

## REACTIVE INTERMEDIATES IN THE ROOM TEMPERATURE RHODOPSIN PHOTO-REACTIONS: PICOSECOND TIME-RESOLVED CARS

GEORGE H. ATKINSON\*, FRANK JÄGER and LASZLO UJJ\*

*Department of Chemistry and Optical Science Center,  
University of Arizona, Tucson, Arizona 85721*

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The vibrational spectra of the batho and lumi intermediates in the room-temperature rhodopsin photo-reaction are measured by picosecond time-resolved coherent anti-Stokes Raman spectroscopy (PTR/CARS). The instrumental principles underlying PTR/CARS together with the advantages of PTR/CARS applications in protein reactions are discussed.

*Keywords:* Rhodopsin; coherent anti-Stokes Raman scattering

Vibrational spectra from intermediates, appearing over more than five orders of magnitude in reaction time, are measured with exceptionally high signal-to-noise (S/N) ratios using picosecond time-resolved coherent anti-Stokes Raman scattering (PTR/CARS). Developed in recent years explicitly as an experimental methodology for examining biophysical reactions in trans-membrane proteins such as bacteriorhodopsin (BR) [1] and rhodopsin (Rh) [2], PTR/CARS has been utilized primarily to obtain structural information on intermediates that comprise the room-temperature (RT), protein photo-reactions. Since the CARS measurements are made with pump-probe instrumental configurations, the reaction time after optical initiation of the

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\* Corresponding authors.

photo-reaction is the only experimental parameter that is systematically permitted to vary. The resultant vibrational Raman spectra, therefore, monitor the structural changes that occur in real time as the photo-reaction evolves. Under these time-resolved conditions, PTR/CARS data provide an independent measure of the kinetics associated with the formation and decay of intermediates observed that can be compared with kinetic data derived from transient absorption experiments. It is important to note that PTR/CARS data are unique in that they resolve reaction dynamics in terms of molecular structure changes during the reaction rather than only changes in electronic transitions normally monitored *via* transient absorption. The exception to this point is, of course, the transient infrared absorption spectroscopy recently developed for femto/picosecond time resolutions [3, 4]. As a consequence, PTR/CARS studies have revealed structural aspects of photo-reactions not previously elucidated and thereby permit biochemical/chemical reactions to be defined, often for the first time, in terms of the time-dependent (*e.g.*, picosecond) evolution of molecular structure.

The quality (S/N) of PTR/CARS data has recently been significantly improved by a development in the detection system and the incorporation of both sample and reference compartments within the liquid jet sample. This configuration permits the simultaneous generation of CARS signals from the buffer/water solvent (reference) and the reactive protein suspension (sample). A schematic representation of the PTR/CARS apparatus is presented in Figure 1. The two resultant CARS signals are measured on two separate, horizontal sections of a  $1024 \times 256$  pixel array detector located in the focal plane of a triple (two stages of subtractive (filter) dispersion followed by a single, concave grating spectrograph) spectrometer.

Many aspects of the PTR/CARS methodology have been derived from its application to intermediates in the BR photocycle [5]. The BR<sup>RT</sup> photocycle is especially attractive experimentally since it is reversible (within  $ca \sim 5$  ms) reaction sequence that ensures a long-lived protein sample without significant optical deterioration. The quantitative non-linear, third-order susceptibility ( $\chi^{(3)}$ ) fitting functions for CARS spectra from a protein (BR-570) [1], the quantitative extraction of a CARS spectrum from a binary mixture of two isomeric species in a mixture (dark-adapted BR containing BR-570

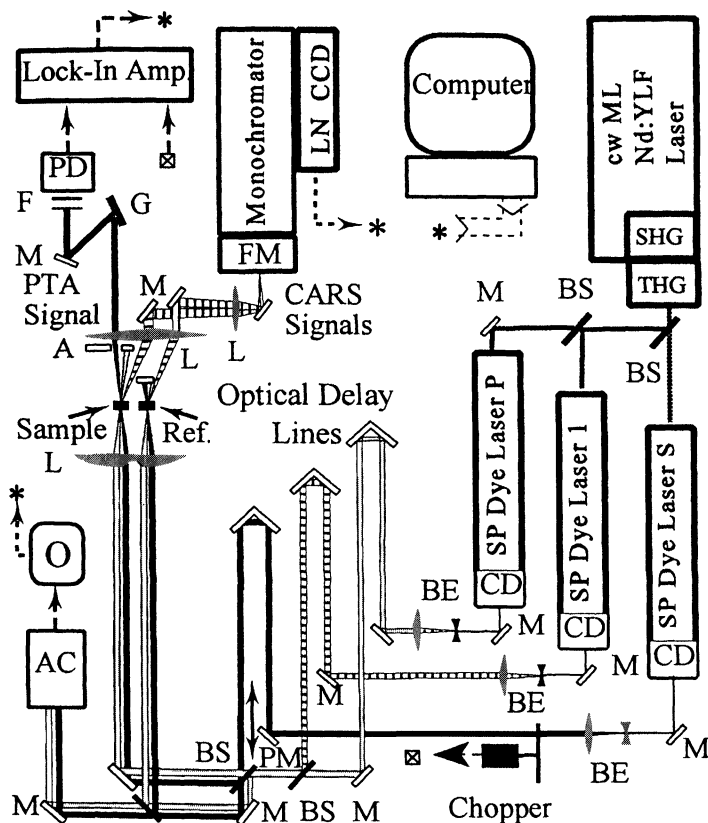


FIGURE 1 Instrumentation used to measure picosecond transient absorption (PTA) and picosecond time-resolved coherent anti-Stokes Raman scattering (PTR/CARS). Spectroscopic data are simultaneously collected from a reference (water and bleached opsin) and rhodopsin sample. Abbreviations include: SHG: second harmonic generator, THG: third harmonic generator, ML: mode locker, M: mirror, BS: beam splitter, CD: cavity dumper, BE: beam expander, AC: auto-correlator, O: oscilloscope, PD: photo diode, A: aperture, PM: phase-matching adjustment, F: optical filter, G: optical grating, and L: lens. Adapted and modified from Chem. Phys. (1996) 212, 421–436.

and BR-548) [6], and the separation of CARS spectrum assignable to a transient intermediate (K-590) from PTR/CARS data recorded from a mixture containing static and transient species [5] are all critical advances made initially *via* studies of the BR photocycle.

Recently, PTR/CARS techniques have been extended to measure CARS data from intermediates having lifetimes in the microsecond

time regime. Instrumentally, this involves the spacial separation of the pump laser pulse ( $\omega_p$ ) from the laser pulses ( $\omega_1$  and  $\omega_s$ ) used to generate CARS signals. By placing the  $\omega_1$  and  $\omega_p$  downstream in the flowing liquid jet sample from the  $\omega_p$  pulse, CARS data from intermediates appearing later in the photo-reaction can be recorded. The precise time delay is determined by sample flow rate, the focal volume of the  $\omega_1$  and  $\omega_s$  pulses within the sample and the physical distance between the  $\omega_p$  and  $\omega_1/\omega_s$  pulses.

These PTR/CARS experiments maintain the short pulsed (typically 3 ps), low-energy excitation conditions which avoid optical perturbations (*e.g.*, secondary photochemistry) while monitoring the vibrational structure of RT intermediates having nanosecond to microsecond lifetimes. In the case of the BR<sup>RT</sup> photocycle, these new PTR/CARS techniques have been used to establish that the structure of K-590<sup>RT</sup> remains unchanged between 3 ps and 1 ns of the BR<sup>RT</sup> photocycle, but undergoes a structural rearrangement (especially in the hydrogen-out-of-plane or HOOP modes) over the 1 ns to 100 ns interval preceding the formation of L-550 [7]. These PTR/CARS data have led to the identification of two structures assignable to K-590<sup>RT</sup>, one in the picosecond time regime (ps/K-590) and a subsequently-formed configuration appearing in the nanosecond time period (ns/K-590).

One of the major advantages of PTR/CARS has been realized through its application to studies of the Rh<sup>RT</sup> photo-reaction. The irreversibility of the Rh<sup>RT</sup> photo-reaction has long presented difficult experimental barriers to the measurement of time-resolved vibrational data. When Rh<sup>RT</sup> dissociates *via* the detachment of the retinal chromophore from the protein upon the formation of the meta II intermediate, the sample denatures into an unbound retinal and the apo-protein opsin [8]. Only the exceptionally high S/N and rapid vibrational data acquisitions offered by PTR/CARS have provided the experimental opportunity to record high quality vibrational data from the Rh<sup>RT</sup> intermediates while tolerating the rapid loss of sample. These PTR/CARS techniques have been utilized to measure extended (700–1700 cm<sup>-1</sup>) vibrational Raman spectra from batho<sup>RT</sup>, lumi<sup>RT</sup>, and partially for BSI<sup>RT</sup>, all for the first time [9, 10]. These RT data also make it feasible to identify and characterize temperature effects and to elucidate the vibrational structure of these intermediates for the first

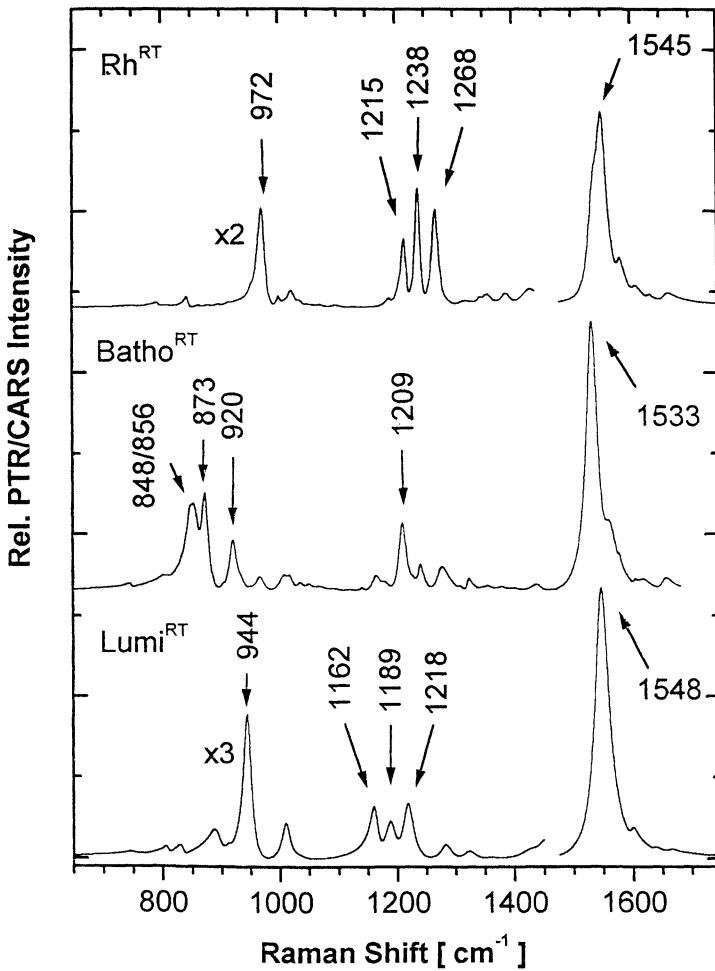


FIGURE 2 Background-free CARS spectra of rhodopsin at room temperature ( $\text{Rh}^{\text{RT}}$ ) and two of the initial photochemical intermediates ( $\text{batho}^{\text{RT}}$  and  $\text{lumi}^{\text{RT}}$ ).

time. The PTR/CARS spectra of  $\text{batho}^{\text{RT}}$  and  $\text{lumi}^{\text{RT}}$ , together with the picosecond resonance CARS spectrum of  $\text{Rh}^{\text{RT}}$ , are presented in Figure 2. PTR/CARS techniques have also recently been used to measure the vibrational spectrum from a picosecond intermediate in the truncated RT photo-reaction of an artificial  $\text{Rh}^{\text{RT}}$  pigment containing a 7-membered ring [11].

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