

## Pump–Dump–Probe Spectroscopy of Bacteriorhodopsin: Evidence for a Near-IR Excited State Absorbance

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Bacteriorhodopsin (bR) is a light-harvesting retinal protein (26 kDa) found in the purple membrane of *Halobacterium halobium*.<sup>1</sup> The photoactivity of bR derives from a retinal that efficiently photoisomerizes from its *all-trans* to its *13-cis* form<sup>2</sup> ( $\Phi = 0.6^3$ ) and drives the unidirectional transport of protons across the membrane. The photoisomerization of retinal in bR has been studied by femtosecond time-resolved absorbance<sup>4</sup> and fluorescence<sup>5</sup> spectroscopy. Whereas time-resolved fluorescence studies probe the excited state population, time-resolved absorbance studies probe both ground and excited electronic states and can, therefore, probe intermediates along the entire reaction pathway. The enhanced information content afforded by transient absorbance measurements comes at a cost in spectral complexity: negative-going features arise from the bleach of the ground state and from stimulated emission from the excited state; positive-going features arise from absorbance from intermediates and photoproducts. When these features overlap, details relating to the reaction pathway can be obscured. Transient absorbance spectra of bR recorded over a broad spectral range<sup>4d,f</sup> provided indirect evidence for a previously uncharacterized near-IR excited state absorbance whose presence complicates the interpretation of the transient spectra. This suggestion motivated our development of a 3-color femtosecond pump–dump–probe technique whose dump pulse can alter the course of a photoinduced reaction and thereby unveil overlap-

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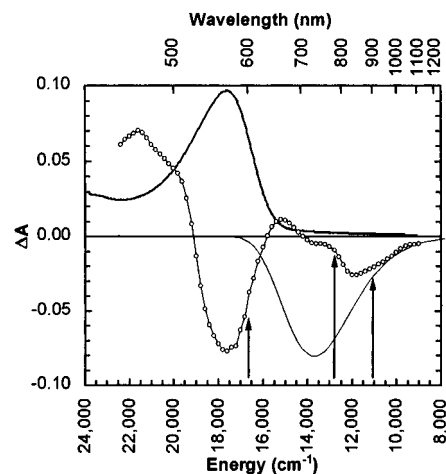
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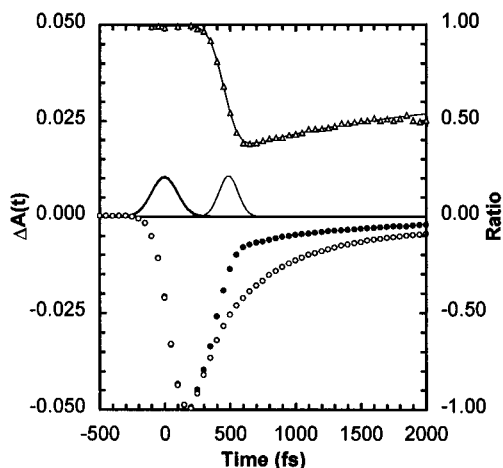
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(6) The pump pulse was generated by frequency doubling the signal output of a home-built BBO-based optical parametric amplifier (OPA). The probe pulse was derived from a broadband continuum that was generated by focusing the output of a second OPA into a 3-mm sapphire crystal. The dump pulse was generated by a regeneratively amplified Ti:sapphire laser system (Clark-MXR Inc., Dexter, MI) whose femtosecond pulses (1 kHz; 0.8 mJ; 120 fs; 780 nm) also powered the two OPAs. The pump, dump, and probe pulses were focused to spot sizes of approximately 200, 160, and 120  $\mu\text{m}$  in diameter, respectively. For the kinetic measurements, the pump and dump pulse energies were adjusted to 300 and 660 nJ, respectively. The bR sample (Sigma) was suspended in Hepes buffer, pH 7.4, and loaded into a 1 mm path length sample cell. The absorbance of the sample was 0.3 at 600 nm.



**Figure 1.** Time-resolved absorbance spectrum of light-adapted bR recorded 0.316 ps after photoexcitation (○). For comparison, a scaled equilibrium absorbance spectrum and a negative-going stimulated emission spectrum are shown (—). The stimulated emission spectrum was constructed from spontaneous emission spectra published elsewhere.<sup>4a,5,7</sup> The vertical arrows indicate the photon energy of the femtosecond pump (600 nm; 16 667  $\text{cm}^{-1}$ ), dump (780 nm; 12 820  $\text{cm}^{-1}$ ), and probe (900 nm; 11 111  $\text{cm}^{-1}$ ) pulses.

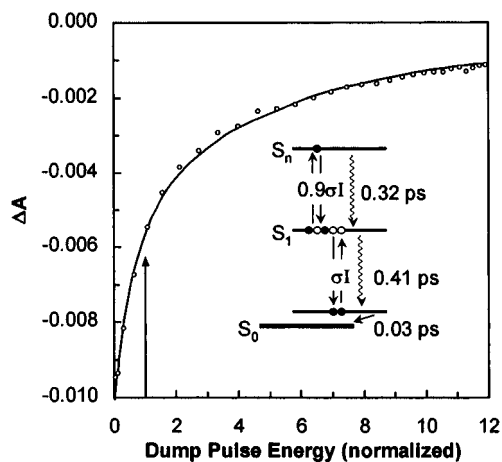


**Figure 2.** Time dependence of the stimulated emission measured at 11 111  $\text{cm}^{-1}$  (900 nm) with (●) and without (○) the dump pulse. The pump and dump pulses are illustrated by their respective instrument response functions (—). The pump, dump, and probe pulses were polarized parallel to one another. The pump pulse excited <30% of the bR within the illuminated volume. The dump pulse was adjusted to 2 times the flux of the pump pulse ( $\text{J cm}^{-2}$ ) and was delayed 450 fs. The ratio of the two traces ( $\Delta$ ) is plotted in the upper half of the figure.

ping features in the transient absorbance spectrum. In this communication, we use pump–dump–probe spectroscopy to reveal and characterize a near-IR excited state absorbance of bR.

Tunable femtosecond pump, dump, and probe pulses were used to excite the bR, perturb the excited state population, and probe the stimulated emission, respectively.<sup>6</sup> The time-resolved absorbance spectrum of bR, recorded without the dump pulse, is shown in Figure 1. The negative-going feature near 12 000  $\text{cm}^{-1}$  (833 nm) arises from stimulated emission and is proportional to the excited state population. This feature is expected to be centered near the peak of the fluorescence emission spectrum, 14 000  $\text{cm}^{-1}$  (714 nm),<sup>4a,5,7</sup> a region where the transient absorbance is quite small. This result suggests that

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**Figure 3.** Pump-induced stimulated emission at 750 fs as a function of dump pulse energy. For these measurements, the pump pulse polarization was oriented at the magic angle ( $54.74^\circ$ ) relative to the dump and probe pulses. A four-state model, illustrated in the inset, was used to fit the dump pulse energy dependence of the stimulated emission (—). The dump pulse energy is normalized relative to that used to acquire the kinetic traces in Figure 2 (vertical arrow).

an excited state absorbance cancels much of the stimulated emission in this vicinity.<sup>4f</sup> A femtosecond dump pulse centered near  $14\,000\text{ cm}^{-1}$  should, therefore, transfer some of the excited state population ( $S_1$ ) back down to the ground state ( $S_0$ ) and some to an even higher excited state ( $S_n$ ). In Figure 2, we show the time dependence of the stimulated emission measured with and without a delayed dump pulse. In the absence of the dump pulse, the stimulated emission decays via two channels: production of the *13-cis* photoproduct and regeneration of the *all-trans* ground state. The dump pulse transfers population out of the first excited state and is manifested as a loss of stimulated emission intensity. According to a least-squares analysis of the ratio of the two curves, the dump pulse transfers approximately two-thirds of the  $S_1$  population into other electronic states. According to the analysis that follows, approximately half of that is sent “upstairs” and the other half “downstairs”. The rate constant for the recovery of the ratio is well described by  $k = (1.46 \pm 0.1\text{ ps})^{-1}$ , which corresponds to the difference between the rates of relaxation from the first,  $k_{0-1} = (0.41 \pm 0.02\text{ ps})^{-1}$ ,<sup>8</sup> and higher,  $k_{1-n} = (0.32 \pm 0.02\text{ ps})^{-1}$ , excited electronic states. As expected, the relaxation from  $S_n$  is faster than the relaxation from  $S_1$ .<sup>9</sup>

To examine these electronic energy levels further, we measured the intensity of the stimulated emission at 750 fs as a function of dump pulse energy (Figure 3). The rate at which the dump pulse depletes the  $S_1$  population is related to the extinction coefficients that radiatively couple the electronic energy levels and the rates of nonradiative relaxation among them. To explain the saturation curve, we invoke a 4-state model with two levels in  $S_0$  and one level each in  $S_1$  and  $S_n$

(8)  $k_{0-1}$  is determined only approximately because the decay of the undumped excited state population is not first order.<sup>4d,f,5</sup>  $k_{0-1} = (0.41 \pm 0.02\text{ ps})^{-1}$  is the weighted average of the rates from a biexponential fit.

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(inset of Figure 3). The need for two levels in  $S_0$  arises because of the large ( $4000\text{ cm}^{-1}$ ) Stokes shift of the fluorescence emission that ensues upon photoexcitation from  $S_0$  to  $S_1$ . The corresponding anti-Stokes shift caused by  $S_1$  being dumped down to the upper level of  $S_0$  causes the absorbance spectrum to shift out of resonance with the dump pulse, thereby trapping the population in the ground state. The saturation curve was modeled by solving numerically the differential equations that couple the four levels. The absorbance and stimulated emission extinction coefficients coupling  $S_0$  and  $S_1$  at 780 nm were assumed to be the same numerical value,  $\epsilon_{780} = 4.6 \times 10^4\text{ L mol}^{-1}\text{ cm}^{-1}$ .<sup>10</sup> The extinction coefficient coupling  $S_1$  and  $S_n$  was made to be  $0.9\epsilon_{780}$ , as required by the numerical value of  $\epsilon_{780}$  and the magnitude of  $\Delta A_{780}$  extrapolated to 0 fs. The nonradiative relaxation rates from  $S_n$  to  $S_1$  and  $S_1$  to  $S_0$  were set at the experimentally determined values. Three parameters were varied in the least-squares fit of the data: the  $S_1$  population without a dump pulse (determines the magnitude of  $\Delta A$  at zero dump intensity), the calibration for the dump pulse flux (determines the slope of the saturation curve near zero dump intensity), and the relaxation rate from the upper to the lower level of  $S_0$  (determines the dynamics of the anti-Stokes shift). The calibration for the dump pulse flux obtained from the least-squares fit was within 20% of the estimate based on focused spot size and pulse energy. This good agreement validates our estimate for  $\epsilon_{780}$ . The best-fit anti-Stokes relaxation rate of  $(0.03 \pm 0.01\text{ ps})^{-1}$  is consistent with the Stokes shift dynamics estimated in earlier studies.<sup>4c,f,5</sup> The ability of the 4-state model to describe the saturation curve out to dump pulse energies much greater than that used to obtain the data in Figure 2 suggests that these electronic levels dominate the photophysics of bR.

The excited state absorbance characterized here accounts for the large apparent Stokes shift of the stimulated emission spectrum.<sup>4d,f</sup> Because this feature overlaps with the photoproduct absorbance, the ground state absorbance, and the stimulated emission, the interpretation of the transient absorbance dynamics becomes more complicated. However, having characterized this feature, we are now working toward a new interpretation of the transient absorbance dynamics with an aim to characterize in detail the reaction pathway(s) leading from the *all-trans* to the *13-cis* isomer of retinal in bR.

Other excited state pump–dump–probe techniques such as fluorescence depletion have been used to study gas-phase photodissociation dynamics.<sup>11</sup> Here we have extended this technique to femtosecond transient absorption spectroscopy. These techniques are quite general and allow one to probe incisively photon-induced reaction pathways.

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(10) The integrated oscillator strengths for stimulated emission and ground state absorbance were assumed to be the same; given  $\epsilon_{570} = 6.2 \times 10^4\text{ L mol}^{-1}\text{ cm}^{-1}$  for absorbance and an estimated fluorescence emission spectrum, it is straightforward to calculate  $\epsilon_{780}$  for stimulated emission.

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