Molecular Mechanism of the Differential Photoelectric Response of Bacteriorhodopsin

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In order to determine the molecular origin of the differential photocurrent from bacteriorhodopsin (bR), the photoelectric response of bR film deposited on an indium tin oxide (ITO) conductive glass electrode under CW excitation is compared with that under pulsed laser excitation at different pH and with opposite membrane orientation with respect to the ITO electrode surface. The characteristics (sign and magnitude) of the dominant component of the differential photocurrent (appearing on the millisecond time scale) are found to correlate with the process of proton release into, or uptake from, the aqueous solution during the photocycle under different experimental conditions. This suggests that the differential current results mainly from the change in the H^+ concentration at the bR−ITO electrode interface.

Introduction

Bacteriorhodopsin (bR) is a protein present in the purple membrane (PM) isolated from *Halobacterium salinarium*.[1] In the native purple membrane, the bR molecules are arranged in trimers, forming a two-dimensional hexagonal crystal lattice in PM.[2] The purple color is due to the retinal chromophore, which is covalently bound to Lys-216 through a protonated Schiff base (PSB). Upon light absorption, bR undergoes a photoreaction that can be presented as a cycle consisting of a series of discrete spectral intermediates, with lifetimes varying from picoseconds to milliseconds. As a result of the photocycle, light energy is converted into a proton gradient across the protein membrane. This electrochemical energy is used for the synthesis of ATP from ADP.[3−5]

In recent years, the displacement photocurrent from bR has been studied extensively (for a review, see ref 6), which has provided crucial information concerning the charge movements observed in purple membrane. When excited by a pulsed laser, at least two major components, B1 and B2,[7] have been observed. B1 is a fast component observed on the nanosecond time scale,[8−10] and B2 is a component observed on the microsecond time scale. A slower component in the millisecond time range, B3, has also been observed.[11,12] The B1 component is believed to be associated with the retinal photoisomerization, accompanying the formation of the K intermediate and the fast charge separation in the photoexcitation of bR molecule.[13,14] This has been supported by the fact that no B1 was observed from modified *all-trans*-retinal which cannot photoisomerize.[15] The B2 component is mainly due to the proton translocation across the membrane, and a good correlation between the lifetime of B2 and that of the L−M transition has been found in the pH range from 2.4 to 11.0 when measured under high ionic strength (such as >40 mM KCl) in a polyacrylamide gel.[12] Compared to B2, B3 is small in magnitude observed from gel immobilized bR.[12] However, the origin of B3 has not yet been assigned.

It was reported recently that oriented bR films show differential photocurrent response upon CW light excitations.[16−19] Two transient photocurrent peaks were observed with opposite signs, corresponding to turning the CW light source on and off. In addition, an imaging device based on this photoelectric response from bR LB films has been fabricated.[16] However, the molecular mechanism responsible for the differential photoelectric response in bR is not yet understood. It was reported that the direction of the differential current (more precisely, the first current peak) was opposite to that of the vectorial proton translocation across the membrane.[17] Thus, it was proposed that the primary charge separation resulting from the photoisomerization could be responsible for the differential photocurrent. However, since photoisomerization occurs very rapidly, its contribution to the differential photocurrent is expected to be small. Evidently, a detailed understanding of the differential photocurrent requires a detailed study of the charge movements on the various time scales during the photocycle.

In this article, we present comparative results of experiments carried out under both pulsed and CW laser photoexcitations on different time scales and under different experimental conditions, in order to identify the intermediates or processes in the bR photocycle responsible for the observed differential photocurrent. The photocurrent is studied upon changing the pH and changing the membrane orientation on the electrode surface. A correlation is found between the slow photocurrent components observed under pulsed laser excitation (the B3 component) and that from CW laser excitation (the D1 component). This strongly suggests that it is this slow component in the pulsed excitation experiments that is responsible for the differential photocurrent under the CW excitation.

Material and Methods

Purple membrane was isolated and purified from *Halobacterium salinarium* strain ET 1001 according to a standard procedure described previously.[20] The orientation of the bR film on an ITO conductive glass electrode was obtained by the electrophoretic sedimentation (ES) method as follows: several drops of bR suspension were placed between two ITO electrodes ~1 mm apart, and a 5 V dc electric field was applied for 10 s, resulting in an oriented bR film. BR films with controllable membrane orientation patterns can be prepared by controlling the pH of the bR suspension, since the permanent dipole moment of a PM patch changes sign as the pH is changed.[21] Opposite membrane orientation can be confirmed by observing the polarities of both B1 and B2 photocurrent components under pulsed laser excitation. Two types of oriented films were studied with either the predominant cytoplasmic (CP) or extracellular (EC) surface facing ITO directly, giving orientation

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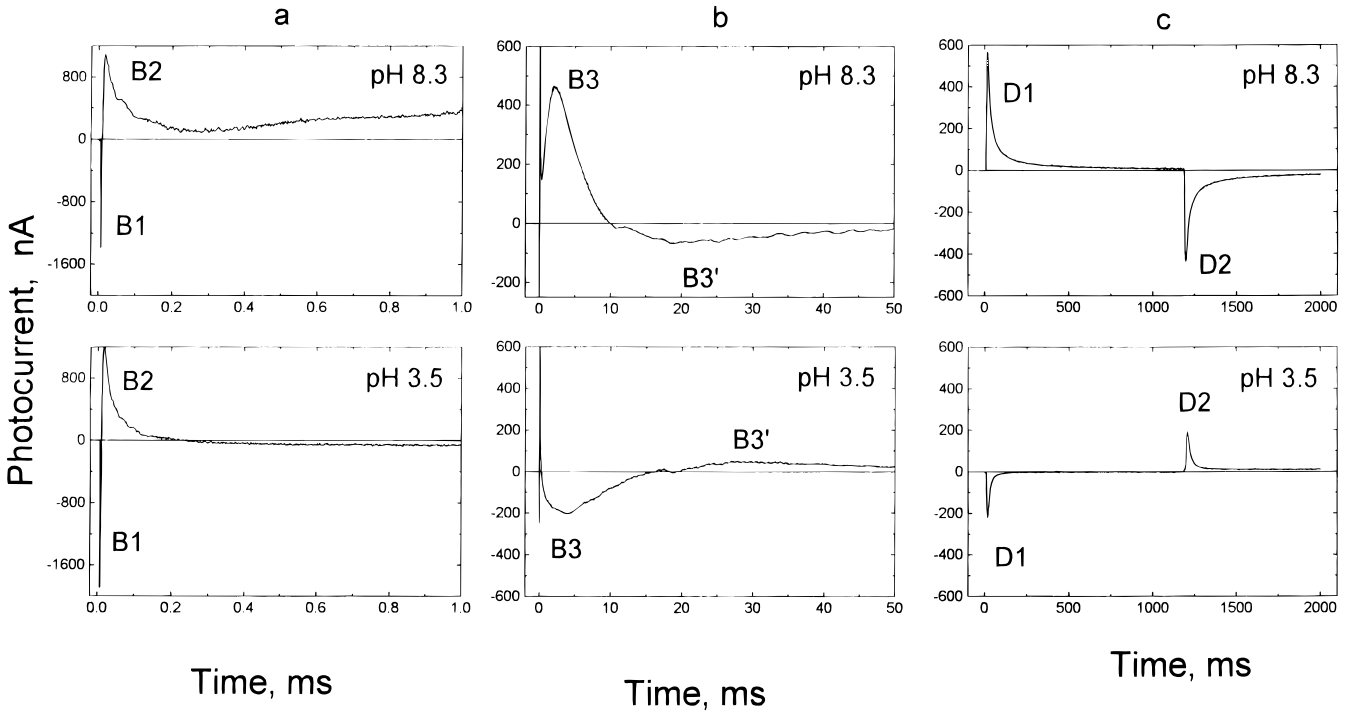

Figure 1. Photocurrent components from an oriented bR film at various time scales at two typical pHs. The measurements were carried out under identical conditions of 10 mM KCl and 1 mM K_2HPO_4 at pH 8.3 for upper curves and pH 3.5 for lower curves, respectively. (a) Fast photocurrent components (B1 and B2) observed under pulsed laser excitation. (b) Slow photocurrent components (B3 and B3′) observed under pulsed laser excitation. (c) Differential photocurrent component (D1 and D2) observed under CW excitation.

patterns of ITO/CP−EC and ITO/EC−CP, respectively. The optical density of the film was controlled to be between 0.1 and 0.5 at 570 nm. The bR-deposited ITO electrode was separated from a blank counter ITO electrode by 8 mm in a plastic cuvette (10 × 10 × 45 mm), which is filled with the desired aqueous electrolyte solution. The bR films were light adapted by exposing them to visible light (>532 nm) before recording the photocurrent signal.

The photoelectric measurement was carried out by using either a pulsed laser or a CW laser as the excitation source. The pulsed laser is from the second harmonic (532 nm) of a Nd:YAG laser (Quanta-Ray; DCR-3, Spectra Physics Co., Mountain View, CA), and the CW source is an argon ion pumped dye laser (Innov 70, Coherent Co., Santa Clara, CA). The photocurrent was measured with a Keithley Model-428 current amplifier (Keithley Instruments Inc., Cleveland, OH) with a time resolution of ∼2 μs. The output from the current amplifier is digitized by a personal computer through an AT-MIO-16E-2 I/O. The experiment was controlled through a computer program running under the Lab-Windows (National Instruments Co., Austin, TX) environment on a PC. The measuring system is calibrated so as the positive current represents positive charges moving from the electrolyte solution toward the ITO/bR side.

Results and Discussion

Figure 1 shows the photocurrent components resulting from an oriented bR film from the pulsed excitation (a and b) and from turning on and off the CW excitation light (c) at pH 8.3 (top) and 3.5 (bottom), on various time scales. In Figure 1a, the photocurrent at the two pHs under pulsed laser excitation is shown in the 0−1.0 ms time range. Figure 1a reveals that there are two components in the photoelectric signal: B1, a very fast component (<1 μs), which is always in the opposite direction to the B2 signal; the latter occurs on the microsecond time scale which is thought to result from the deprotonation of the PSB and the proton transport to the surface. The comparison between the B1 and B2 signals at low pH and at high pH demonstrates that the current directions for B1 and B2 are independent of pH. The slight difference observed in the amplitude of B1 and B2 when the pH is changed agrees with a previous report.[22] A significant difference is found at the long time scale part of the B2 decay which goes above zero at high pH and below zero at low pH, showing the existence of a slower component, the B3 component.

Figure 1b shows the photocurrent on the 0−50 ms time range. It shows the B3 and B3′ components at a relatively long time scale, along with compressed current profile resulting from the B1 and B2 components. The pH affects markedly the polarity of B3. It can be seen that B3 has reversed direction at high pH versus low pH and so does B3′. However, there is no photocurrent sign change in B1 and B2. In addition, it should be noticed that the sign of B3′ is always in a direction opposite to that of B3, at both low pH and high pH.

Figure 1c shows the differential photocurrent under different pHs. Two transient photocurrent peaks are denoted as D1 and D2; D1 appears as the CW light source is turned on, and D2 appears when the excitation source is turned off. It can be seen that D2 is always opposite to D1, independent of pH. It is of interest to see that the current component D1 (or D2) has a sign at low pH opposite to that at high pH, indicating that the differential current direction is pH dependent. From the results in Figure 1b,c, we conclude that D1 correlates with B3 both in polarity and in amplitude and that D2 correlates with B3′ in a similar way. From an electrochemical point of view, D1 and D2 show the behavior of the charging and discharging of a chemical capacitor, consisting of ITO/bR−ITO. The charging/discharging processes could occur from light-induced increase or decrease in the proton concentration at extracellular surface at high pH or opposite at low pH.

The orientation of bR in Figure 1 is such that the extracellular (EC) side of membrane is facing the ITO surface (ITO/EC−

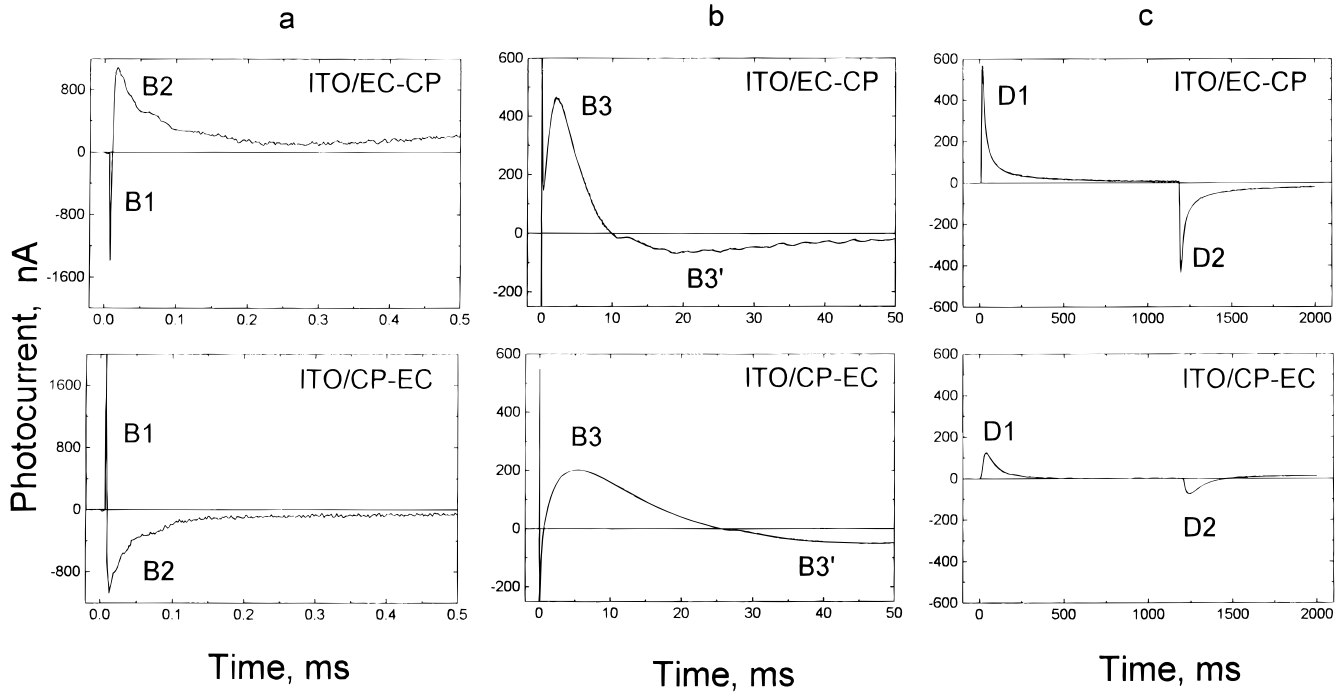

Figure 2. Membrane orientation effect on the photocurrent components. Identical experimental conditions were used as in Figure 1 at pH 8.3. (a) B1 and B2 components. (b) B3 and B3′ components. (c) D1 and D2 components. Two types of membrane orientation were used and noted as ITO/EC−CP (extracellular side facing the ITO surface) for the upper curves and ITO/CP−EC (cytoplasmic side facing the ITO surface) for the lower curves.

CP pattern), which is confirmed from the photoelectric measurements and the current direction detected from both the B1 and B2 components, as we mentioned above. Positive signal suggests that the concentration of positive charges increases at the bR deposited ITO electrode side. Figure 2 shows the membrane orientation effect on the sign of the photocurrent components produced at constant pH on the various time scales. Two types of membrane orientation were used; the upper curves are those for the ITO/EC−CP pattern, and the lower curves are the ITO/CP−EC pattern. According to Figure 2a, the B2 component has an opposite sign for the two opposite orientations, showing that its direction is in agreement with the vectorial proton translocation, i.e., from the CP side to the EC side. B1 shows similar polarity reversal as the membrane orientation changes. Further, randomly oriented bR film shows that the magnitudes of both B1 and B2 decrease while that of B3 remains unchanged (data not shown here). The results support that B2 is correlated with the proton movement within the membrane and that the oppositely oriented membrane patches in the random orientation result in cancellation of the magnitude of B2. The contribution of proton transfer to the B2 component has also been reported in a previous publication.[23] The fact that B1 and B2 have opposite signs for their current suggests that the part of the photocycle prior to the deprotonation step (the M formation) leads to opposite polarizations of the membrane surface attached to the ITO electrode.

Figure 2b shows that B3 has the same photocurrent sign for the two opposite orientations of the membrane. This indicates that the sign of B3 is independent of the bR film orientation or independent of the membrane configuration with respect to each other. Figure 2c shows that the signal of the D1 (and also D2) component is also independent of membrane orientation. Furthermore, for the ITO/CP−EC pattern, it is also found that at low pH no sign change is observed for the B1 or B2 component, whereas B3 and D1 change their signs from positive to negative (data not shown). All these results indicate that both B3 and D1 components are independent of the membrane orientation but sensitive to the pH in solution. In addition, Figure 2b,c shows that both the kinetics and magnitudes of B3 and D1 (as well as B3′ and D2) change in a similar way for opposite film orientations, that is, higher in photocurrent peak maximum and faster in rise and decay for the ITO/EC−CP orientation pattern. This further proves that B3 and B3′ in the pulsed excitation share the same molecular mechanistic origin as the D1 and D2 in the CW excitation, respectively. In other words, the main contribution to the differential photocurrent (D1) observed under CW excitation is mainly from the same origin of B3 rather than from either B1 (due to photoisomerization) or B2 (due to proton transfer from the PSB to the extracellular surface).

Since B3 follows B2 in time, it must arise from a process occurring after the M formation. At high pH, the rise time (hundreds of microseconds to milliseconds) of the B3 signal under pulsed excitation experiments coincides with that of the proton appearance in solution as determined by Grzesiek and Dencher[24] using a pyranine dye in solution. This suggests that B3 results from the proton accumulation near the ITO/bR side in aqueous solution. The pH dependence of the B3 and D1 signals also supports this proposal. It is found that bR releases a proton into the aqueous phase at pH $>$ 6, whereas proton uptake from the solution occurs at pH $<$ 5 with a delayed proton release.[25] This explains the observed change in sign of the B3 and D1 signals as the pH changes from 8.3 to 3.5. A detailed pH dependence of the sign of the D1 and B3 components show a reverse of sign at pH ∼5.5. Further, it has been reported that the net pH change in the aqueous phase of the bR vesicle-containing solution depends on the pH of the medium.[26,27] These results confirm our proposal that the differential photocurrent arises from the production of pH gradient at the two electrodes caused by the change in the hydrogen ion concentration at the ITO−membrane interface resulting from the proton release from or uptake by the membrane at the ITO−membrane interface. The independence of the sign of B3 and D1 on the membrane orientation can be explained by the rapid equilibrium between

the membrane surface and the interfacial solution between the membrane and the ITO surface. This is due to the fact that the membrane is porous and heterogeneously deposited.

Our conclusions are consistent with those of a recent study by Robertson et al.[19] It is proposed that the local pH change between the bR membrane surface and the ITO electrode gives rise to the differential photocurrent D1 component. However, these authors found that the film oriented with the cytoplasmic side facing the electrode surface (ITO/CP−EC pattern) produced a current opposite to that from the randomly oriented one. On the basis of this, one would expect negative D1 at high pH. However, this contradicts our result that D1 is always cathodic rectified at neutral and high pH (Figure 1c, upper curve, and Figure 2c). The cathodic rectification of the differential photocurrent has also been shown in Miyasaka et al.'s work,[16,17] although no low-pH study had been carried out in these reports. It is possible that the texture of the membrane and the nature of the interface dictate whether or not rapid equilibration of the proton concentration at the membrane−electrode interface takes place. If it does, then the sign of the photocurrent signal will be independent of the membrane orientation, as observed by Miyasaka et al. and in the present work. If the membrane texture is such that it does not allow for rapid proton equilibration, then the orientation of the membrane with respect to the electrode surface does determine the sign of the photocurrent. The net proton concentration change in a certain time interval is determined by the pH-dependent steady state established between the proton release and proton uptake processes.

The rise and decay of the differential current observed when the light is turned on and off is described as follows. As the light is turned on, the $[H^+]$ changes at the ITO− membrane electrode surface. This change creates a pH gradient between the two electrode surfaces which gives rise to a voltage that causes the electrons to flow from one electrode to the other. The polarization thus created can be neutralized by the ionic movement within the electrolyte solution which leads to the decay of the voltage and thus current. As a result, a charging of the chemical capacitor takes place. When the light is turned off, and the steady-state concentration of H^+ established by the proton pump of the photocycle ceases, a current in the opposite direction takes place which decays to zero as soon as the ionic concentration at the electrodes equilibrate with that in solution, i.e., when the chemical capacitor is completely discharged. It is interesting to point out that if the change in the proton concentration is indeed the cause of the observed differential current, this experimental technique could be used to determine the proton release and uptake by membranes in general.

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