Charge Displacement in Bacteriorhodopsin During the Forward and Reverse bR-K Phototransition

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ABSTRACT Dried oriented purple membrane samples of *Halobacterium salinarium* were excited by 150 fs laser pulses of 620 nm with a 7 kHz repetition rate. An unusual complex picosecond electric response signal consisting of a positive and a negative peak was detected by a sampling oscilloscope. The ratio of the two peaks was changed by 1) reducing the repetition rate, 2) varying the intensity of the excitation beam, and 3) applying background illumination by light of 647 nm or 511 nm. All of these features can be explained by the simultaneous excitation of the bacteriorhodopsin ground form and the K intermediate. The latter was populated by the (quasi)continuous excitation attributable to its prolonged lifetime in a dehydrated state. Least-square analysis resulted in a 5 ps upper and 2.5 ps lower limit for the time constant of the charge displacement process, corresponding to the forward reaction. That is in good agreement with the formation time of K. The charge separation driven by the reverse phototransition was faster, having a time constant of a 3.5 ps upper limit. The difference in the rates indicates the existence of different routes for the forward and the reverse photoreactions.

Introduction

Bacteriorhodopsin (bR) is the single protein of the purple membrane found in *Halobacterium salinarium* and related species. Based on the retinal chromophore attached to it via a protonated Schiff-base, this protein utilizes the energy of light to build up a proton electrochemical potential for ATP synthesis. In the dark-adapted state of bR, a thermodynamic equilibrium is maintained between the retinal molecules in all-*trans* and 13-*cis* conformation, whereas in the physiologically important light-adapted state, every chromophore is in all-*trans* form. In both states photon absorption initiates a photocycle of different intermediates; in light-adapted bR this results in a pump of protons across the membrane (for reviews see Stoeckenius et al., 1979; Oesterhelt and Tittor, 1989; Birge, 1990; Mathies et al., 1991; Lanyi, 1992).

The primary events of the photocycle of light-adapted bR, studied by ultrafast absorption kinetic experiments, can be summarized as follows (Nuss et al., 1985; Sharkov et al., 1985; Polland et al. 1986; Petrich et al., 1987; Mathies et al., 1988; Dobler et al. 1988; Pollard et al., 1989):

$$bR_{570} + h\nu \rightarrow bR^*(trans) \overset{100-200\,fs}{\rightarrow} bR^*(cis) \overset{300-500\,fs}{\rightarrow} J_{625} \overset{3-5\,ps}{\rightarrow} K_{590} \cdots,$$

where the indices indicate the absorption maxima of the corresponding forms, and the asterisk denotes excited states. The retinal chromophore is in all-*trans* conformation in both the ground state and the Franck-Condon excited state. Via a barrierless transition completed in less than 200 fs, it isomerizes to a 13-*cis* excited state by twisting around the 13–14 double bond (Dobler et al., 1988; Mathies et al., 1988). The isomerized excited state molecules decay to the J intermediate. This is a ground state in the electronic sense, but it is still unrelaxed because of its highly distorted retinal conformation and "hot" vibrational structure (Doig et al., 1991). For this reason, J is also short-lived and transforms to a K intermediate in 3–5 ps. Time-resolved Raman spectroscopy data support the idea that both J and K intermediates have a 13-*cis* retinal chromophore (van den Berg et al., 1990). Recent absorption kinetic studies carried out on site-directed mutants of bR indicate that the rate of the isomerization depends markedly on the protein environment of the chromophore (Song et al., 1993).

The room temperature lifetime of the K form is several microseconds, although there is a contradiction in the literature on the formation of a somewhat different KL intermediate in the 10 ps-10 ns time range (Midler and Kliger, 1988; Doig et al., 1991; Delaney et al., 1993). On dehydration this lifetime can be extended to the millisecond region (Váró and Lanyi, 1991). Reverse photoreaction from K to bR was demonstrated by accumulation of K at low temperature (Birge et al., 1989, and references cited there) by photoequilibrium measurements at room temperature (Govindjee et al., 1990) and by double-pump excitation (Bazhenov et al., 1992). Low temperature absorption kinetic experiments of 30 ps time resolution indicate that the overall rate of the reverse photoreaction is higher than that of the forward one (Kryukov et al., 1981, Iwasa et al., 1984). However, the detailed path and kinetics of the K→bR back photoreaction are still unknown.

Because bR functions as a proton pump, the investigation of charge movement processes in the course of the primary

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events also has crucial importance. Excitation of electrically oriented purple membrane samples makes possible the detection of such charge movements by direct electric methods (for a review, see Keszthelyi and Ormos, 1989). A general observation is that every step of the photocycle is also manifested as a charge separation process (Keszthelyi and Ormos, 1980). Picosecond electrical studies in recent years demonstrated the occurrence of an ultrafast phase of the electric signal (Groma et al., 1984, 1988; Trissl, 1985; Simmeth and Rayfield, 1990). Because the highest time resolution of these studies is 5 ps (Simmeth and Rayfield, 1990), the assignment of this signal to the first intermediates of the photocycle is not unambiguous. Interestingly, the polarity of the picosecond signal, which is opposite to the overall proton translocation, is identical for light-adapted and dark-adapted bR samples and for those reconstituted with retinal analogs, regardless of whether they are in all-*trans* or 13-*cis* conformation (Trissl and Gärtner, 1987). On the other hand, however, excitation of the K intermediate by double flash resulted in an electric signal of opposite polarity and a time constant less than 30 ps (Ormos et al., 1983; Trissl et al., 1989). It was recently demonstrated that bR in blue and acid purple forms also has early charge separation of direction opposite to that of the purple membrane (Fukuzawa et al., 1994).

We describe the electric response signal of bR generated by a train of 150 fs laser pulses. A single pulse of such a short length does not reexcite the K intermediate; however, a continuous train generates a high population of K. This makes possible the simultaneous detection of the charge movement processes corresponding to both the forward and the reverse photoreaction at room temperature, using a nondestructive excitation intensity. A statistical analysis of the experimental traces, taking into account the response of the measuring instrument, resulted in the characterization of that process by a time resolution higher than that shown in the previous studies.

MATERIALS AND METHODS

Purple membrane strain S9 of *H. salinarium* was prepared by standard methods (Oesterhelt and Stoeckenius, 1974). Electrically oriented purple membranes were deposited and dried onto indium-tin-dioxide thin layer (surface resistance 20 Ohm/square) (Optilab Kft., Budapest, Hungary), as described by Váró and Keszthelyi (1983). The thickness of the dried purple membrane layer was approximately 25 μm. This layer was incorporated into a modified SMA panel mount jack receptacle (model R 125 464; Radiall, Rosny-sous-Bois, France). This coaxial system has an inner electrode of 1.27 mm diameter, separated by a 4.1 mm diameter insulator tube from the outer electrode. The inner electrode and the insulator tube were cut down at the plane of the mounting flange of the receptacle. A hole of 30 μm in depth and 4.1 mm in diameter was then drilled into the insulator and the inner electrode, perpendicular to the plane of the flange. The size of the dried purple membrane layer deposited onto indium-tin-dioxide was trimmed to fit into this hole. In this way the indium-tin-dioxide layer had a mechanical contact to the outer electrode, forming a capacitor with the inner one. This capacitor was almost completely filled with the oriented purple membrane sample, ensuring a good capacitive junction between the intramembrane charge motion and the receptacle.

The excitation pulses were generated by a homemade colliding-pulse mode-locked dye laser operating at a repetition rate of 120 MHz. The output of this laser was amplified by a copper vapor laser (model Cu 25; Oxford Lasers, Oxford, UK) pumped six-pass dye amplifier (Nickel et al., 1989) and compressed by an SF-10 prism-pair. This system emitted a train of 620 nm pulses with a 7 kHz repetition rate. Autocorrelation measurements, performed using noncollinear second harmonic generation in a KDP crystal, resulted in a 150 fs pulse-width of the final output. The maximum average intensity at the place of the sample was 100 mW/cm^2 (14 μJ/cm^2 pulse energy density). The nonamplified colliding-pulse mode-locked pulses passed through the amplifier and also reached the sample, but their average power was less than 1% of the amplified ones.

Background illumination was produced by the 647 nm line of a cw krypton ion laser (model 165; Spectra Physics, Mountain View, CA) with 1100 mW/cm^2 intensity or the 511 nm line of the copper vapor laser with 140 mW/cm^2 intensity.

The light-induced electric response signal of the sample was detected by a Tektronix 11801A digital sampling oscilloscope (Wilsonville, OR) equipped with a 40-GHz SD-30 sampling head (equivalent rise-time <8.8 ps). The sample was directly connected to the 50 Ohm input of the sampling head via an SMA connector to minimize the dispersion of the electric signal. The oscilloscope was triggered by a fast metal-semiconductor-metal photodiode driven by a fraction of the exciting laser. To get a good signal-to-noise ratio, 32–128 traces of 512 points were averaged.

Numerical processing and analysis of the experimental data were performed in the framework of the MATLAB program (The MathWorks, Inc., Natick, MA). For least-square fitting we used the UMSOLVE nonlinear minimization routine.

RESULTS AND DISCUSSION

The electric response signal of bR, induced by the 7 kHz train of the 620 nm laser pulses (Fig. 1), consists of a positive and a negative peak in the picosecond range. (The ringing on the decaying phase of the traces is attributable to electric reflection artifacts.) This shape is in sharp contrast to the previously published traces, having simply a rising phase (negative in our orientation) corresponding to the real charge separation process and a decaying phase determined by the RC constant of the measuring circuit (Groma et al., 1984, 1988; Trissl, 1985; Simmeth and Rayfield, 1990). All of the reported traces, however, were detected at a low repetition rate (typically 10 Hz) of the exciting laser, ensuring that a majority of the molecules were rested in the bR ground state. In contrast to this, our high repetition illumination maintains a considerable fraction of molecules in the photocycle. A plausible explanation is that the anomalous positive peak is caused by a charge movement process corresponding to the excitation of a photointermediate of bR.

To verify the validity of the above explanation, we reduced the repetition rate of excitation by mechanical chopping. The solid line in Fig. 1 *A* shows the response signal when 10 Hz chopping was applied. In this case, a train of 40 pulses with the original 7 kHz repetition rate reached the sample in every 100 ms. This caused a marked reduction in the height of the positive peak and an increase in that of the negative one, as expected for a reduced fraction of cycling molecules.

As additional evidence for the crucial role of the fraction of cycling molecules in the shape of this complex response

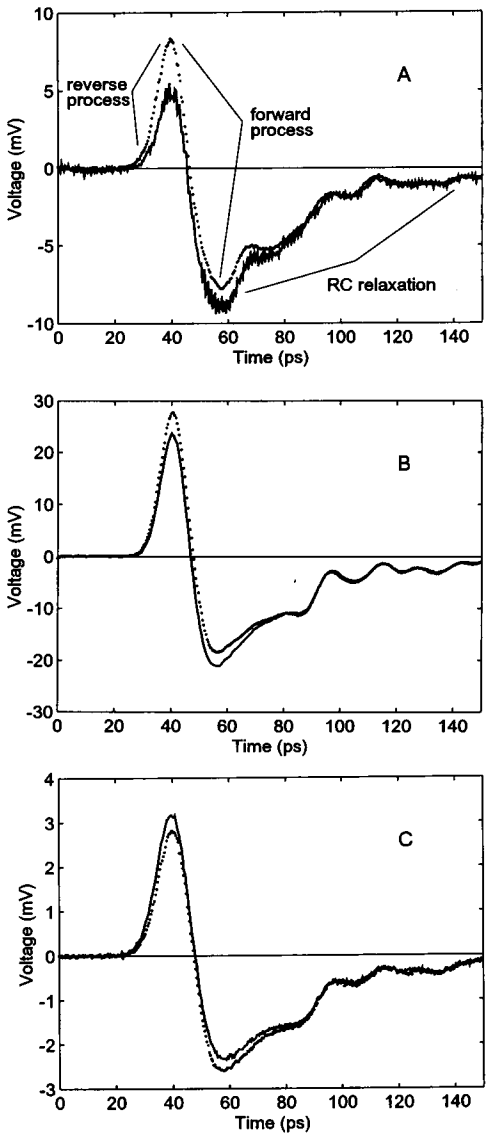

FIGURE 1 Light-induced electric response signal of bR excited by a 620 nm amplified CPM laser of 150 fs pulse-width. (*A*) Dotted line shows excitation with 7 kHz repetition rate. Solid line indicates excitation with chopped laser. A train of 40 pulses with the original repetition rate passed through the hole of the chopper at every 100 ms. (*B*) Dotted line shows normal excitation (~70 mW/cm^2). In addition to the normal excitation, a background illumination with a 647 nm krypton ion laser of 1100 mW/cm^2 intensity was applied (——). (*C*) Dotted line indicates normal excitation (~7 mW/cm^2). In addition to the normal excitation, a background illumination with the 511 nm line of the copper vapor laser of 140 mW/cm^2 average intensity was applied (——). The ringing on the decaying phase of the traces is attributable to electric reflection artifacts.

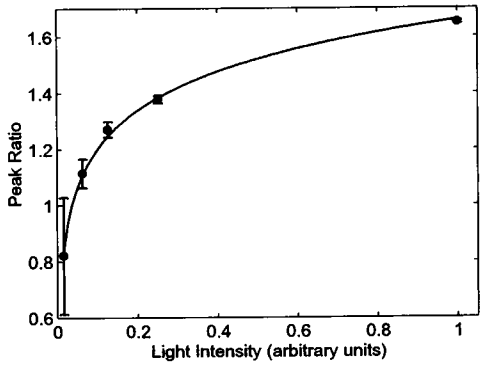

FIGURE 2 Effect of the excitation light intensity on the ratio of the positive and negative peak (height/height) of the electric response signal. Unit intensity corresponds to ~100 mW/cm^2 at the sample.

signal, the intensity of the exciting laser was gradually reduced by neutral density filters. Fig. 2 shows the ratio of the height of the positive and the negative peak at different laser intensities. The relative weight of the positive peak monotonically increases with increasing light intensity, again in accordance with our explanation.

The following details have to be taken into consideration in order to determine which intermediate of the photocycle is responsible for the positive peak. Under a (quasi)continuous laser illumination, the long-lived intermediates M, N, and O have the highest population beyond the bR ground state. The M form can be excluded as a source of the positive peak because of the lack of its absorption at 620 nm. In addition, at <75% humidity dried samples produce practically no N and O intermediates (Váró and Keszthelyi, 1983; Váró and Lanyi, 1991). Instead the photocycle is completed via a shunting branch from the M intermediate to the bR ground state. On the other hand, under the same conditions the K intermediate, which normally decays to L in several microseconds, has a considerable population even at 1 ms after the excitation. This means that with a 7 kHz repetition rate there is a relatively good chance to excite the K and the L intermediates. There is no chance, however, for the excitation of the extremely short-lived J intermediate. The existence of the photo-induced reverse reaction from K to bR is a well known phenomenon (Kryukov et al., 1981; Iwasa et al., 1984; Birge et al., 1989; Govindjee et al., 1990; Bazhenov et al., 1992), whereas no similar reaction has been reported for L. Clearly, the corresponding charge movement should have the opposite direction than that of the forward reaction. Such a reversed electric signal was demonstrated experimentally by double flash experiments (Ormos et al., 1983; Trissl et al., 1989). Considering these observations, we attribute the anomalous positive peak of the electric signal to the K→bR reverse photoreaction. However, some minor contribution from the L, N, or O forms cannot be completely excluded.

If the two opposite components of the electric signal originate from the photoequilibrium of the bR and K states, then extra background illuminations of wavelength higher or lower than 620 nm of the exciting laser should also alter the ratio of the peaks. Fig. 1 *B* demonstrates this phenomenon. In this case the sample was illuminated by the 647 nm line of a cw krypton ion laser in addition to the exciting laser. At this wavelength, the ratio of the extinction coefficients of K and bR is higher than at 620 nm. Consequently, this extra illumination shifts the equilibrium toward a lower K population, manifested in the reduction of the positive

peak and the increase of the negative one. The opposite effect was observed by background illumination with the 511 nm line of the copper vapor laser, which increases the population of K in the photoequilibrium (Fig. 1 *C*). The observed dependence of the peak ratio on the background illumination completely rules out the L and N forms as potential sources for the reverse reaction, inasmuch as their absorption spectra are blue-shifted with respect to bR.

The kinetics of the electric response signal was analyzed by statistical methods with use of the following model. Both the bR→K and the K→bR transition are presumed to be single, first-order reactions. Although the excited state of bR decays to K via the J form, the reported 300–500 fs lifetime of this intermediate (Nuss et al., 1985; Petrich et al., 1987; Mathies et al., 1988; Dobler et al. 1988; Pollard et al., 1989) is much less than the time resolution of our measurements. Hence the real charge separation process within the purple membrane can be described by two exponentials of opposite sign. This light-induced electric transient also relaxes via an exponential process determined by the capacitance of the sample holder and the 50 Ohm input of the oscilloscope. The detected trace on the oscilloscope is built up as a convolution of these three exponentials and the response function of the measuring system (Groma et al., 1988). We have only partial information about this instrumental response function: the 8.8 ps upper limit of the oscilloscope rise-time and the nominal 18 GHz frequency limit of the SMA standard. For this reason, in the model it was simply described by a Gaussian function, the half-width of which was considered as an unknown parameter (see below). The laser excitation was considered to occur instantaneously.

Using the above model, a least-square fitting of the experimental traces was carried out to estimate its unknown parameters. These parameters are the time constants and amplitudes of the two charge separation processes, the time constant (RC constant) of the relaxation, and the half-width of the instrumental response. Because the relaxing parts of the traces are highly corrupted by error attributable to electric reflections and because this range does not contain too much useful information, the fit was performed in two steps. First, the complete signal was fitted with all of the free parameters to determine the RC constant. Then this value was kept constant, and the informative slice of the signal was analyzed in further detail. The first step resulted in an RC constant of 31 ps, falling into the 20–40 ps range calculated from the geometry of the sample holder.

As presented in Fig. 3 *A*, the quality of the fitting with the optimal parameters on the most informative slice of the experimental trace is rather good. The corresponding parameters are 3.2 ps time constant and 930 mV amplitude for the bR→K transition, 2.9 ps time constant and −914 mV amplitude for the K→bR transition, and 9 ps for the half-width of the instrumental response. These data, however, should be handled with some care for the following reasons. 1) Nine picoseconds is clearly an underestimation of the half-width of the instrumental response because the sampling head alone has a rise-time of ~8.8 ps, and some dispersion certainly takes place on the SMA sample holder and connector. Consequently, the time constants (2.9 and 3.2 ps) were overestimated to obtain the optimal fit. 2) The two time constants are very close to each other. This gives a high uncertainty in the amplitudes if their signs are opposite. 3) The model is highly nonlinear, which means that there is no standard way for the estimation of the parameter errors. In addition, the values of the time constants are in the range of the instrumental time resolution, making the error estimation even more difficult.

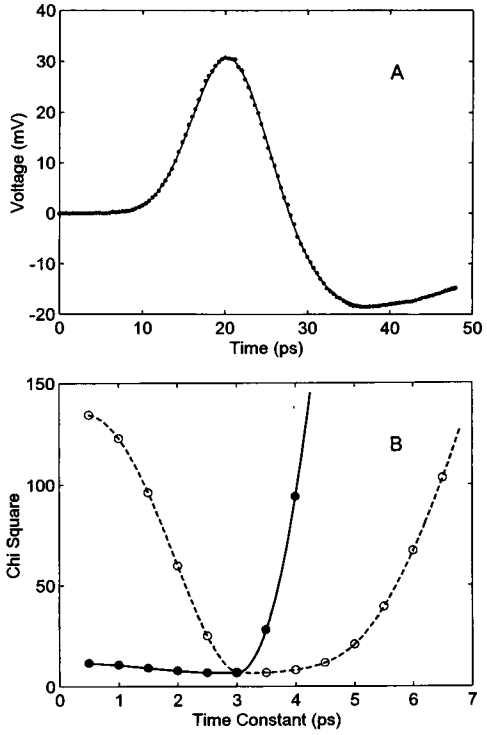

FIGURE 3 Results of the model fitting to the measured electric response signal. (*A*) Dotted line indicates experimental data. Solid line indicates fitting with the optimal model parameters. Time constants: 2.9 ps and 3.2 ps; amplitudes: −914 mV and 930 mV; RC constant: 31 ps; half-width of the measuring system response: 9 ps; χ^2 of the fit (unweighted): 6.6. (*B*) Variation of χ^2 of the fit when the time constant of the faster (●) and the slower (○) process was fixed to different values, whereas all of the other parameters were optimized by the fitting procedure.

To overcome the above difficulties and to find safe upper limits for the time constants, we varied the two time constants separately over a set of fixed values, whereas the other parameters were optimized. The corresponding variation of χ^2 (the sum of the square of the unweighted residuals) is presented in Fig. 3 *B*. As expected, the response of χ^2 to the variation of the smaller time constant (K→bR transition) is asymmetric and hardly changes below the time constant value where χ^2 has a minimum, but it increases sharply above that. There is no specific theoretical value of χ^2 that would imply a "natural" upper and lower limit for the corresponding time constant. As an arbitrary criterion, we selected the points at which χ^2 reached a value twice as

much as its minimum. This resulted in an upper limit of 3.5 ps for the time constant of the faster process (K→bR transition). This limiting value is also justified by the sudden increase of the χ^2 curve around this point (Fig. 3 *B*, *solid line*). The corresponding fitted half-width of the instrumental response is 8.2 ps. This unrealistic low value also indicates that the time constant is conservatively overestimated. No lower limit of this time constant can be determined because of the flat characteristics of the χ^2 curve below the minimum.

If the time constant of the faster process is >3.5 ps, the calculated time constant of the slower process is just slightly higher than the other. The actual difference depends on the stopping criteria of the iteration process. Correspondingly, the absolute values of the amplitudes are unrealistically high (>1000 mV). The amplitude of the faster process is always negative, and its absolute value is a bit lower than that of the slower one to follow the shape of the experimental curve. (Note that a negative amplitude corresponds to the rise of a positive signal.) As the time constant of the faster process approaches zero, the time constant of the slower one increases to 4.3 ps, the amplitudes of the two exponentials are well separated to −57 mV and 72 mV, respectively, and the half-width of the instrumental response is 9.8 ps. All of these values look rather realistic.

On the basis of the same criteria applied for the faster process, the upper limit for the slower process (bR→K transition) is 5 ps. In this case the χ^2 curve is more symmetric, hence a lower limit of 2.5 ps can also be specified. Below the lower limit the two time constants are again very close, the absolute values of the amplitudes are >1000 mV, and the half-width of the instrumental response is >10 ps. Above the higher limit the time constant of the faster process is <0.1 ps, the amplitudes take values around −40 mV and 60 mV, respectively, and the half-width of the instrumental response is <9.4 ps.

In the above statistical analysis the lower limit for the slower process and the upper limit for the faster process are overlapped in the 2.5–3.5 ps range. Hence merely by this statistical justification the reverse process can be even faster than the forward one. Note however that in every case the positive phase of the signal precedes the negative one. Elementary function analysis of the applied model shows that this shape can take place only if the faster process has negative amplitude and the slower one has positive amplitude. Earlier studies showed that with our orientation the bR→K transition has positive amplitude (Groma et al., 1984, 1988). Hence the observed signal shape implies that the reverse reaction should be the faster one.

The 5 ps upper limit given for the time constant of the forward charge separation process is identical to that found previously by Simmeth and Rayfield (1990). By the presented statistical methods, however, we were also able to specify a lower limit. The 2.5–5 ps range coincides well with that found for the rise-time for the K intermediate by optical methods (Nuss et al.; 1985, Sharkov et al., 1985; Polland et al. 1986; Petrich et al., 1987). Hence we can attribute this charge movement to the J→K transition. From this type of measurement, it cannot be determined whether there is an earlier charge separation process corresponding to the rise of the J intermediate because every process faster than 3 ps is masked by the back photoreaction. Note that the biphasic nature of the electric signal observed in this study cannot be explained simply by a two-step forward reaction of opposite-charge separation processes. First, this would contradict the previous observation of Simmeth and Rayfield et al. (1990), who found a monophasic signal using an electrical detector of even somewhat higher time resolution than ours. Second, in such a case the variation of the peak ratio with the repetition rate and the intensity of the excitation as well as with background illumination would be hard to explain.

The sign of the charge movement corresponding to the reverse photoprocess is opposite to that of the forward reaction, as expected logically and from the previous experiments (Ormos et al. 1983; Trissl et al. 1989). The absolute value of the amplitude of the reverse process was found to be smaller. Because the amplitude is proportional to the population of the corresponding form, the absorption cross-section, and the quantum yield of the photoreaction, this alone is not enough to estimate the ratio of the electric dipole moment change in the forward and reverse process.

What is important is that the charge displacement in the reverse photoreaction is faster than in the forward one. This is in agreement with previous optical studies (Kryukov et al., 1981; Iwasa et al., 1984). Our direct electric method significantly extended the time resolution of the reverse photoreaction, giving a 3.5 ps upper limit for its time constant. The difference in the rates of the forward and reverse photoreactions strongly indicates that the detailed routes of the bR→K and K→bR transitions are probably different, and the two forms do not share a common single minimum excited state (Kryukov et al., 1981).

Our experiments were carried out on dehydrated samples simply to avoid the difficulties of measuring an ultrahigh-frequency signal in the presence of water. One might suppose that as the absence of water molecules strongly alters the slow phase of the bR photocycle (Váró and Keszthelyi, 1983; Váró and Lanyi, 1991), it also has some effect on the mechanism of the early charge separation processes. This problem is to be investigated in our laboratory.

SUMMARY

By exciting dried oriented purple membranes with a continuous 7 kHz train of 620 nm femtosecond laser pulses, we found a condition during which the charge displacement process from the forward and the reverse bR-K photoreaction can be observed simultaneously. The modification of the electric response signal shape by the variation of the frequency and the intensity of the exciting laser as well as by application of different background illumination favors the idea that the origin of the reverse phase of the signal is really the K→bR back reaction.

Statistical analysis of the electric response signal resulted in a 5 ps upper and a 2.5 ps lower limit for the forward reaction. This is coincidental with the formation time of the K intermediate, determined by optical methods. Charge displacement processes from the earlier phases of the forward reaction cannot be excluded.

The rate of the charge separation in the reverse photoprocess is higher than in the forward one. This study resulted in a 3.5 ps upper limit for its time constant. This difference in the rates indicates that the forward and reverse routes of the bR-K phototransition are somewhat different.

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