ELECTRIC RESPONSE OF A BACK PHOTOREACTION IN THE BACTERIORHODOPSIN PHOTOCYCLE

P. ORMOS, ZS. DANCSHÁZY, AND L. KESZTHELYI, *Institute of Biophysics, Biological Research Center, Hungarian Academy of Sciences, Szeged H-6701, Hungary*

ABSTRACT The electric response of a back photoreaction in the bacteriorhodopsin photocycle was investigated. The proton pumping activity of green flash excited bacteriorhodopsin stops if the M_{412} form is illuminated by blue light (Karvaly and Dancsházy, 1977). In the present work a fast negative displacement current signal was measured in an oriented membrane suspension system, indicative of back movement of protons from M_{412} to BR_{570}. Quantitative evaluation of the data shows that there are at least two steps in the back reaction, with different rate constants. The temperature dependence of the rate constants shows simple linear Arrhenius behavior between 5° and 40°C. The rate constants were slower by a factor of 1.8 in D_2O suspension. The relevance of the protein electric response signals (PERS) observed in this paper to the early receptor potential is discussed.

INTRODUCTION

In our previous studies it was shown that upon simultaneous green and blue light illumination of bimolecular lipid membrane containing bacteriorhodopsin (BR) the blue light decreases the photopotential that is generated (Karvaly and Dancsházy, 1977). It was proposed that excitation of the M_{412} intermediate causes rebinding of protons or inhibition of their release from the purple membrane (Ormos et al., 1978). In kinetic measurements it was shown that this blue light effect appears very fast and is connected with the M_{412} intermediate (Dancsházy et al., 1978). On dried oriented purple membrane layers Hwang et al. (1977) showed that excitation of M_{412} causes charge movement in the membrane in a direction opposite to that induced by the excitation of BR_{570}. Spectroscopic investigation of the back photoreaction of M_{412} at low temperature revealed that regeneration of BR_{570} takes place through several intermediates (Litvin and Balashov, 1977). In flash photolysis experiments of this back photoreaction, existence of one intermediate has been demonstrated with a lifetime of ~200 ns at room temperature (Kalisky et al., 1978). Recently it has been established that a transient photoelectric response can be detected on oriented untreated purple membrane suspension, and its different components can be assigned to different steps in the BR photocycle (Keszthelyi and Ormos, 1980). Using this system the signal amplitude due to charge movement in the purple membrane can be measured and the charge displacement calculated.

In the present work we studied the photoelectric signal induced by the blue light excitation of M_{412} using the system and methods described in the paper of Keszthelyi and Ormos (1980).

MATERIALS AND METHODS

Orientation of purple membranes and photoelectric measurements were carried out as described by Keszthelyi and Ormos (1980). After the first green laser flash the blue flash (duration, 2 μs; wavelength, 400 nm with 20-nm bandwidth, 2 mJ energy) was triggered with variable delay time by a switching unit built in our institute. The green and blue light beams were reflected by a semitransparent mirror to the suspension at the same position.

RESULTS

In Fig. 1 the photoelectric signal obtained upon successive green and blue flash illumination is presented. Delay time of the blue flash was chosen to be 600 μs when the M_{412} concentration was highest. As can be observed in Fig. 1, the characteristics of the signal induced by blue flash is markedly different from that of the green one: it contains a large negative signal with decay time longer than the "green signal," as well as some positive signal. It is well known that the blue light has two effects: it excites the M_{412} form but it is also capable of exciting BR_{570} (with appropriate efficiency), generating a similar signal as for the green light. The method of the separation of the signal corresponding to the excitation of the M_{412} form is illustrated in Fig. 2: (*a*) green and blue signals together, (*b*) excitation by blue light only, i.e., the excitation of BR_{570}, (*c*) the difference of signal *a* and *b*. We considered the difference signal *c* as the signal associated with the excitation of the M_{412} form. It admittedly contains an error because signal *b* is larger than that part of signal *a* which corresponds to the blue excitation of BR_{570}; in the given measurement, however, the green flash drives 20% for BR_{570} to M_{412}, i.e., 80% of BR_{570} is available for the blue excitation, hence the subtracted signal has an error of 20%. From the amplitudes of the respective signals, however, it can be seen that the error after the subtraction in the determined signal of M_{412} is not >5%. In this estimation additivity between the responses of BR_{570} and M_{412} to blue light is used, substantiated by the fact that the blue light excited two different populations of molecules, their signals are independent. (During the time of blue flash of ~2 μs practically no M_{412} form is produced to be reexcited.)

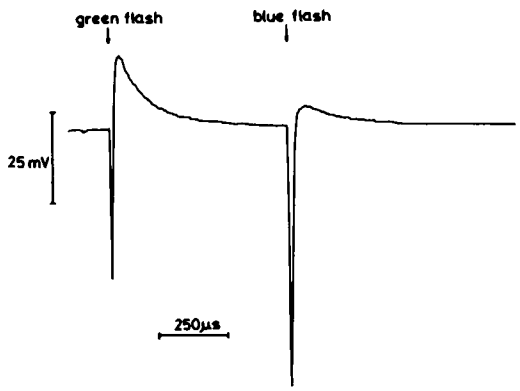

FIGURE 1 Photoresponse of oriented PM suspension upon successive green and blue flash excitation. Optical density of the suspension, OD, = 1.8. Orienting voltage, U_0, = 10 V. Distance between the electrodes, *D*, = 8 mm.

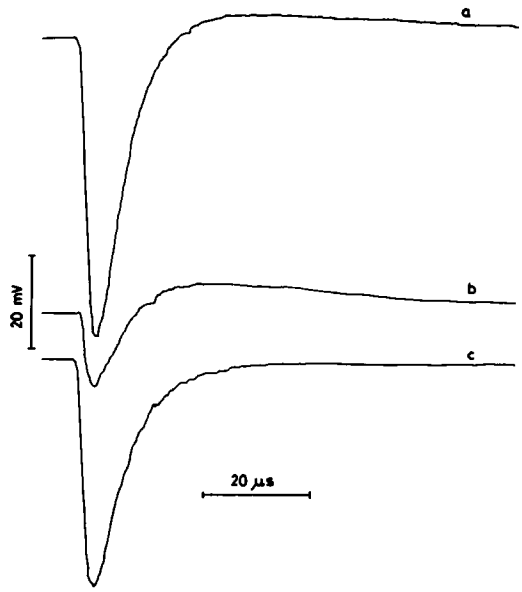

FIGURE 2 Decomposition of the blue flash induced photosignal to obtain the one due only to excitation of M_{412}: (*a*) photoresponse, to the blue flash followed the green one with Δt = 600 μs delay time, (*b*) photoresponse of the blue flash, when no previous green one was applied, (*c*) difference of the above two signals (*a*–*c*), representing the response of M_{412}.

At room temperature the resulting difference signal consists of one exponential with negative sign and a time-constant of about τ_2, = 10 μs. At low temperature between 5° and 10°C, however, a fast component could be distinguished by visual inspection with positive sign and a time-constant of τ_1, ~2 μs, being at the limit of our time resolution (Fig. 3). The time constant decreases, naturally, with increasing temperature. At temperatures >10°C the fast component was not recognizable at all.

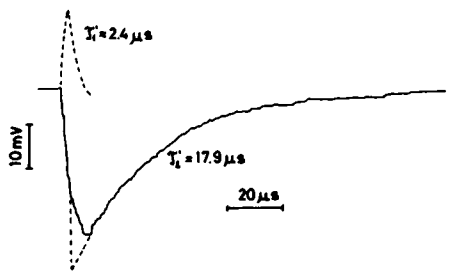

FIGURE 3

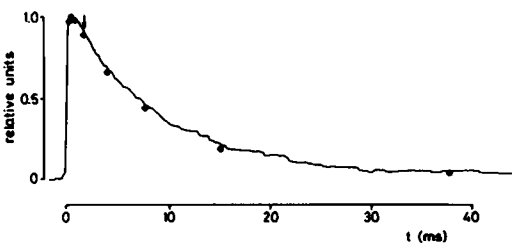

FIGURE 4

FIGURE 3 The blue light induced signal (see Fig. 2 *c*) at 5°C to demonstrate the two exponential components. Dashed lines show the decomposition of the photoresponse into the two processes.

FIGURE 4 Correlation of the time course of M_{412} decay and the dependence of the amplitude of the blue light induced difference photosignal on the delay time of the blue flash. (—) time course of the change in absorbance at 400 nm, (●) the amplitude of the blue light effect at different delay times. The signals have been normalized at the point marked by the arrow.

In Fig. 4 the correlation between the M_{412} spectral decay and the amplitude of the blue-light induced difference electric response signal (Fig. 2 *c*) obtained with different delay times between the green and blue flashes is represented. It is considered to be a direct proof that the measured difference electric response signal of blue light is due to the M_{412} form.

The temperature dependence of the time constant τ_2, between 5° and 40°C, was also measured. An Arrhenius plot of the time-constants gave a straight line with an activation energy of $(1.30 \pm 0.15) \times 10^{-20}$ J (equivalent to 1.9 ± 0.3 kcal/mol^{-1}) and a frequency factor of $(1.6 \pm 0.6) \times 10^6$ s^{-1}.

The hydrogen isotope effect on this blue-light induced regeneration reaction path has also been investigated. The rate of the main reaction is slowed down by a factor of 1.8 when the purple membranes were resuspended in D_2O.

DISCUSSION

In understanding the observed phenomena we follow the explanation of Keszthelyi and Ormos (1980). According to this, the electric signals represent displacement of protons, the exponential components correspond to the successive transitions in the photochemical cycle. Until the formation of M_{412} the protons first move backwards (during the K → L transition) and then forward (during the L → M reaction) with respect to the pumping direction.

It seems to be proper to repeat and partially extend the argumentations of the paper of Keszthelyi and Ormos (1980) regarding the statement that the displaced charges are protons.

It is a well established fact that bacteriorhodopsin is a light driven proton pump (Stoeckenius et al., 1979). According to resonance Raman spectroscopy measurements (Lewis et al., 1974) the Schiff base linkage is protonated in BR_{570} and that of the M_{412} intermediate is unprotonated. That means a real displacement of protons.

The investigations of Hess and Kuschmitz (1978), and our unpublished results in the UV absorption range, revealed that tyrosin and tryptophan side chains get unprotonated and protonated with the time constants of the photocycle, again demonstrating the displacement of protons inside the proteins.

The quantitative evaluation of the protein electric response signal (PERS) was in accord with the displacement of the protons from Schiff base in the direction of positive external electrodes (except the K → L transition), reaching the surface in the M → O transition and uptake from the other side in the O → BR transition which is the reprotonation step of the Schiff base. The position of the protonated Schiff base (1.5 nm from the internal surface) is in good accord with the results of the structural investigations of bacteriorhodopsin (Ovchinnikov, 1979).

The pH sensitivity of the photoelectric activity of bacteriorhodopsin as determined by Drachev et al. (1977) offers additional support to our explanation that the displaced charges are protons.

It is known from the studies of Witt and Zickler (1973) that ion fluxes in the water phase equilibrate the asymmetric charge of thylakoids within ~10 μs. Long living components of the PERS signal are possible only if the protons inside the protein are well protected from the conducting external medium. This equilibration is precisely the reason that the charging of the membrane is not observed, i.e., the time curve of PERS of M → O and O → BR

transitions coincide with the light absorption signal. From this it follows that the protons which moved away from the Schiff base during K → L and L → M transition reside at a distance of ~0.4 nm from it in the M_{412} form.

The presence of two components in the signal due to the photoinduced back reaction $M_{412} \rightarrow BR_{570}$ means that the regeneration of BR_{570} takes place through at least two thermally activated processes, after electronic excitation of M_{412}. A tentative correlation of the components of the electric signal was performed as follows: the first, fast process correlates with the one described by Kalisky et al. (1978). In that work, however, no further reaction (and intermediate) was observed. Litvin and Balashov (1977) reported two more successive reactions and intermediates, with absorption maxima (565 and 585 nm, respectively) near 570 nm, and therefore difficult to distinguish spectroscopically from BR_{570}. Our results suggest that the main signal we have found is due to one of the last reactions (possibly the $P_{565} \rightarrow P_{585}$ reaction) using the nomenclature of Litvin and Balashov (1977), and during the other ($P_{585} \rightarrow P_{570}$) no significant charge displacement occurs.

For quantitative evaluation of the data the theory described by Keszthelyi and Ormos (1980) was used. The basic photoresponse (caused by the excitation of BR_{570}) is due to a $d_1 = -0.15$ nm backward motion of two protons in the K → L reaction, and a $d_2 = 0.5$ nm forward motion in the L → M reaction (if we tentatively accept that two protons are always moving simultaneously). For the numerical evaluation of the back reaction it was determined in an absorption kinetic control measurement that in the present case 33(±2)% of the M_{412} form was converted back by the blue flash (the rest regenerated into BR_{570} via the M → O → BR path). With this information the value of the blue light induced charge displacements could be immediately calculated.

The equation for the calculation

$$V_i(t) = \frac{N R Q d_i}{D} k_i f/k_1, \ldots, k_i, t/, \tag{1}$$

where $V_i(t)$ is the measured voltage on the R measuring resistance due to the i-th reaction; d_i the displacement of charge in the i-th reaction; D the distance of the electrodes; $f/k_1, \ldots\ldots, k_i, t/$ the Buteman function for the i-th component; $k_i = 1/\tau_i$. With the values $N = 7 \times 10^{14}$; $R = 50$ kΩ; $D = 8$ mm, the following results were obtained: in the first, fast process, two elementary charges move forward a distance of $d_{1'} = 0.03$ nm, and then they move backward to $d_{2'} = -0.35$ nm. The absolute error in these numbers is 30% due to the difficulty in determining N (the number of photocycles triggered by the green flash), the relative error of $d_{2'}$, with respect to d_1 and d_2 is not >5%. The relative value of $d_{1'}$, however, remains a rough estimate because, as already mentioned, $\tau_{1'}$ was at the limit of our time-resolution.

The interpretation of the results is obvious: the blue light induced fast regeneration of M_{412} into BR_{570} is accompanied by the return of the protons to their original position ($d_1 + d_2 + d_{1'} + d_{2'} \simeq 0$). An important consequence of this finding is that by the formation of the M_{412} form the protons are still not released from bacteriorhodopsin. They are, however, already displaced to a certain extent. It is interesting to note that the sequence of charge dislocation after both the green and the blue light excitation (in the "green" case of course only the displacements until the formation of M_{412} are considered) is similar, only opposite in direction: the charge

movement begins with a fast small reverse movement with respect to the main displacement. Further investigation of this fact should give better insight into the process caused by the light absorption.

The results presented here show striking similarity with those of Cone (1967), but on a completely different system. In Cone's report early receptor potentials (ERP) of rat eye are presented. Exciting rhodopsin in its main absorption band gives an ERP signal similar to the presented photoelectric signal on bacteriorhodopsin, and exciting the meta-II-rhodopsin (which is regarded as the equivalent to M_{412} in the rhodopsin photocycle) gives the same type of response with opposite sign and with somewhat smaller time-constants. This observation further supports our view that the charge displacement signals in BR reported here and by Keszthelyi and Ormos (1980) are very similar to the ERP signals of rhodopsin (see also the paper of Hong and Montal, 1979, Trissl, 1979, and Hong, 1978, and a recent paper of Bolshakov et al., 1979, in which the electric responses of rhodopsin and bacteriorhodopsin are compared) and thus they should be interpreted in a similar way. This note concerns the amplitudes of the two components of the ERP signal mostly. According to Eq. 1, the amplitude of a displacement current signal linearly depends on the rate constant. The disappearance of the second component in case of pH change or cooling is surely the result of the decrease of the rate constant resulting in the decrease of the amplitude of the signal.

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