J|A|C|S
ARTICLES
Published on Web 07/02/2002

Following Evolution of Bacteriorhodopsin in Its Reactive Excited State via Stimulated Emission Pumping

Sanford Ruhman,*,† Bixue Hou,† Noga Friedman,‡ Michael Ottolenghi,† and Mordechai Sheves‡

Contribution from the 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 April 5, 2002

Abstract: New information concerning the photochemical dynamics of bacteriorhodopsin (BR) is obtained by impulsively stimulating emission from the reactive fluorescent state. Depletion of the excited-state fluorescence leads to an equal reduction in production of later photoproducts. Accordingly, chromophores which are forced back to the ground state via emission do not continue on in the photocycle, conclusively demonstrating that the fluorescent state is a photocycle intermediate. The insensitivity of depletion dynamics to the "dump" pulse timing, throughout the fluorescent states lifetime, and the biological inactivity of the dumped population suggest that the fluorescent-state structure is constant, well-defined, and significantly different than that where crossing to the ground state takes place naturally. In conjunction with conclusions from comparing the photophysics of BR with those of synthetic analogues containing "locked" retinals, present results show that large-amplitude torsion around $C_{13}{=}C_{14}$ is required to go between the above structures.

Introduction

Ultrafast storage and fixation of absorbed photon energy is common to most natural photosynthetic systems and is thought to be a determining factor in their high efficiency. Bacteriorhodopsin (BR), the light-energized proton pump embedded in the purple membrane of *Halobacterium salinarum*,[1−3] has been investigated using femtosecond pump−probe spectroscopy for over a decade, with the intention of deciphering the initial stages of translating photon energy into biological activity.[4,5] Indeed, dramatic spectral changes do take place in BR on the subpicosecond time scale. Aside from an instantaneous ground-state bleach, and sub-100-fs spectral shifts, strong absorption and emission bands appear, peaking at 460 and 950 nm, respectively. Both bands are assigned to the fluorescent excited state "I_{460}", where 460 refers to the absorption peak of the intermediate in nanometers. Both bands then decay concertedly with a ∼0.5 ps time scale, while an excess red absorption buildup is associated with "J_{625}", believed to be the first reactive ground-state intermediate.[6−10]

The absorbing moiety in BR is a retinal molecule, bound by a protonated Schiff base (PSB) linkage to the protein. Knowledge that isomerization of this prosthetic group from *all-trans* to *13-cis* is essential to the biological activity in BR strongly influenced the interpretation of early femtosecond investigations. These portray the retinal as a light-activated spring, which upon absorption of a photon isomerizes impulsively, transforming the photon energy into a mechanical strain within the protein. Subsequently, the strain was envisioned to drive the ∼10-ms photocycle to completion, culminating in ejection of a proton from the cell. From the available pump−probe data, a <200-fs time scale for partial isomerization was determined,[7−9] and the brevity of this process was suggested to be essential for the high efficiency of the biological action (0.6).[11,12]

More recent studies do not support this scenario. Theoretical models of internal conversion in retinal PSB highlight the necessary activity of more than one internal degree of freedom.[13] Experimentally, the initial ∼200 fs of spectral evolution in photoexcited BR is found to be virtually identical to that observed for an analogue containing a "locked" retinal group which is prohibited from twisting around $C_{13}{=}C_{14}$ by a five-membered ring (BR5.12).[14,15] This indicates that the said torsion

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

(1) Ottolenghi, M., Sheves, M., Eds. *Isr. J. Chem.* **1995**, *35*.
(2) Lanyi, J., Ed. *Biochim. Biophys. Acta Bioenerg.* **2000**, 1460.
(3) Haupt, U.; Tittor, J.; Oesterhelt, D. *Annu. Rev. Biophys. Biomol. Struct*. **1999**, *21*, 367.
(4) Kochendoerfer, G. G.; Mathies, R. A. *Isr. J. Chem.* **1995**, *35*, 211.
(5) Stuart, J. A.; Birge, R. R. *Biomembranes* **1996**, *2A*, 33.
(6) Hurley, J.; Ebrey, T. G.; Honig, B.; Ottolenghi, M. *Nature* **1977**, *270*, 540.
(7) Mathies, R. A.; Brito Cruz, C. A.; Pollard, T. W.; Shank, C. V. *Science* **1988**, *240*, 777.
(8) Dobler, J.; Zinth, W.; Kaiser, K.; Oesterhelt, D. *Chem. Phys*. **1988**, *144*, 215.
(9) Kobayashi, T.; Terauchi, M.; Kouyama, T.; Yoshizawa, M.; Taiji, M. *Proc. SPIE-Int. Soc. Opt. Eng*. **1990**, *1403*, 407.
(10) Sharkov, A. V.; Pakulev, A. V.; Chekalin, S. V.; Matveets, Yv. A. *Biochim. Biophys. Acta* **1985**, *808*, 94.
(11) Govindjee, R.; Balashov, S. P.; Ebrey, T. G. *Biophys J*. **1990**, *58*, 597.
(12) Tittor, J.; Oesterhelt, D. *FEBS Lett.* **1990**, *263*, 269.
(13) Garavelli, M.; Negri, F.; Olivucci, M. *J. Am. Chem. Soc*. **1999**, *121*, 1023.
(14) Zhong, Q.; Ruhman, S.; Ottolenghi, M.; Sheves, M.; Friedman, N.; Atkinson, G. H.; Delaney, J. K. *J. Am. Chem. Soc.* **1996**, *118*, 12828.
(15) Ye, T.; Friedman, N.; Gat, Y.; Atkinson, G. H.; Sheves, M.; Ottolenghi, M.; Ruhman, S. *J. Phys. Chem. B* **1996**, *103*, 5122.

10.1021/ja026426q CCC: $22.00 **© 2002 American Chemical Society**

is not involved in the fastest responses of BR to photon absorption—a conclusion which is supported by studies using other experimental methods as well.[16,17] The 0.5-ps decay of the fluorescent state is the stage at which dynamics of the native and locked BRs diverge, the former continuing in the photocycle to "J_{625}", while the latter repopulates the initial ground state exponentially in ~20 ps. This suggests that decay from "I" to "J" might reflect the stage of retinal isomerization in the native protein.

Much still remains to be learned concerning the crucial primary light-driven events in BR, particularly regarding the formation and decay of the fluorescent "I_{460}" state. In light of the spectral invariance of its bands, is it really a "state", characterized by a specific structure and energy? If so, how is the structure stabilized, and how does it differ from the relaxed ground state, or from the structure of the reactive conical intersection through which internal conversion takes place? If the 0.5-ps decay of the fluorescent state reflects barrierless $C_{13}=C_{14}$ isomerization, why is it not accompanied by continuous shifts in the central frequencies of the excited-state absorption and emission? Does the decay of "I_{460}" reflect a change in the absorption and emission cross sections of the *whole* excited population, or rather a reduction in the density of excited states? Finally, does "I_{460}" lie exclusively along the reactive or nonreactive pathways, or precede bifurcation into the two?

Addressing these questions experimentally is challenging. Direct femtosecond pump−probe experiments have not given them irrefutable answers. Spectroscopies, which have contributed to deciphering later stages of the photocycle such as FTIR and picosecond CARS,[18−20] are not applicable on subpicosecond time scales. Here we report application of a three-pulse ultrafast spectroscopic scheme, which provides novel complementary information concerning the excited-state dynamics. This is not the first application of multiple excitation pulse schemes to follow the evolution of excited retinal proteins,[21] or to study BR specifically.[22,23] It is unique in that the delayed excitation or "dump" pulse is introduced at a wavelength where, for BR, "stimulated emission pumping" (SEP) is conducted exclusively, without simultaneous excitation to higher excited states. This drastically simplifies the interpretation and allows conclusions to be drawn which hinge upon this selectivity.

In the present work, two 30-fs pulses are sequentially applied: one, at 535 nm, to initiate the reaction, and a variably delayed NIR pulse centered at 1070 nm, to selectively stimulate emission (but not absorption) from the fluorescent state. A central working hypothesis in this scheme is that 1070 nm lies outside the range of overlap between emission and absorption bands of "I_{460}".[24] Probe pulses follow the evolution of transmission at two frequencies: 900 nm to probe the fluorescent-state population, or 650 nm to probe the variations in the absorption of the "K_{590}".

SEP has the potential for addressing the issues described above in the following ways. For purposes of illustration, consider two extreme scenarios for the 0.5-ps decay of "I_{460}". In the first, which we coin "dynamical", all the excited states evolve continuously, leading to an exponential reduction in emission and absorption cross sections. In contrast, a "kinetic" scenario envisions the emitting population to be arrested in a specific configuration, characterized by a constant structure and cross section, but the concentration of "I_{460}" decays exponentially due to a thermally activated rate process. In the later case, the probability of stimulating emission should be independent of the delay, whereas in the former the "dump"-induced percent depletion should diminish with the spectral features of "I_{460}". Conducting SEP at various delays has the potential of differentiating between these possibilities—which are not discernible by simple pump−probe data. The same approach, when probing is shifted to follow the formation of later intermediates, can shed light on the questions of both the structure and the identity of the fluorescent state as a photocycle intermediate, by quantitative comparison of "I_{460}" depletion with that observed in the formation of "K". Finally, impulsive stimulated Raman spectroscopy can be employed to extract frequencies of Raman-active modes in the excited state, which would be invaluable for appreciating structural changes in its formation.[25]

Experimental Section

Halobacterium salinarum was grown from the S9 strain, and purple membranes containing bacteriorhodopsin were isolated as previously described.[26] Potassium phosphate buffer was used to adjust the pH to 7. Laser pulses are produced by a homemade ~30-fs multipass amplified Ti:sapphire laser system, generating 600-μJ pulses at 1 kHz. The 1070-nm pulses were derived in a two-stage NOPA (Clark MXR) equipped with a booster consisting of 2 mm of type II phase matched BBO, pumped by 120 μJ at 790 nm, producing 40 μJ in the IR. Initiation of the cycle is conducted by frequency doubling part of the NIR output in 100 μm of BBO, producing a pulse at 535 nm. Probing pulses at 900 and 650 nm were both generated by isolating portions of a white continuum generated in sapphire with 50-nm fwhm interference filters. Prism pairs were used to eliminate residual chirp in all pulses except those at 1070. All pulses were linearly polarized in parallel to each other. Sample preparation and handling, and all other data collection schemes, were as described elsewhere. Pump and probe intensities were reduced to ensure linear signal dependence, while the suspension concentration was varied to obtain pump transmission of ~70%. Scans were collected with dumping pulses alone to test for contributions from two-photon absorption in the IR, with negative results. Figure 1 presents a transient difference spectrum observed in BR 200 fs after photoexcitation,[15] along with an absorption spectrum of BR ground state and intensity spectra of the laser pulses. The 535-nm spectrum was measured directly, while that at 1070 nm was simulated assuming an identical frequency bandwidth.

Results and Discussion

Initial experiments tested the dependence of dumping dynamics on the delay between the 535- and 1070-nm pulses by probing the emission at 900 nm. Figure 2 shows the raw dumping data along with a reference scan taken without IR irradiation. The inset presents $R(t)$, the ratio of stimulated emission signals with and without dumping for the various SEP delays. A nine-point Savitzky−Golay smoothing of the data is

(16) Akiyama, R.; Yoshimori, A.; Kakitani, T.; Imamoto, Y.; Schichida, Y.; Hatano, Y. *J. Phys. Chem. A* **1997**, *101*, 412.
(17) Song, L.; El-Sayed, M. A. *J. Am. Chem. Soc.* **1998**, *120*, 8889.
(18) Maeda, A. *Isr. J. Chem.* **1995**, *35*, 387.
(19) Althaus, T.; Eisfeld, W.; Lohrmann, R.; Stockburger, M. *Isr. J. Chem.* **1995**, *35*, 227.
(20) Atkinson, G. H.; Ujj, L.; Zhou, Y. *J. Phys. Chem.* **2000**, *104*, 4130.
(21) Yan, M.; Rothberg, L.; Callender, R. *J. Phys. Chem. B* **2001**, *105*, 856.
(22) Gai, F.; Mcdonald, C.; Anfinrud, P. A. *J. Am. Chem. Soc.* **1997**, *119*, 6201.
(23) Logunov, S. L.; Volkov, V. V.; Braun, V.; El-Sayed, M. A. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 8475.
(24) Hasson, K. C.; Gai, F.; Anfinrud, P. A. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 15124.
(25) Gershgoren, E.; Vala, J.; Kosloff, R.; Ruhman, S. *J. Phys. Chem. A* **2001**, *105*, 5081.
(26) Oesterhelt, D.; Stoeckenius, W. *Methods Enzymol.* **1974**, *31*, 667.

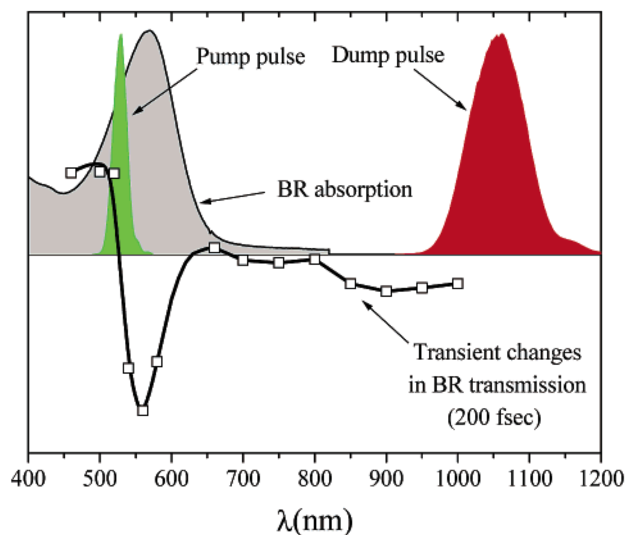

Figure 1. An overlay of normalized spectra, including absorption of light-adapted BR, a difference transmission spectrum in BR 200 fs after photoexcitation, and spectra of pump and dump pulses used in this study. The pump spectrum is directly measured, and that of dump is calculated from the former, assuming conservation of bandwidth in frequency.

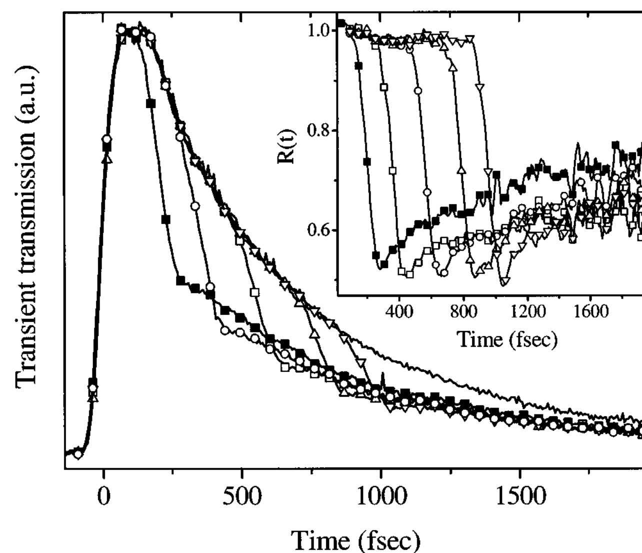

Figure 2. Pump−dump probe data presented for a 900-nm probe and various dumping delays with identical dump fluences. Included is a scan taken without dump. (Inset) The ratio of emission signals with and without dump pulse for the data in the main figure.

implemented to eliminate high-frequency noise. We find that for SEP delays larger than ~200 fs, the postdump portions of the scans converge at longer delay times. In contrast, when the IR pulse comes earlier, the sample experiences a lesser ultimate depletion. Also, the ratio plots are identical within the noise margins, regardless of the dump timing, over the range from >200 to 1000 fs. Finally, stimulated emission scans exhibit an excess depletion immediately after the SEP which recovers within a few hundred femtoseconds. This stage of emission reconstitution is identical for all dumping delays beyond 200 fs.

These results provide striking indications concerning the underlying dynamics within the protein and agree perfectly with the kinetic scenario. Following a brief initial period of dynamical change, the dumping probability and dynamics of emission recovery are identical throughout the lifetime of the fluorescent state. This outcome can arise only if the emitting excited proteins remain unchanged as long as the "I_{460}" state is populated! This finding is compatible with, but farther reaching than, the observed conservation of absorption and emission spectra of the fluorescent state. Thus, despite the indications that the 0.5-ps relaxation of the fluorescent state reflects the process of isomerization, *the emitting population appears photophysically to reflect a stationary "state" which is of constant structure and energy.*

The partial recovery of the emission, observed in all the dumping data, warrants additional comment. Similar trends recorded by Gai et al. were assigned to a replenishment of "I_{460}" from higher lying excited states which were populated by their 800-nm secondary pump pulse.[22] This explanation is not applicable to the present results since the dumping pulses stimulate emission exclusively. Accumulation of ground-state population by the vertical impulsive dumping process is most likely responsible for the excess emission reduction. Due to the vertical nature of optical transitions, down-pumped population will initially absorb precisely at the dumping frequency and further reduce the net emission cross section. Structural rearrangement on the ground state may then rapidly dissipate this absorption, partly replenishing the excess emission. Another mechanism which could further contribute to emission replenishment is "dynamical hole burning" in the emission band.[27]

Accepting the unchanging nature of the fluorescent state over its lifetime raises the intriguing question of whether the dumped population continues on to produce ground-state photoproducts or repopulates the reactant. A previous study has proposed that the constant spectrum of the "I_{460}" bands results from a stage of population accumulating at the reactive curve, crossing to the ground state itself.[28] However, if "I_{460}" lies precisely above the crossing seam, dumping in the IR should, at least partially, lead onward in the photocycle, just as curve crossing would.

To test this, we have chosen to follow dump-induced changes in the absorption of "K_{590}", the intermediate formed exponentially from "J" with a ~4-ps formation time. Following a later intermediate is aimed at allowing all irregular transients to die out before assessing the change in population of the photocycle. Accordingly, three pulse experiments, probing once with 900 nm to record changes in the fluorescent state and then under identical conditions probing at 650 nm to follow "K" formation, were conducted, taking special care to ensure that probes at both wavelengths equally overlap the irradiated volume in the sample. Results are shown in Figure 3. The mainframe portrays the 900-nm probing data, with the 650-nm results displayed to scale in the inset.

A qualitative answer can be obtained directly from the data. Clearly, the process of dumping reduces the ultimate concentration of "K" and does so more efficiently when the dumping is performed earlier. A quantitative analysis is required in order to learn if *all* the dumped population is excluded from the photocycle. For this we have ignored reorientation of the protein on a 10-ps time scale, allowing comparison of fractional reduction in "I_{460}" emission and in "K_{590}" absorption directly on the level of signal, without the need of translating first to species concentrations. Designating the fractional reduction of

(27) Loring, R. F.; Yan, Y. J.; Mukamel, S. *J. Chem. Phys.* **1987**, *87*, 5840.
(28) Haran, G.; Wynne, K.; Xie, A.; He, Q.; Chance, M.; Hochstrasser, R. M. *Chem. Phys. Lett.* **1996**, *26*, 389.

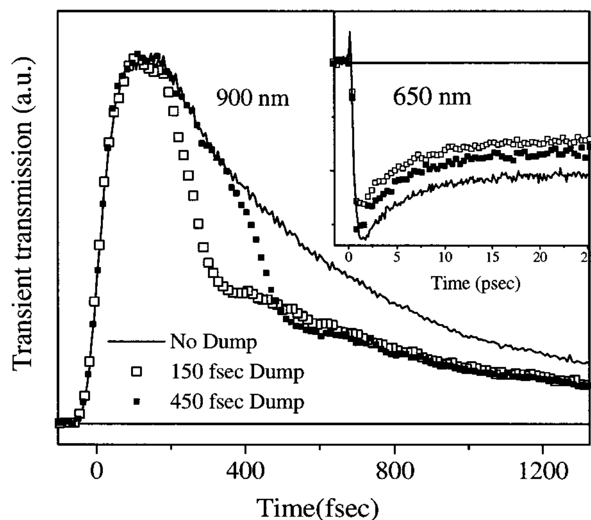

Figure 3. Data as in Figure 2, with two dumping delays, along with pump–probe data without dump pulses for reference. (Inset) Data taken with identical pump and dump cycles, probing at 650 nm for much longer delays for following the formation of "K_{590}".

emission 300 fs after the dump as $R(300)$, the fractional depletion in fluorescent population can be calculated: $\Delta = R(300)\exp(-t/0.5)$. t is the delay in picoseconds from the peak of the stimulated emission to the dump, and the exponential describes the reactive decay of the emitting population with time. $R(300)$ is used as a criterion for emission depletion in order to eliminate the influence of nascent ground-state population. Using this method, we calculate Δ to be 32% and 22% for the two dump delays, respectively. These are identical within error to the degrees of "K" depletion recovered from the 650-nm probing data. We conclude that *all of the dumped population is excluded from the photocycle, and it returns within the time required for the formation of "K" to repopulate the relaxed ground state.*

Finally, to test the linearity of the induced emission and estimate the cross section for dumping, precise measurements of $R(300)$ at a series of IR fluences were conducted, probing emission depletion at 900 nm. Analysis was complicated by the parallel polarization of all pulses. After ensuring that no more than 5% of the protein sample is electronically excited at 535 nm, $R(300)$ is simulated as a function of the dump fluence using eq 1:

$$R = \frac{\int_0^{\pi/2} \cos^4(\theta)\exp(-\cos^2(\theta)\varphi 3\sigma)\sin(\theta)\,\mathrm{d}\theta}{\int_0^{\pi/2} \cos^4(\theta)\sin(\theta)\,\mathrm{d}\theta} \qquad (1)$$

θ is the angle between the transition dipole and the field vectors, φ is the fluence in inverse square centimeters, and σ the emission cross section at the dump frequency in square centimeters. Disregarding slight shifts in dipole orientation, the fourth power of the cosine represents the probability of chromophore excitation and subsequent detection in emission, assuming an initially isotropically oriented sample. The decaying exponential represents the fluence-dependent depletion of all excited proteins at a specific field-dipole angle. After numerical evaluation of $R(\sigma,\varphi)$, the function was fit within an error of <3% to $R(\sigma,\varphi) \sim \exp(-1.97\sigma\varphi)$.

The pulse fluences, 12% of which was transmitted through a 50-μm pinhole, were 100, 200, and 400 nJ per pulse and caused 24, 42, and 62% emission depletion, respectively. These results agree within 10% error with the predictions of eq 1, with $\sigma = (4 \pm 0.5) \times 10^{-17}$ cm^2. This value is consistent with that obtained from the transient spectrum of Figure 1, considering that the BR peak cross section for absorption is $\sim 2.6 \times 10^{-16}$ cm^2.[5]

We turn here to consider the ramifications of our findings. The fluorescent population does, remarkably, behave as a homogeneous stationary state, altering neither in absorption spectrum nor in emission cross section. This state of affairs need not be the case, and its significance is emphasized by the earlier finding that, in contrast, the analogous evolution in excited rhodopsin gives rise to continuous dynamical spectral shifts and periodical spectral modulations of nascent products, indicative of coherent wave packet motion throughout! A simple dynamic scenario, which is compatible with the arrested spectral evolution observed here, would be a barrier en route to the potential crossing configuration, which stops the structural changes before the isomerization takes place. Accumulation of population near the barrier, and thermal activation onward, might explain the observations.[28,29] However, just such a scenario has been proposed and then refuted by experiments conducted at drastically reduced temperatures, with no change in the decay rates of "I_{460}".[30] A rate of 2 ps^{-1} is extremely rapid for an activated structural evolution in so large a molecule, so this mechanistic picture leads to new riddles.

While the stationary nature of the fluorescent population is not trivial to explain, our findings do rule out earlier suggestions that two separable fluorescent components are being observed, one leading back to the ground state and a second representing isomerizing chromophores.[24] Such a scenario would lead to drastic changes with delay time on the effects of SEP. Consistent with this is the finding that multiexponential emission decays, observed before in our laboratory as well, appear to result from excessive excitation fluence. The conclusions of the recent study of Logunov et al.,[23] at least insofar as they are represented in the schematic potential diagram presented, disagree with ours and envision a population which continuously evolves on a relatively flat and featureless fluorescent-state potential en route to "J". The reported dependence of excited-state pumping dynamics on the delay between the pump pulses could stem from involvement of "hot" reconstituted ground-state population, known to absorb weakly at 400 nm when relaxed at 300 K.

Another conclusion of utmost importance is the direct proof that *"I_{460}" is a photocycle intermediate*. While this is often assumed, it has not previously been demonstrated directly,[15] and the contrary has been proposed.[31] The major evidence that the fluorescent state lies along the photocycle has been derived from kinetic analysis of the disappearance of the "I_{460}" bands and the buildup of "J". But the demonstration in this laboratory that similar kinetics are observed near the "J" peak at 660 nm, both for BR and BR5.12, which is prohibited from twisting, has already proven that analysis irrelevant. Accordingly, the results presented here prove for the first time that formation of "I_{460}" either precedes divergence into reactive and nonreactive pathways or lies along the former.

(29) Ben-Nun, M.; Molnar, F.; Lu, H.; Phillips, J. C.; Martinez, T. J.; Schulten, K. *Faraday Discuss.* **1998**, *110*, 447.
(30) Logunov, S. L.; Masciangioili, T. M.; Karmalov, V. F.; El-Sayed, M. A. *J. Phys. Chem. B* **1998**, *102*, 2303.
(31) Kobayashi, T.; Kim, M.; Taiji, M.; Iwasa, T.; Nakagawa, M.; Tsuda, M. *J. Phys. Chem. B* **1998**, *102*, 272.

In view of these conclusions concerning the fluorescent state, it is crucial to understand its structure, the process of its formation, and how crossing to the ground electronic surface is achieved. The two first points are related, since the process of formation involves motion along degrees of freedom which are displaced from the relaxed ground-state geometry. The demonstrated lack of reactivity in the dumped population indicates that, if the ground potentials phase space can be divided into reactant and product subspaces, the "I_{460}" structure must belong to the former. Also, from our study of BR5.12, it is evident that no $C_{13}{=}C_{14}$ torsion is needed for its formation. Theoretical studies identify this mode as a prerequisite for reaching the reactive conical intersection through which the ground potential is accessed.[13] If "I_{460}" formation does not involve any such torsion, and if we interpret the constant photophysical nature of the fluorescent state to indicate a stationary structure, then the stage of large-amplitude double bond torsion required for accessing the ground state must coincide with "I_{460}" decay.

The initial period during which variations in SEP *are* observed, before the said stationary behavior sets in, coincides with a phase of spectral modulations, which were recently observed in this laboratory for the first time.[32] Thus, from present results, we reconfirm that rapid dynamic changes do precede formation of the fluorescent state. The most useful dynamic information for identifying the geometrical changes which accompany its formation could be provided by vibrational spectra. Ongoing impulsive dumping experiments using even shorter IR pulses are being conducted for that purpose and should lead to precise identification of the displaced modes, bringing this approach full cycle.

Finally, this discussion, coming after decades of BR study using ultrafast lasers, demonstrates that insight into the detailed function of systems this complex is gained incrementally. Nonetheless, in our present effort new and decisive indications concerning excited-state dynamics in BR have been uncovered and may serve to reject or continue study of models which arise from the large-scale calculations which have been dedicated to BR photochemistry. At a time when realistic computer simulations for proteins are coming of age, such new indications should prove invaluable.

(32) Ye, T.; Gershgoren, E.; Friedman, N.; Ottolenghi, M.; Sheves, M.; Ruhman, S. *Chem. Phys. Lett.* **1999**, *314*, 429.

Conclusions

Using impulsive stimulated emission pumping, we have demonstrated directly for the first time that the fluorescent state in bacteriorhodopsin is a photocycle intermediate, lying either before the bifurcation into reactive and nonreactive pathways or along the former. The measured insensitivity of depletion dynamics to the "dump" pulse timing, throughout the fluorescent state's lifetime, and the biological inactivity of the dumped population suggest that the fluorescent state structure is constant and significantly different than the natural crossing to the ground state. In conjunction with conclusions drawn from comparing the photophysics of BR with those of synthetic analogues containing "locked" retinals, these results suggest that large-amplitude torsion around $C_{13}{=}C_{14}$ is required to go between the above structures.

Acknowledgment. We thank Prof. E. Riedle, and Mr. J. Piel for help in setting up the IR booster amplifier. This work was supported by the Human Frontier Science Program (HFSP) and the Israel Science Foundation (ISF). The latter is administered by the Israel Academy of Sciences and the Humanities. The Farkas Center is supported by the Minerva Gesellschaft, GmbH, Munich, Germany. M.S. holds the Katzir−Makineni Chemistry Chair.

JA026426Q