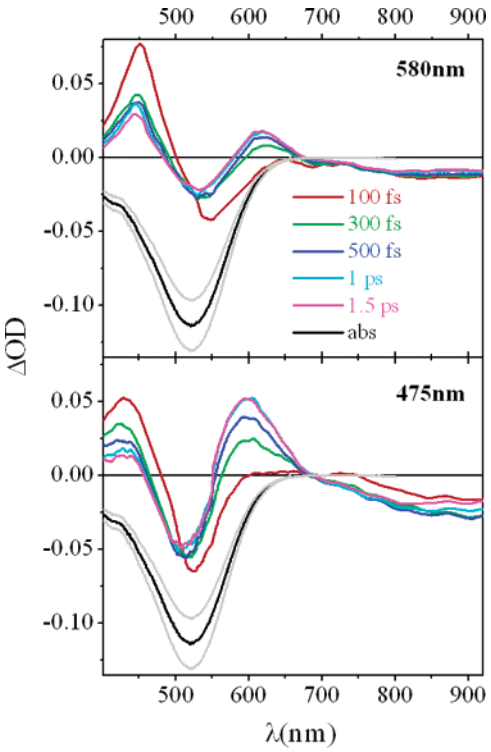


Figure 2. Time-corrected multichannel data obtained with 475- and 580-nm excitation pulses for selected time delays below ≤1.5 ps. A bleach spectrum representing the absorption of the reactant molecules excited by the pump pulses is depicted for comparison. The gray curves above and below represent the uncertainty of this measure.

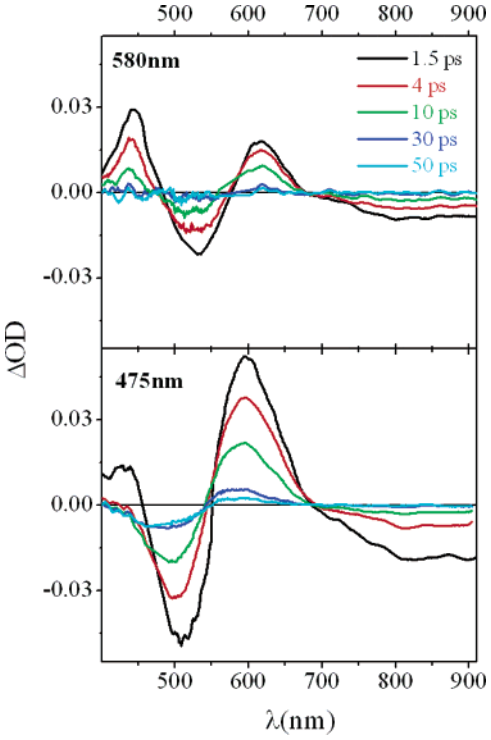


Figure 3. Same as Figure 2 for pump probe delays $1.5 \leq t \leq 50$ ps.

data, leading to an increased OD in the red at the expense of the blue absorption component, with the conservation of an isosbestic point at 515 nm over most of this period.

The end result of this decay is however different when exciting on the red and blue edges of the reactant absorption. In the latter case this phase nearly erases the absorption component to the blue of the bleach feature, giving rise to a

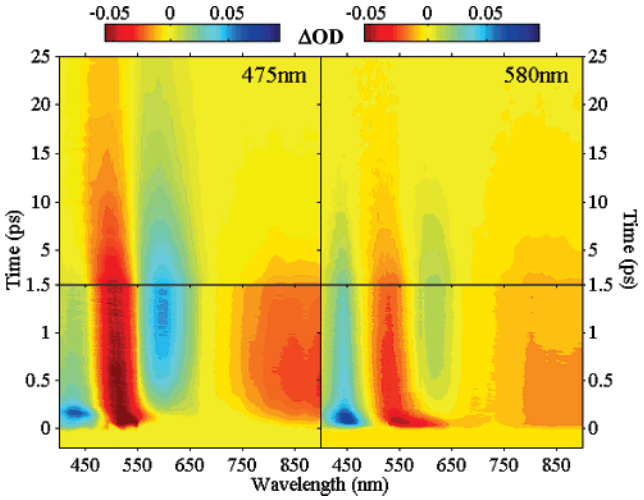


Figure 4. Contour map of transient difference spectra corrected for probe group delay dispersion from −0.5 to 20 ps, shown on a split time axis with an expanded scale up to 1.5 ps. Notice the different color-coded scales of ΔOD pertaining to the different experiments.

prominent absorption peaking at ~590 nm. When exciting to the red, a similar shift leads from a situation of dominant excited-state absorption to the blue peaking near 450 nm, to one where a balanced degree of absorption appears on both sides of the bleach feature centered at 530 nm. An additional difference is a delayed buildup of emission in the range from 700 to 800 nm during the initial ~100 fs of probe delay observed following blue absorption, a counterpart of which is undetected when exciting at 580 nm. At long delay times a spectral feature resembling the derivative of the ground-state absorption bleach which outlives the emission is observed clearly in the blue excitation data, which is much weaker or absent when exciting in the red.

In view of the separable timescales for spectral evolution apparent in both data sets, global fitting to a parallel compartment model producing decay-associated spectra (DAS) was conducted after singular value decomposition (SVD) was employed for noise filtering and data reduction.[35] The spectral evolution was modeled with three and four decay components for red and blue excitation experiments, respectively, keeping the associated spectra constant. The fourth ~60-ps component was necessary when exciting in the blue to model the slowly decaying bleach component described above and observed in Figures 3 and 4. The resulting spectra and the decay times recovered are summarized in Figure 5. As the full delay range was obtained with short and long experimental runs, without the option for pump normalization, the fitting was first conducted for these runs independently. An iterative adjustment process was later conducted to obtain a unified set of parameters which optimally fit both short and long data sets.

The induced transmission changes following blue and red photoexcitation are demonstrated to be well fit from 100 fs with two of the decay constants close to 0.150 and 10.5 ps in both cases. The intermediate decay time obtained by global fitting is somewhat different in the two cases, being best fit to 1.2 and 2 ps for 475 and 580 nm excitation, respectively. These time constants were arrived at by an iterative process which started with unrestricted global fitting of the raw data following SVD to a triexponential decay. Then a common decay constant was sought for the ~10-ps decay stage which would fit both data sets equally leading to the 10.5-ps decay constant fit to both. The sub-picosecond decay was found from both data sets

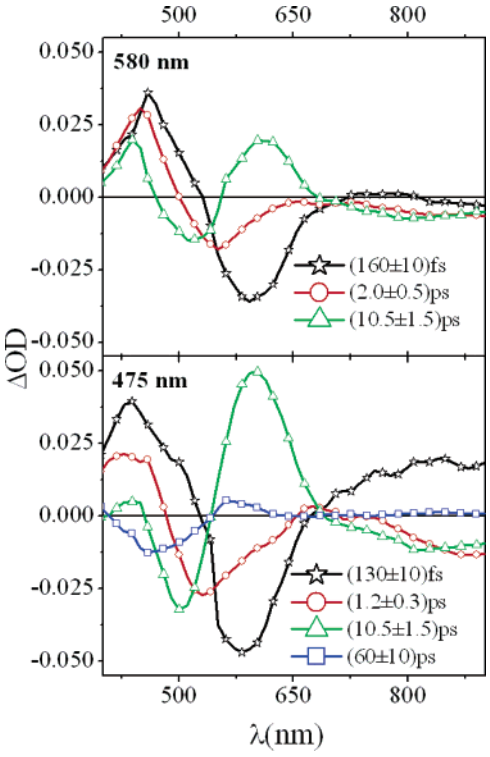


Figure 5. DAS derived by global fitting of the data at both excitation wavelengths to a multiexponential functional form with the depicted decay constants. The black DAS are obtained from the short time data sets and match a global delay time (Figure 4) of 100 fs.

without any imposed constraints to be different by no more than 20% (130 vs 160 fs).

A longer 60-ps decay was found necessary only in the 480-nm excitation experiments where long life time differential spectral components were detected as described above. The quality of fit afforded by these functions using the tabulated constants to the raw data is displayed in Figures 6 and 7. The former presents slices of raw data along with the predictions of the model for both data sets at various representative wavelengths. Figure 7 shows the deviation from data to model in a color-coded contour plot, exhibiting a good match between the two over the full range of delays and probe wavelengths. The resulting DAS accordingly provide a reasonable basis for discussing the various phases of spectral evolution, and the underlying dynamics.

Discussion

The target of our investigation is to asses whether the excited-state dynamics of the retinal chromophore is correlated with its absorption maximum as the dynamics was shown to be quite different in RPSB (440 nm in methanol solution) and in BR (570 nm). Achieving this would optimally involve translating the transient absorption data of chromophore I into a dynamic model covering the full duration of the photochemical event, including all relevant transient species with their associated dynamically evolving spectra. Not only are we unable at this time to achieve this goal for the compound under study but a similar comprehensive model for the transformations in BR and RPSB is not available. In the present case analysis of our data even questions the use of the reactants absorption spectrum, the one species associated spectrum which is in general known with high precision, to simulate the pump induced bleach. Accordingly the comparison between the photoinduced dynamics will to an extent be conducted on a phenomenological level,

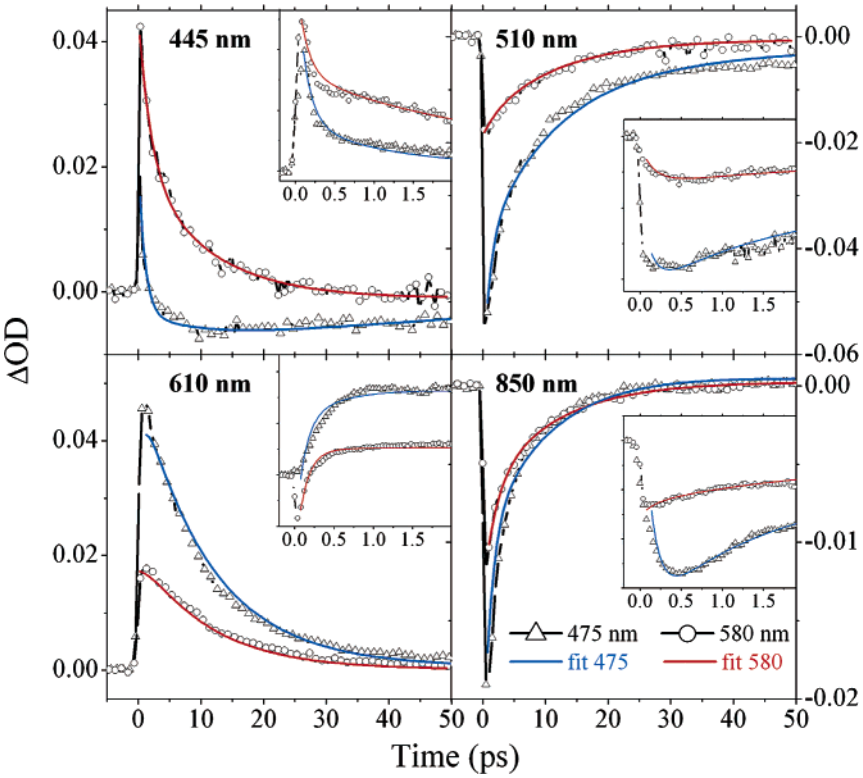


Figure 6. Cuts in the multichannel data taken at wavelengths representing significant features in the transient spectra. The colored curves present the fit provided by the DAS and decay times from Figure 5 are shown for comparison.

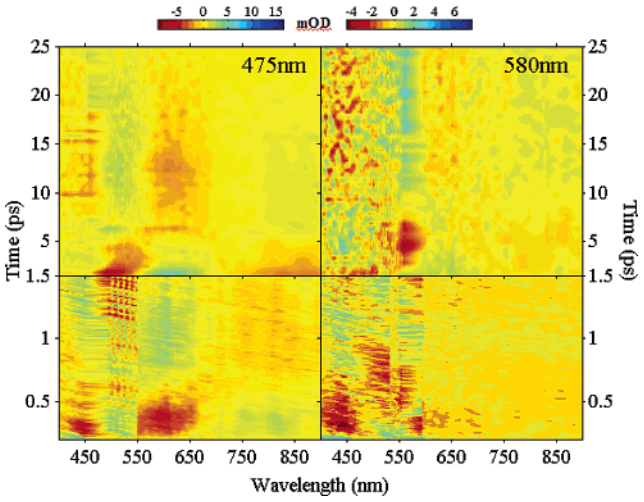


Figure 7. A color-coded contour map presenting the residuals after fitting the maps presented in Figure 4 with the multiexponential model summarized in Figure 5.

as reflected by the analysis in terms of DAS and not species associated spectra[35].

Wavelength-Dependent IC Kinetics. We concentrate first on 580-nm pumping results, deferring until later the effects of tuning the excitation pulse spectrum within the reactants absorption band. The red-shifted absorption maximum displayed for chromophore I in Figure 1 gives rise to a matching spectral shift in the excited-state emission of chromophore I which as in the case of BR extends much farther into the IR than that from alcohol solutions of RPSB. Disregarding the dramatic sub-picosecond spectral evolution described above, IC kinetics are presumably reflected primarily in the stimulated emission decay which is well described as biexponential with 2- and 10.5-ps decay times as shown in Figure 5. Since 580-nm pumping induced changes below 650 nm that must reflect both ground-state absorption bleach overlapping with excited-state absorption decay concertedly with the stimulated emission, the assignment of both to IC seems straightforward and provides a basis of comparison to the divergent reference points of BR and RPSB. By use of this criterion, we conclude that despite the spectral tuning achieved in chromophore I, which closely mimics the opsin shift in BR, the red shift in the absorption maximum of compound I does not catalyze IC as observed in BR but actually inhibits it as reflected in the 10.5 ps here vs 4[20] and 7 ps[18] obtained in gated fluorescence and transient transmission experiments respectively. It is difficult to decide which of these literature sources is more relevant for comparison, just as the source of the discrepancy between them is not clear. In both cases however the rates are higher than that observed here of $\sim$0.1 ps^{-1}. Solvent properties have been shown to mildly effect the late stages of RPSB IC but not enough to induce such a large difference.

Akin to BR,[36] and completely unlike RPSB in ethanol,[17,18] the difference spectrum during the first $\sim$40 fs of delay following excitation of chromophore I consists of excess transmission all the way from 900 nm to the ground-state bleach, making it impossible to separate the two contributions. To the blue of the ground-state bleach an instantaneous buildup of a sharply peaked excited-state absorption is also common to observations in BR. The sub-picosecond component of spectral evolution observed in our experiments, represented by the black curve in the right-hand panel of Figure 5, matches neither the trends in BR where excited-state absorption shifts rapidly to higher photon energies nor those for RPSB, which is initially characterized by induced absorption from the ground-state bleach up to the near IR, with a delayed buildup of emission. Comparison with literature results on RPSB is problematic as the reported measurements involve 400-nm excitation which lies significantly below the 450-nm absorption peak. They may therefore be more analogous to our 475-nm experiments where

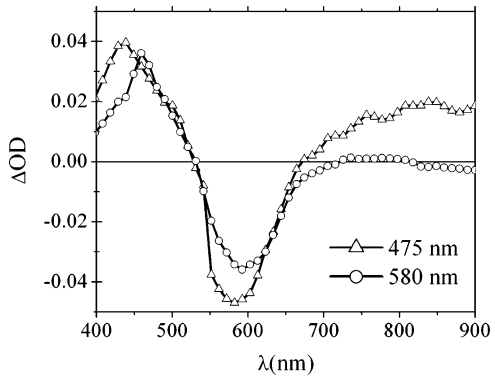


Figure 8. Spectra associated with the sub-picosecond (~150 fs) decay component extracted from data after excitation with pulses at 475 and 580 nm.

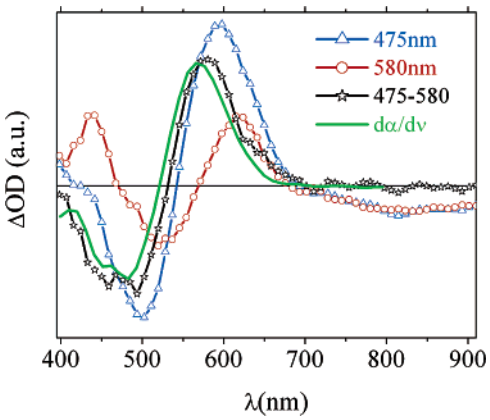


Figure 9. Subtraction of transient absorption spectra obtained from blue and red excitation at a delay of 6 ps, along with a normalized first derivative of the ground state absorption of compound I for comparison. See text for details.

delayed fluorescence buildup is in fact observed. The spectrum associated with the ~150-fs decay depicted in black in Figure 5 demonstrates that, while the emission is nearly unchanged, a rapid shift in absorption to the red with an apparent isosbestic point near 515 nm takes place during the initial few hundreds of fs following photoexcitation.

The isosbestic point nearly coincides with the peak of the reactant's absorption, opening the possibility that the observed red shift in absorption is actually due to ground-state bleach dynamics. In this scenario excitation initially induces excess bleach near the pumping wavelength, later relaxing to reproduce a negative contribution from the full ground state band. Excitation experiments on the blue edge of the absorption band can test this possibility. A comparison of DAS related to the sub-picosecond decay components in both 580- and 475-nm experiments is presented in Figure 8 and are nearly identical in the range from 400 to 650 nm. To the red a positive absorption component appears in the 475-nm excitation DAS, but this is probably related to the delayed appearance of fluorescenece which is modeled by a short-lived absorption band in this spectrum, which is compensated by emission bands in the slower decaying DAS as seen in Figure 5. The fact that the initial evolution in the blue half of the probed range is identical regardless of the excitation wavelength allows its assignment to the excited state and not to a dynamic or static hole burning in the ground state.

Sub-Picosecond Spectral Evolution and Sample Inhomogeneity. As discussed in the introduction, a stage of spectral evolution on similar time scales has been reported in almost all retinal proteins[37] and in RPSB solution, making its assignment a separate focus of interest in these systems. In BR it has been interpreted in terms of structural rearrangement along displaced high-frequency modes of the chromophore,[38] and in some cases to rapid dielectric response of the protein binding pocket.[39] A recent time-resolved fluorescence study of RPSB in various solvents envisions an alternative scenario where simultaneous excitation of, and subsequent rapid IC between closely spaced excited singlet states.[20] Such a scenario has recently been suggested for explaining the isomerization dynamics and transient spectroscopy observed in photexcited BR and RPSB.[40] Both are assumed to be optically coupled to the ground state, and a combination of vibrational and electronic relaxation in the combined manifold leads to a rapid stage of fluorescence narrowing and red shifting. The existence of an isosbestic point during this stage of spectral evolution after red and blue excitation is consistent with a stage of electronic relaxation between two excited surfaces. During the same period only a weak change in the intensities of emission are observed, so if the band shifting is due to electronic relaxation, both states must be optically coupled to the ground state almost equally as suggested by Zgrablic et. al.,[20] very unlike polyenes such as carotenoids.[41,42,43]

Unraveling this question could be aided by subtracting ground state contributions to the transient spectra, in order to establish if absorption from excited states exhibits double peaked structures, indicative of more than one absorbing species. As described in the experimental our excitation fluence determinations are estimated to be accurate to ±15%, and this limitation could mar the results of this procedure. However, examination of the data obtained at both wavelengths (580 and 475 nm) raises doubts of whether such an approach is meaningful. At all delay times, including stages that are dominated by the 10.5-ps relaxation component where emission in both experiments is isospectral and isotemporal, an excess of transmission persists near the central wavelength of the excitation pulses used. This is apparent in Figure 4 where, from 1 ps onward, the trough of excess transmission in shades of red is centered at 490 nm after blue excitation with a significantly expressed absorption only to longer wavelengths, while the red absorption leads to a similar trough centered at 525 nm, sandwiched by two discernible absorption ridges. Figure 9 presents a subtraction of the two data at 6-ps delays where emission amplitude is virtually identical in both, along with a scaled first derivative of the absorption spectrum displayed in Figure 1. A possible interpretation is that this spectrum is inhomogeneously broadened by subpopulations that interconvert on timescales longer than the few tens of ps explored by IC.

If in fact this interpretation is correct, it would be surprising on a number of levels. First, width of the absorption spectrum is on par with that of BR and even narrower than that of RPSB in ethanol as shown in Figure 1. If a significant part of that is inhomogeneous the homogeneous width must require it to be even narrower than that of BR or RPSB in alcohol solutions still. Breaking down the mechanisms of broadening for chromophore I in TFE to its individual contributions from vibronic displacements and electronic dephasing has not been systematically conducted. Accordingly the suggestion that this band is inhomogenously broadened by slowly equilibrating structural changes is in principle an open question. As stated in the experimental section it is known that retinal protonated Schiff base does isomerize in thermal equilibrium to ca. 80% all trans and 20% 13 cis.[44] However in analogy with other polyene PSB compounds, these isomers should exhibit almost identical electronic spectra and such a relative minor cis component

should not induce significantly altered kinetic traces as seen here at the two wavelengths explored. Another source for ground-state inhomogeneity may involve multiple chromophore−solvent structures having different absorption maxima.

Other interpretations of the difference of ΔOD in the range 400−650 nm throughout the excited-state lifetime would include the generation of vibrationally excited ground state reactants/products that naturally absorb to the red of their relaxed analogues. This interpretation is in conflict with the existence of this difference in transmission immediately after excitation and before photoproducts should be generated. Another possibility would be that, despite the electronic relaxation which is reflected in the DAS in Figure 8, tuning the excitation wavelength alters the initial population of different excited states, possibly explaining the similarity, albeit with opposite sign, of the different spectra in Figures 8 and 9. Further investigation is required to decide definitely between these possibilities. One thing is certain and that is that tuning the excitation wavelength within the intense visible absorption band of compound I in TFE produces significantly different spectral evolution reflecting photon energy effects on its photochemical dynamics.

Comparing Chromophore I Photochemistry with That of RPSB and BR. Perhaps the most important issue raised by this aspect of our results is the question of whether similar effects exist in the photochemistry of RPSB in alcohol solution, which as explained in the introduction is the constant yardstick by which protein effects on retinal chemistry within rhodopsins is measured. While experiments are underway in our laboratory to test this point, comparing our findings with previous studies of RPSB photoisomerization in methanol solution on longer timescales is instructive. The detection of the reactant bleach that outlives the excited-state is consistent with generation of mono-cis products that exhibit a reduced extinction coefficient relative to the all trans reactants.[21] The relative prominence of this feature in the case of "blue" excitation is also compatible with some reports indicating an increased isomerization quantum yields and product distribution as well.[45] A similar wavelength dependence was however not detected in photoisomerization experiments on mono-cis conformers.[46] The excess absorption that is observed up to the latest delays studied is inconsistent with long-term product buildup but may be associated with unstable single bond isomers that are observed even after seconds when unprotonated Schiff bases are studied, and which may have nanosecond lifetimes even in the protonated analogues[21].

It is noteworthy that, in the current study, in RPSB in alcohol solutions,[17,18,19,20] and in most ion-pumping rhodopsins,[26,29] IC kinetics are well fit to a biexponential decay, the first ∼1 ps, and a second a few picoseconds in time scale. In the case of BR, a similar finding with somewhat shorter decay times was also reported and possible origins of the separable timescales discussed.[47] In the case of the solution-phase RPSB, a combination of excited-state and ground-state cooling, with its effects on IC were suggested to be responsible for the multiexponential decay.[19] In other cases parallel schemes with reactive and unreactive branches in the excited-state have been invoked for explaining IC kinetics in retinal proteins. The possibility that IC back reactions in retinal proteins can influence ultrafast pump−probe experiments must always be considered since the quantum yield of primary photochemistry is less than 70% even in the most efficient transformations. While transient changes in broad and unstructured electronic bands can provide apparent lifetimes of reactive intermediates, they cannot disclose the underlying changes in chemical bonding and molecular geometry, leading to the ambiguity in interpretation discussed above.

Recent studies based upon ultrafast vibrational spectroscopic methods can help in sorting out the ambiguity in assigning the decay components with a dynamic scheme. Transient IR probing was recently used to follow primary events in Halorhodopsin.[48] The results indicate that the 2- and 8-ps decay components previously observed in pump probe data relate to separate IC channels, with only the former leading to photoproducts. Also in a femtosecond-stimulated Raman probing study on BR, nonlinear emission signals exhibited a quarter picosecond decay of the excited population out of the Franck Condon region,[49] a time scale close to the leading IC decay proposed in some visible pump probe experiments,[47] and in recent UV probing experiments on BR.[50] It is tempting to associate the multiexponential decay kinetics in these various systems to a similar mechanism. Verification of this supposition will however require the application of similar methods to the various retinal proteins and their archetypical model system—the isolated RPSB in solution. Only then will we be able to ascertain if this kinetic similarity reflects analogous dynamics.

Finally, while we have concentrated here on the temporal aspects of IC in this model system, another aspect of protein effects on retinal photochemistry is not covered here at all and that is the quantum yield and selectivity of double bond isomerization. Tuning of the topology and vertical energy gaps between the optically active states is likely to effect these aspects as well, and this should be the target of future work. In particular, the notion presented in several studies that the quantum efficiency for isomerization should diminish with a reduction in IC rates could thereby be tested.

Conclusions

A retinal schiff base analogue which closely mimics the opsin shift in BR has been investigated with femtosecond multichannel pump−probe spectroscopy. Results demonstrate that the red shift afforded in the model system does not hasten internal conversion relative to RPSB in solution, and stimulated emission takes place with biexponential kinetics and characteristic timescales of approximately 2 and 10.5 ps. This shows that interactions between the prosthetic group and the protein, which lead to the opsin shift in BR, are not directly involved in reducing the excited-state lifetime by nearly an order of magnitude. A subpicosecond phase of spectral evolution which consists of a discontinuous red shifting of induced absorption features is detected and assigned to electronic relaxation within the excited-state manifold. This interpretation is based on the appearance of identical spectra changes following excitation anywhere within the reactants intense visible absorption band, and is bolstered by the isosbestic point observed throughout. The fact that analogous stages of rapid spectral change are observed nearly in all retinal proteins and free RPSBs underlines the importance of these findings. Finally, a transient excess bleach component which tunes with the excitation wavelength is detected in the data and tentatively assigned to inhomogeneous broadening in the ground-state absorption band. Finding out if the pronounced excitation wavelength dependence of both the dynamics and the transient spectra is relevant to photoinduced dynamics in native RPSB in solution as well is the focus of continued study in our lab.

Acknowledgment. We thank Prof. N. P. Ernsting, Dr. L. Lustres, O. Shoshana, O. Nahmias, and E. Mastov for technical assistance. This work was supported by the Israel Science

Foundation, which is administered by the Israel Academy of Sciences and the Humanities, and by the A.M.N. Fund for the Promotion of Science, Culture and Arts in Israel. The Farkas Center is supported by the Minerva Gesellschaft, GmbH, Munich, Germany. M. S. holds the Katzir-Makineni Chair in chemistry.

References and Notes

(1) Spudich, J. L.; Yang, C. S.; Jung, K. H.; Spudich, E. N. *Annu. Rev. Cell Dev. Biol.* **2000**, *16*, 365.
(2) Beja, O.; Aravind, L.; Koonin, E. V.; Suzuki, M. T.; Hadd, A.; Nguyen, L. P.; Jovanovich, S.; Gates, C. M.; Feldman, R. A.; Spudich, J. L.; Spudich, E. N.; DeLong, E. F. *Science* **2000**, *289*, 1902.
(3) Andersen, L. H.; Nielsen, I. B.; Kristensen, M. B.; El Ghazaly, M. O. A.; Haacke, S.; Nielsen, M. B.; Petersen, M. Å. *J. Am. Chem. Soc.* **2005**, *127*, 12347.
(4) *Biochim. Biophys. Acta.* 2000, *1460*, special issue.
(5) Nakanishi, K.; Balogh-Nair, V.; Arnaboldi, M.; Tsujimoto, K.; Honig, B. *J. Am. Chem. Soc.* **1980**, *102*, 7945.
(6) Tajkhorshid, E.; Suhai, S. *Theor. Chem. Acc.* **1999**,*101*, 180.
(7) Houjou, H.; Inoue, Y.; Sakurai, M. *J. Am. Chem. Soc.* **1998**, *120*, 4459.
(8) Rajamani, R.; Gao, J. *J. Comp. Chem.* **2001**, *23*, 96.
(9) Kloppmann, E.; Becker, T.; Ullmann, G. M. *Proteins: Struct. Funct. Bioinf.* **2005**, *61*, 953.
(10) Hoffmann, M.; Wanko, M.; Strodel, P.; Konig, P. H.; Frauenheim, T.; Schulten, K.; Thiel, W.; Tajkhorshid, E.; Elstner, M. *J. Am. Chem. Soc.* **2006**, *128*, 10808.
(11) Blatz, P. E.; Mohler, J. H.; Navangul, H. V. *Biochemistry* **1972**, *11*, 848.
(12) Baasov, T.; Sheves, M. *J. Am. Chem. Soc.* **1985**, *107*, 7524.
(13) Albeck, A.; Livnah, N.; Gottlieb, H.; Sheves, M. *J. Am. Chem. Soc.* **1992**, *114*, 2400.
(14) Harbison, G. S.; Smith, S. O.; Pardoen, J. A.; Courtin, J. M. L.; Lugtenburg, J.; Herzfeld, J.; Mathies, R. A.; Griffin, R. G. *Biochemistry* **1985**, *24*, 6955.
(15) Nakanishi, K.; Crouch, R. *J. Isr. Chem.* **1995**, *35*, 253.
(16) Rohrig, U. F.; Guidoni, L.; Rothlisberger, U. *Chem. Phys. Chem.* **2005**, *6*, 1836.
(17) Hamm, P.; Zurek, M.; Roschinger, T.; Patzelt, H.; Oesterhelt, D.; Zinth, W. *Chem. Phys. Lett.* **1996**, *263*, 613.
(18) Hou, B.; Friedman, N.; Ruhman, S.; Sheves, M.; Ottolenghi, M. *J. Phys. Chem. B* **2001**, *150*, 7042.
(19) Hamm, P.; Zurek, M.; Roschinger, T.; Patzelt, H.; Oesterhelt, D.; Zinth, W. *Chem. Phys. Lett.* **1997**, *268*, 180.
(20) Zgrablic, G.; Voïtchovsky, K.; Kindermann, M.; Haacke, S.; Chergui, M. *Biophys. J.* **2005**, *88*, 2779.
(21) Freedman, K. A.; Becker, R. S. *J. Am. Chem. Soc.* **1986**, *108*, 1245.
(22) Koyama, Y.; Kubo, K.; Komori, M.; Yasuda, H.; Mukai, Y. *Photochem. Photobiol.* **1991**, *54*, 433.
(23) Doukas, A. G.; Aton, B.; Callender, R. H.; Ebrey, T. G. *Biochemistry* **1978**, *17*, 2430.
(24) Del Zoppo, M.; Castiglioni, C.; Gerola, V.; Zuliani, P.; Zerbi, G. *J. Opt. Soc. Am. B* **1998**, *308*, 15.
(25) Losa, A. M.; Galván, I. F.; Martín, M. E.; Aguilar, M. A. *J. Phys. Chem. B* **2006**, *110*, 18064.
(26) Lutz, I.; Sieg, A.; Wegener, A. A.; Engelhard, M.; Boche, I.; Otsuka, M.; Oesterhelt, D.; Wachtveitl, J.; Zinth, W. *PNAS* **2001**, *98*, 962.
(27) Zadok, U.; Khatchatouriants, A.; Lewis, A.; Ottolenghi, M.; Sheves, M. *J. Am. Chem. Soc.* **2002**, *124*, 1184.
(28) Olivucci, M.; Lami, A.; Santoro, F. *Angew. Chem., Int. Ed.* **2005**, *44*, 5118.
(29) Schmidt, B.; Sobotta, C.; Heinz, B.; Laimgruber, S.; Braun, M.; Gilch. P. *Biochim. Biophys. Acta* **2005**, *1706*, 165.
(30) Lenz, M. O.; Huber, R.; Schmidt, B.; Gilch, P.; Kalmbach, R.; Engelhard, M.; Wachtveitl, J. *Biophys. J.* **2006**, *91*, 255.
(31) Sheves, M.; Albeck, A.; Friedman, N.; Ottolenghi, M. *Proc. Natl. Acad. Sci. U.S.A.* **1986**, *83*, 3262.
(32) Shoshana, O.; Pérez, Lustres, J. L.; Ernsting, N. P.; Ruhman, S. *Phys. Chem. Chem. Phys.* **2006**, *8*, 2599.
(33) Backus, S.; Peatross, J.; Huang, C. P.; Murnane, M.; Kapteyn, H. C. *Opt. Lett.* **1995**, *20*, 2000.
(34) Kovalenko, S. A.; Dobryakov, A. L.; Ruthmann, J.; Ernsting, N. P. *Phys. Rev. A* **1999**, *59*, 2369.
(35) van Stokkum, I. H. M.; Larsen, D. S.; van Grondelle, R. *Biochim. Biophys. Acta* **2004**, *1657*, 82.
(36) Mathies, R. A.; Brito Cruz, C. H.; Pollard, W. T.; Shank, C. V. *Science* **1988**, *240*, 777.
(37) Schmidt, B.; Sobotta, C.; Heinz, B.; Laimgruber, S.; Braun, M.; Gilch, P. *Biochim. Biophys. Acta*. **2005**, *1706*, 165.
(38) Cembran, A.; Bernardi, F.; Olivucci, M.; Garavelli, M. *J. Am. Chem. Soc.* **2003**, *125*, 12509.
(39) Kennis, J. T. M.; Larsen, D. S.; Ohta, K.; Facciotti, M. T.; Glaeser, R. M.; Fleming, G. R. *J. Phys. Chem. B* **2002**, *106*, 6067.
(40) Cembran, A.; Bernardi, F.; Olivucci, M.; Garavelli, M. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 6255.
(41) Kohler, B. E. *Chem. Rev.* **1993**, *93*, 41.
(42) Hudson, B. S.; Kohler, B. E.; Schulten, K. *Excited States* **1982**, *6*, 1.
(43) Polívka, T.; Sundström, V. *Chem. Rev.* **2004**, *104*, 2021.
(44) Sheves, M.; Baasov, T. *J. Am. Chem. Soc.* **1984**, *106*, 6840.
(45) Donahue, J.; Waddell, W. *Photochem. Photobiol.* **1984**, *40*, 399.
(46) Becker, R. S.; Freedman, K. *J. Am. Chem. Soc.* **1985**, *107*, 1477.
(47) Feng, G.; Hasson, K. C.; McDonald, J. C.; Anfinrud, P. A. *Science* **1998**, *279*, 1886.
(48) Peters, F.; Herbst, J.; Tittor, J.; Oesterhelt, D.; Diller, R. *Chem. Phys*. **2006**, *323*, 109.
(49) McCamant, D. W.; Kukura, P.; Mathies, R. A. *J. Phys. Chem. B* **2005**, *109*, 10449.
(50) Schenkl, S.; Mourik, F. V.; Friedman, N.; Sheves, M.; Schlesinger, R.; Haacke, S.; Chergui, M. *PNAS* **2006**, *103*, 4101.