PICOSECOND AND NANOSECOND COMPONENTS IN BACTERIORHODOPSIN LIGHT-INDUCED ELECTRIC RESPONSE SIGNAL

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ABSTRACT Numerous investigations on the primary events of the bacteriorhodopsin photocycle indicate that the first steps of the energy transformation process take place in the 500 fs-5 ps region. These processes are known to be followed by others in the μs and ms regions. Recent observations indicate also the existence of nanosecond intermediate(s). Here we are reporting on direct measurements of the light-induced electric response signal of purple membrane carried out in the ps and ns regions. The laser flash-induced electric response of dried oriented purple membrane samples were detected by an ultrafast sampling oscilloscope. The measured kinetic curves were analyzed by exponential fitting and by a simulation-optimization method taking into account the time characteristics of the measuring setup. This analysis revealed a two phase real charge separation process. The first phase (tau = 21 ± 2 ps) coincides well with the overall bR-$\mathcal{J}$ K transition. The second phase (tau = 6 ± 0.5 ns) can be correlated with the nanosecond optical transitions reported by several workers, or may be an optically silent charge movement inside the protein moiety or on the surface of the membrane.

INTRODUCTION

Bacteriorhodopsin (bR), the single protein of purple membrane of *Halobacterium halobium* transforms light energy into electrochemical potential. In the course of this process the retinal chromophore undergoes a photocycle (1), accompanied by a step-by-step motion of protons inside the membrane (2). This charge movement can be detected as an electric signal.

Recent investigations into the primary steps of the photocycle show that the excited state of bR decays into the $\mathcal{J}$ form with a time constant of 430–700 fs, followed by the 3–5 ps formation of the K intermediate (3–6). It is generally accepted that the lifetime of K is several microseconds in room temperature. On the other hand, Yoshizawa and coworkers observed a transient different from K at 596 nm at 150 ps after excitation (7). Similarly, Kuschmitz and Hess found absorbance changes occuring within 200 ns after excitation in the 280–430 nm region (8). Transitions on the same time scale were reported from resonance Raman studies by El Sayed and coworkers (9) and by Stern and Mathies (10). These results together indicate the existence of an intermediate (called KL) between K and L. Very recently Midler and Kinger (11) carried out a more detailed study on this form. They found a formation time of KL <10 ns.

The primary events of charge separation were also studied by direct electric methods. Light induced electric response signals of bR with a time resolution of 30–100 ps were reported from two independent laboratories (12–13). The particular importance of this type of investigation is that the actual light energy-electric energy transformation occurs in this or an even shorter time scale. Here we achieved a better time resolution for the first phase of charge separation and also found a new electric component in the nanosecond range.

MATERIALS AND METHODS

The basic scheme of the experimental setup was the same as reported earlier (12) with improvements for better time resolution. Glass disks of 5-mm diameter were covered with an optically transparent and electrically conducting SnO_2 thin layer. Purple membrane fragments, isolated by standard methods, were electrically oriented and dried onto the center of this substrate (14). The conductive pin of a standard 18 GHz SMA short circuit terminator (Radiall, France) was removed and the top of its house was replaced by the sample. By this way an outer ring of SnO_2 not covered by purple membrane was directly connected to the house of the terminator. The inner electrode was a golden pin, one side of which was fitted to the hole of a female SMA. The other side was cone shaped with a 2.5 mm diameter base. This electrode was put into an SMA male-female adaptor. By screwing the sample to the adaptor, the SnO_2 layer and the golden electrode formed a capacitor filled with purple membranes. The adaptor was connected to the sampling head of a 14 GHz sampling oscilloscope (Tektronix Inc., Beaverton, OR model 7704 equipped with 7S11 sampling unit, S-4 sampling head and 7111 sampling time base, equivalent rise time 25 ps).

The sample was excited by a home made N_2 laser driven double-resonator quenched fluorescein dye laser, equipped with a three-stage amplifier (15). This laser system fired 5 $\mu\mathcal{J}$ single pulses of 570 nm with 10 Hz repetition rate. The half width of pulses, determined by streak camera (Hamamatsu Photonics K.K., Japan, model C979) was 22 ± 2 ps.

The sampling oscilloscope was triggered by the laser pulses via a vacuum photodetector (Instrument Technology, UK, model HSD50, rise time 200 ps). The horizontal deflection of the oscilloscope was controlled by and its digitized output signal was acquired by a microcomputer (Sinclair Spectrum, England, with home made interfaces). An inherent characteristic of this sampling technique is that instability in the energy of laser pulse causes vertical as well as horizontal error in the signal. To reduce this effect a fraction of laser light was detected by a PIN photodiode and input to a discriminator of a nuclear spectrometer (KFKI, Hungary, model NK 225). The output of the discriminator was followed by the computer. A point corresponding to a particular time position was accepted and the horizontal deflection was incremented only if the pulse intensity fell into a preset range.

The slow phases of the measured signal were analyzed by least square fitting of exponentials. The fast phase is a convolution of different processes and was analyzed by a parameter optimizing simulation method (see Results and Discussion for details). Both types of calculation were carried out by a global optimizing program GLOBAL (16) using a stochastic nonderivative method (17) on an Olivetti M24 personal computer.

RESULTS AND DISCUSSION

The light-induced electric response signal of bacteriorhodopsin is shown in Fig. 1 on different time scales. In the simplest case of this type of experiment the measured signal has two phases of opposite direction: one is determined by the real charge separation process and the second by the discharging of the measuring circuit. Supposing a first order reaction in charge separation and an RC circuit comprising the capacitance of sample and the 50 Ohm impedance of the SMA connectors, both phases are exponential, and in our case the faster one belongs to the charge separation. It is clear from Fig. 1, however, that the signal has a two-phase decay. To exclude the possibility that a part of the signal is due to some experimental artifact (e.g., photovoltic response of the SnO_2 electrode) we repeated the experiment with a sample containing dried but not oriented purple membranes. No signal on this sample was observed showing that the only sources of the signal are charge separation processes on purple membranes. The decaying part of the signal was numerically analyzed by exponential fitting. Using two exponentials we got very good fit (Fig. 1 *A* and *B*). The average of fitting parameters of several traces resulted in time constants of 450 ± 50 ps and 6 ± 0.5 ns with an amplitude ratio of 3:1.

The kinetic description of the rising phase is more complicated since its time range coincides with that of laser pulse (22 ps) and the oscilloscope rise time (25 ps). Hence the measured signal is a convolution of the laser excitation, the real charge separation process, the 450 ps decay process and the oscilloscope response. (The contribution of the 6 ns process is negligible.) Describing the laser pulse and the oscilloscope response by a Gaussian distribution of known half width, the real time distribution of charge separation can be determined. First we tried to solve the problem by direct deconvolution with a discrete Fourier transformation method but this led to high numerical instability. Much better results were achieved by the following.

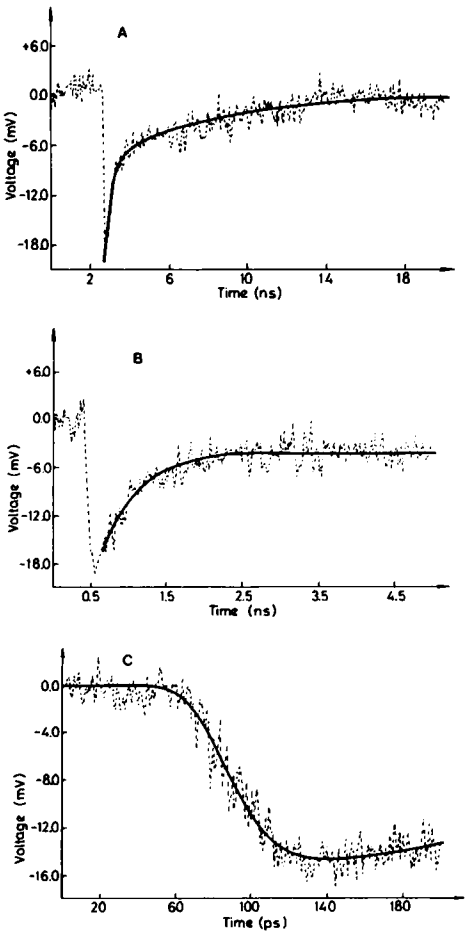

FIGURE 1 Light-induced electric response signal of purple membrane on different time scales. All traces are averages of three scans. Dashed lines are the experimental curves. Continuous line; (*A*) Fitting of the decaying part with two exponentials. Time constants found for this curve: 340 ps and 6.2 ns, amplitudes: 16 and 8 mV respectively. (*B*) Fitting of the decaying part with one exponential. The slowest process considered as baseline. This fit results in a more exact time constant of 520 ps for the faster decay (amplitude 20 mV, baseline 4 mV). (*C*) Fitting of the first phase of the experimental curve by a parameter optimizing simulation method (see text). Time constant found for this curve: 19 ps. Average values of time constants are 21 ± 2 ps, 450 ± 50 ps, and 6 ± 0.5 ns.

The measurable output signal was simulated from the components described above, supposing a first order primary charge separation process. Simulation was carried out by the numerical solution of the system of differential equations describing the convolution problem using a simple Euler method. The simulation has three free parameters: the time position of the maximum of the laser pulse, the time constant of the charge separation and a multiplicative normalizing constant. The GLOBAL program was used to optimize these parameters for the best least square fit of the measured and the simulated curve. As shown in Fig. 1 *C* this type of analysis results in a very good fit. The

time constant of charge separation determined by this method is 21 ± 2 ps.

The result of the kinetic analysis of the measured signal is three exponential process with time constants of 21 ps, 450 ps, and 6 ns. The 21 ps process is clearly a real charge separation and it is close to the 5 ps formation time of the K intermediate. The difference can be due to the unknown high frequency characteristics of the electrical conductivity of the SnO_2 thin layer, not taken into account in the calculations. Although the simulation of this phase gave a rather good fit with a single exponential process, the possible contribution of the bR − J transition cannot be excluded with our time resolution. (A simulated signal with a time constant <10 ps is practically indistinguishable from the response function of the measuring system to an instantaneous electric signal.)

The two slower signals can be interpreted in two ways. One explanation is that the 450 ps process is a real backward charge movement with an amplitude of ~⅔ of the forward process and the 6 ns process is the decay of the RC circuit. Alternatively, the 6 ns process can be a real forward charge movement (following or parallel with the first one) and 450 ps is the RC constant (note that the sign of the amplitudes of two convoluting exponentials depends on the ratio of their time constants!). Supposing that the sample forms an ideal plane capacitor its calculated capacitance is ~5 pF (diameter = 2.5 mm, thickness = 0.01 mm, estimated from the optical density, relative dielectric constant = 1). With the 50 Ohm wave resistance this gives an RC constant of 250 ps. This estimation contains some uncertainty (e.g., the unknown dielectric constant of purple membrane is probably higher than 1) but the calculated value is much closer to 450 ps then to 6 ns, hence the first explanation can be rejected.

The existence of a 6 ns charge separation process cannot be interpreted on the basis of the traditional scheme of the bacteriorhodopsin photocycle, which does not contain any intermediate in this time region. It is possible, however, that this electric process is not accompanied by any change in the absorbance spectrum. Such a transition was found in low temperature electric measurements on purple membrane suspension (18). The calculated transition time at 274 K is 500 ns. Recent studies indicate that this component probably does not originate from the protein moiety of purple membranes but rather from the redistribution of the charges in the Gouy-Chapman layer in response to the picosecond electric perturbation (Dioumaev, A.K., D.S. Chernavskii, P. Ormos, G. Váró, and L. Keszthelyi, manuscript submitted for publication). Dried samples at room temperature and humidity contain large amounts of water but the structure of the (quasi)-Gouy-Chapman layer is certainly different from that in a membrane suspension. If our 6 ns signal corresponds to some event in this region this difference can be responsible for the increased speed.

The correlation between the time constants of the optical and electric components can be maintained, however, if one accepts the existence of a KL intermediate with nanosecond formation time (7–11). In their recent study Midler and Kliger (11) found that such an intermediate forms within 10 ns. This value correlates very well with our 6 ns rise time of a charge separation process. Hence our work supports the existence of a KL intermediate forming in a 6 ns first order process and contributing both to the absorbance change of retinal chromophore and to the charge separation process in purple membrane.

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