brief communication

Ultrafast infrared spectroscopy of bacteriorhodopsin

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ABSTRACT Picosecond infrared spectroscopy is developed and used for the first time to study the dynamics of photoexcited bacteriorhodopsin (BR). Both spectral and time-resolved data are obtained. The results open an entirely new approach to investigations of the BR photocycle.

The infrared difference spectrum (K minus BR_{570}) recorded at ambient temperature between 1,560 and 1,700 cm^{-1} is not identical with the spectrum reported for a frozen sample. Three bands of the K state at 1,622, 1,610, and 1,580 cm^{-1} and the bleaching at 1,637 cm^{-1} (C = NH stretch) are seen. These new spectral lines appear in < 10 ps.

INTRODUCTION

Bacteriorhodopsin (BR), a 26 kdal protein in the purple membrane of *Halobacterium halobium* acts as a light-driven proton pump (1). For two reasons BR has found a broad interest in numerous fields of research. Together with the photosynthetic reaction centers found in plants and bacteria, BR is one of the few light to energy converting pigment systems in nature. Furthermore, among the active ion pumping membrane proteins BR is small and simple enough to serve as a model system. Along with halorhodopsin and sensory rhodopsin, BR forms a family of retinal binding systems, having structures that are very similar but have different functions. This evidences that the specific chromophore protein interaction is of great importance.

In BR the retinal chromophore is bound to a lysine residue via a protonated Schiff base. Under illumination, an all-*trans* to 13-*cis* isomerization of the chromophore around the C_{13}–C_{14} double bond initiates a sequence of relaxation processes within the chromophore and the protein envelope. Under normal conditions a reisomerization step back to BR_{570}, the unphotolyzed parent species, completes this photocycle after ~10 ms (2). It is during this cycle that protons are moved across the protein and an electrochemical potential across the cell membrane is generated which is used by the bacterium to drive metabolic processes under anaerobic conditions (for reviews see references 3 and 4).

Some favorable circumstances permit the study of the function of this biological system by various physical methods. The whole purple membrane can be isolated easily without destroying the fundamental function. In addition, the membrane and the protein show a remarkable stability toward different chemical and physical conditions. Finally, the fact that the photoinduced reactions form a closed cycle makes it possible to work with a relatively small amount of sample. This is important especially in experiments involving repetitively pulsed lasers.

The observed time constants of the photocycle span about 11 decades. They reflect the fast isomerization and the subsequent relaxation processes of both the chromophore and the protein. The ultrafast processes use the photon energy to generate a stable intermediate thereby minimizing energy loss enabling the system to function very efficiently.

The optical spectra of complex biological systems are now readily studied with ultrashort time resolution (5, 6) but they do not easily yield the structures of the metastable states they detect. Resonance Raman spectroscopy can also be carried out in a transient manner (5, 6), but the signals give information largely about the structure of the resonant chromophore, which in the case of BR has concerned the retinal (7, 8). Infrared spectroscopy is the newest of the transient spectroscopies and has the advantage that structural changes in the protein are observed on an equal footing with those in the chromophore. Since the control of the protein conformation via optically induced changes in the chromophore is at the heart of the operation of BR and some other proteins, it is certainly of great importance to explore the protein structural changes directly.

In frozen samples of BR, where the intermediates in the photocycle are frozen for pathologically long periods, it has been possible to use Fourier transform spectroscopic methods (FTIR) to obtain infrared spectra of the early intermediates in the photocycle (9–11). The best time resolution achieved by FTIR methods is

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~10 μs. In the present experiments we improve this by ca. six orders of magnitude, thereby enabling studies of the early intermediates at ambient temperature. This is crucial for the understanding and description of the natural function of these systems, since it is known that the photocycles of halorhodopsin and sensory rhodopsin change qualitatively with temperature (Bogomolni, R. A., personal communication).

BR consists of ~250 amino acids (12) and only the chromophore and probably only a few amino acids are strongly affected during the photocycle. Thus there are significant difficulties related to background absorption to be overcome. Even when the additional IR absorption due to the suspending bulk water is minimized by using hydrated purple membrane films, the amide I (≈1,650 cm^{-1}) and amide II (≈1,530 cm^{-1}) bands of the protein backbone cause a strong stationary IR absorption in certain spectral regions. Thus very small changes of the IR absorbance, typical in the order of 10^{-3} (optical density) have to be detected against a strong IR absorbing background. Recently, a laser system was developed in this laboratory, which unites the required high detectivity in the infrared with a time resolution in the picosecond regime (13) and in the present work this approach is modified to enable the first picosecond transient IR studies of BR.

MATERIALS AND METHODS

The experiment required the construction of an optical source for exciting the sample (pump), a carbon monoxide continuous wave IR source (CO-3i-WTVD; Laser Photonics, Carlsbad, CA) for probing the sample and a fast detector for measuring the transmitted IR. Fig. 1 shows the apparatus. A modelocked, Q-switched frequency doubled Nd:YAG laser (Quantronix, 116, Smithtown, NY) pumps two dye lasers which are cavity dumped to yield single, short pulses (~25 ps) in the visible at a repetition rate of 500 Hz. The pulses of one of them (DL2 at 544 nm, energy per pulse ~80 nJ, focused to ~100-μm diam.) are used to initiate the photocycle of BR. The beam of the carbon monoxide laser (spectral resolution ~4 cm^{-1}, power ~50 mW) interrogates the excited sample volume. The fast detector consists of the pulses of the second dye laser (DL1), a crystal with a second order nonlinear susceptibility and a photomultiplier. The nonlinear interaction in the crystal between the electromagnetic fields of the dye laser pulses and the probing infrared generates a pulse at their sum frequency which is detected by a photomultiplier. Since in infrared experiments of BR the region below 1,800 cm^{-1} is of great interest, a suitable crystal ($AgGaS_2$; Cleveland Crystals, Cleveland, OH) in combination with a red dye laser (DL1) at 760 nm was required. The photomultiplier signal is linear in the intensity of the infrared beam and therefore a direct measure for the transmitted infrared. Setting the repetition rate of DL2 to half of that of DL1 and feeding the photomultiplier signal into two lock-in amplifiers, locked to the corresponding frequencies, the change in the IR absorbance can directly be measured. When the CO laser is set to a fixed wavelength and the time delay between the pulses of DL1 and DL2 is scanned, the time course of the IR absorbance at that particular wavelength can be recorded. When the time delay is set to a fixed value and the IR wavelength is tuned, a spectrum is obtained. Using deconvolution methods and the well-known parameters of the laser pulses, the achievable time resolution of this system is ~10 ps. The high repetition rate of the laser system (500 Hz) makes it possible to obtain a signal-to-noise ratio sufficient for the experiments described here to be completed at a rate of one data point per 1 or 2 min.

The sample is a thin film of purple membrane (isolated from *H. Halobium* strain ET 1001 as described in reference 14) between two calcium fluoride windows (OD of 1.3 at 570 nm). A purple membrane suspension in distilled water was dried to minimize the amount of water and then rehydrated until the photocycle was reestablished (15). The sample and the kinetics of the photocycle were characterized and controlled by static optical and infrared spectroscopy and by transient absorption spectroscopy before and after the experiment. BR was driven into the light-adapted state BR_{570} by shining sufficient light on the whole sample. The sample was rotated sufficiently fast that a completely relaxed BR 570 portion was accessed by successive laser shots. All experiments were performed at ~20°C.

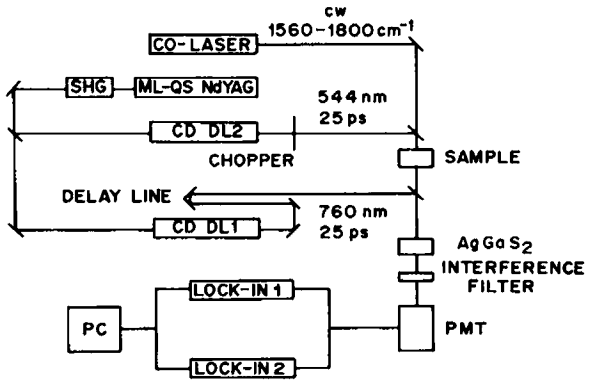

FIGURE 1 Lasersystem for picosecond time-resolved infrared experiments with excitation in the visible. CW: continuous wave; CO: carbon monoxide; ML-QS: mode-locked, Q-switched; CD: cavity-dumped; DL1, DL2: dye laser; PMT: photomultiplier, PC: computer.

RESULTS AND DISCUSSION

Fig. 2 *a* shows the first IR difference spectrum of purple membrane, taken at room temperature on an ultrafast timescale. The time delay between the pump and the probe event was set to 100 ps and data points were collected in the region between 1,700 and 1,560 cm^{-1}. The spectrum displays the net IR-absorbance changes of the sample in that spectral region, measured 100 ps after the excitation. The signals therefore reflect the photoinduced change of transition strength or frequency of any IR-active molecular vibration. The negative (positive) signals correspond to a net increase (decrease) of IR transmission. Spectra of this quality are readily obtained at any delay time.

Fig. 3, *a* and *b* show the kinetic course of the signals at

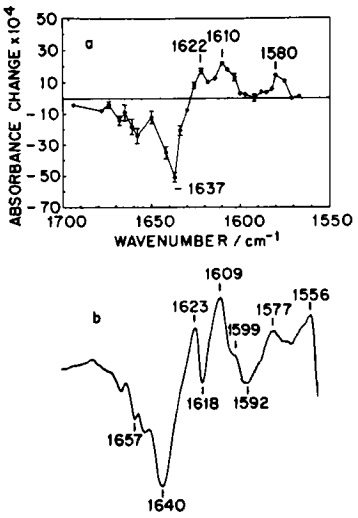

FIGURE 2 (*a*) Infrared difference spectrum of a purple membrane film at ambient temperature. Delay time: 100 ps. Excitation at 544 nm. (*b*) Fourier transform infrared difference spectrum of the BR_{570} – K transition of purple membrane at 77 K (10).

1,641 and 1,610 cm^{-1}. These results indicate that the observed structures appear in < 10 ps.

The first steps in the photocycle of BR were previously studied by transient optical absorption experiments. It is established (16, 17) that the first intermediate in the electronic ground state, J, is reached after 0.5 ps. In this intermediate, the chromophore is already in the 13-*cis* configuration. A relaxation process which possibly incorporates parts of the protein, leads to the red-shifted intermediate K_{610} within 5 ps. Although the subsequent relaxation to the L_{550} state occurs with microsecond time constants, there are possibly (18, 19) intermediate states on the time scale of a few nanoseconds.

The spectrum in Fig. 2 *a* can be interpreted as follows:

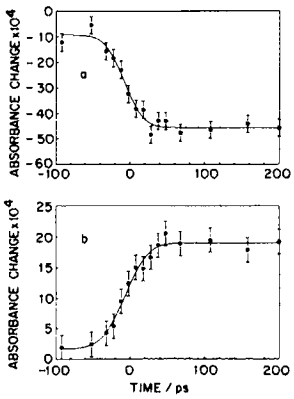

FIGURE 3 IR-absorbance kinetics of the signals at 1,641 cm^{-1} (*a*) and 1,610 cm^{-1} (*b*). Excitation at 544 nm.

the negative signals are due to a bleach of the parent state BR_{570}. The strong band at 1,637 cm^{-1} probably arises from the C = NH stretching vibration of the Schiff base group. In resonance Raman spectra this vibration appears at 1,641 cm^{-1} (20) and in low temperature FTIR spectra at 1,640 cm^{-1} (cf. Fig. 2 *b*). The positive bands at 1,622, 1,610, and 1,580 cm^{-1} are due to the K state after 100 ps on the basis of previous work. These modes are not definitively assignable to particular vibrations of either the chromophore or the protein. Nevertheless, the C = NH stretching mode of the K state surely contributes some to the absorbance change at wavenumbers at ~1,609 cm^{-1} (10, 21).

The rise times of the signals at 1,641 and 1,610 cm^{-1}, as depicted in Fig. 3, *a* and *b*, show that the bleach of BR_{570} and the formation of K are faster than 10 ps, which is consistent with the data from Raman and optical spectra.

The comparison of the room temperature and a typical FTIR spectrum of the BR → K transition, taken at 77 K (10) (see Fig. 2, *a* and *b*), reveals strong similarities in this spectral region. The frequencies of the maxima and minima and the relative intensities in the two spectra are related. This informs us that the restricted environment of the low temperature system permits, in a general sense, the same changes in chemical structure as can occur in ambient samples after 100 ps and are evident in the spectral region explored. However, this behavior may be different at later times, e.g., in the nanosecond region, which is subject of future studies. Differences occur in the region below 1,580 cm^{-1} where the absorption strength in the low temperature spectrum increases again toward the band at 1,556 cm^{-1}, whereas in the room temperature spectrum only the band at 1,580 cm^{-1} exists (unfortunately, 1,563 cm^{-1} is the lowest wavenumber available from this carbon monoxide laser). Since the band at 1,556 cm^{-1} possibly consists of both chromophore (10, 22) and amide II (23) contributions, this difference indicates either a different chromophore structure in the K state or a modified chromophore-protein interaction on this timescale with respect to low temperatures.

The experiments reported here have shown that it is possible to apply picosecond infrared methods to the study of BR dynamics. The time resolution can be readily extended to the subpicosecond region (24) to permit direct observation of structure features of the J → K transition. By extending the wavelength region throughout the infrared, it will also be possible to monitor those spectral regions in which protein contributions dominate. Polarized light studies along the lines carried out for carboxy hemoglobin (25) are now possible, thereby permitting direct measurements of bond motions and protein dynamics. This new approach will

permit the acquisition of structural information that cannot be obtained by any other technique.

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