Temperature jump study of charge translocation during the bacteriorhodopsin photocycle

Hans-Jürgen Butt, Klaus Fendler, András Dér,* and Ernst Bamberg
Max-Planck-Institut für Biophysik, 6000 Frankfurt, Federal Republic of Germany; and *Institute of Biophysics, Biological Research Center, Hungarian Academy of Science, Szeged, Hungary

ABSTRACT Temperature jump experiments were carried out on purple membranes oriented and fixed in polyacrylamide gel. With green background illumination a relaxation of the photocurrent after an infrared laser pulse could be observed. To simulate the temperature jump signals different models of the bacteriorhodopsin photocycle were tested. The parameters of these models were obtained by measuring absorbance changes and photocurrent after excitation with a 575-nm laser flash.

A model with a temperature-dependent branching before the M state turned out to be satisfying. Other models, especially those with a late branching or without branching, could not reproduce the temperature jump measurements.

INTRODUCTION

The cell membrane of *Halobacterium halobium* contains distinct patches the purple membranes. These patches consist of only one protein species and lipids. After light excitation this protein, bacteriorhodopsin (BR), translocates protons through the membrane and thereby passes a series of intermediates. The kinetics of the photocycle has mainly been studied optically (Stoeckenius and Bogomolni, 1982; Parodi et al., 1984; Beach and Fager, 1985; Xie et al., 1987; Sinton and Dewey, 1988). In addition the electric current, associated with the proton transport, has been measured (Drachev et al., 1974; Herrmann and Rayfield, 1976; Trissl and Montal, 1977; Bamberg et al., 1979; Keszthelyi and Ormos, 1980; Fahr et al., 1981; Holz et al., 1988). From these studies the spectral characteristics, the lifetimes of a number of intermediates, and the charge movements associated with the photocycle could be obtained.

Distinct models for the BR photocycle have been proposed. A possible branching in the photocycle and equilibria between intermediates are the main aspects which are different in these models.

Absorbance changes after an infrared laser-induced temperature jump (T-jump) have already been measured with BR (Zubov et al., 1983; Chernavskii et al., 1989). After a green laser flash, Zubov et al. accelerated the photocycle by a subsequent infrared laser pulse. Passive charge transport of ionophores was studied by T-jump measurements on planar lipid membranes (Knoll and Stark, 1977; Brock et al., 1981).

Here we report on measurements of the charge transport of BR after a laser-induced T-jump. Therefore purple membranes were oriented and fixed in polyacrylamide gel. With green background illumination, a relaxation of the steady-state photocurrent after an infrared laser pulse was observed. In addition flash kinetic experiments were performed and the results were used to simulate T-jump signals. By comparing simulations with the measured curves, we tested basic models of the photocycle.

MATERIALS AND METHODS

Bacteriorhodopsin samples

Purple membrane fragments were prepared from *Halobacterium halobium* strains S9 (Oesterhelt and Stoeckenius, 1974). The purple membranes were fixed and oriented in polyacrylamide gel according to Eisenbach et al., 1977, and Dér et al., 1985, with final concentrations of 11% acrylamide, 0.3% *N,N'*-methylene bis-acrylamide, 0.15% ammonium persulfate, 1% tetramethylethylenediamine, and 24 μM BR. Pieces of 1.3 × 7 × 7 mm were cut from the gel and washed for 3 d in electrolyte. It was always made sure that BR was light-adapted.

For the measurement the gel, after incubation in the appropriate buffer for 2 h, was placed in the middle of a 1.3-mm thick cuvette. The cuvette was filled with electrolyte. Platinized platinum electrodes, screened from direct light, were immersed on both sides of the gel. In this way charge translocation through the purple membranes could be measured (see Fig. 1). The cuvette was shielded in a thermostated Faraday-cage which contained two windows of 7 × 7 mm for the light.

To check the electrode behavior, a step voltage of ±1, ±10, and ±100 mV was applied in series to the cuvette, and the resulting current was observed. The current could be described by two exponentials of 5 and 70 s, reflecting electrode capacitance and diffusion polarization. Between pairs of Pt/Pt-electrodes inevitable residual potential differences exist. This potential fluctuated with time constants of a few hours. Slow potential fluctuations could only affect the subtraction procedure which was applied to generate difference signals (see below). However, because traces subtracted from each other were taken within minutes, these fluctuations did not affect the difference signals.

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Samples for control experiments

BR was bleached by illuminating a gel in 1 M hydroxylamine at pH 6 with a halogen lamp (495-nm cut-off filter) for 1 h. To get blue membranes, we suspended a gel in pH 2 buffer and then repeatedly washed in distilled water. Tween-washed membrane fragments, containing halorhodopsin, were prepared from the BR-deficient mutant strain OD 2 according to Bamberg et al., 1984. H,K-ATPase sheets were prepared as described in Rabon et al., 1985. Both were incorporated in polyacrylamide gel like purple membranes.

Temperature jump measurements

For the T-jump measurements (Fig. 1) the electric signal was detected by platinized platinum electrodes, amplified by a current amplifier, filtered at 1,000 Hz, and recorded with a transient recorder (type 4500, Gould Inc., Santa Clara, CA).

As background illumination the light of two halogen lamps (250 W) was focused on the sample. 495-nm cut-off filters and heat protection filters were used for all measurements. The spectral irradiance at 570 nm was checked to be 5 mW/cm^2 per nm. An iodine laser (emission wavelength, 1.32 μm, model 100, Vuman Ltd., Manchester, UK) was applied for the T-jump. At 1.32 μm the absorption coefficient in water is 0.74 cm^{-1}, so an effective and homogeneous heating of the water was obtained. The membrane sheets are then warmed up by the water. With a pulse energy of 3 J and a pulse length of 4 μs, a T-jump of 1°C resulted. The laser beam passed a 1 μm cut-off filter to prevent illumination of BR by the flash lamps of the laser. A few seconds after T-jump, the temperature declined again. Measurements were performed at 5, 9, and 15°C in a pH region from pH 4.1 to pH 8.5. In all cases four signals were averaged.

Flash kinetic measurements

To check, if a certain model reproduced the T-jump signals, it was necessary to know the time constants and current amplitudes of the BR sample. For that reason the kinetics of absorption changes and the photocurrent were measured simultaneously under the same conditions as in the T-jump experiments. A 575-nm laser pulse of 10-ns length and an energy of 300 μJ excited the sample (FL 2001, Lambda Physik, Göttingen, FRG). The excitation light passed through a fiber optic and was directed to the gel. For each temperature (1°–31°C) three measurements with current amplifiers of different time resolutions were done to get the lowest noise possible for each time range. The highest time resolution was 150 ns. For the slowest time range (100 μs–0.4 s) Ag/AgCl-electrodes were used instead of Pt/Pt-electrodes to avoid drift problems. All signals were recorded by a transient recorder (TRD 4070, Krenz, Hirzenhain, FRG). To monitor absorption changes a 250-W halogen lamp was used as a probe source. Having passed through a monochromator, the sample and a narrow band interference filter, a photomultiplier detected the probe light. After further amplification the output was recorded in the transient recorder. The absorption measurements were restricted to times >3 μs due to a laser artefact. The signals were fitted with sums of exponentials. All measurements were averaged 25 times.

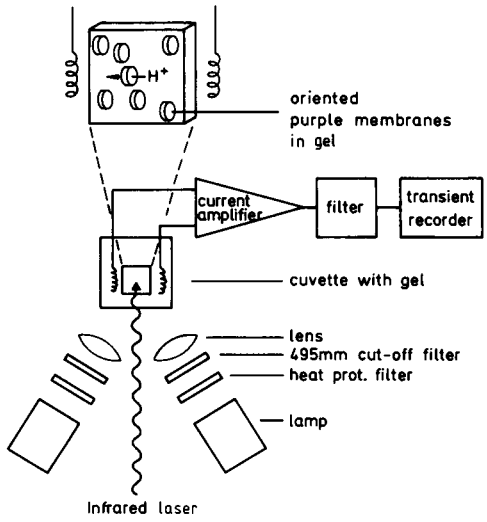

FIGURE 1 Set-up for the T-jump measurements. The arrangement of the purple membranes, the gel, and the electrodes is indicated.

T-jump simulation

Because the measured current is proportional to the pump current I_p generated by BR (Keszthelyi and Ormos, 1983), information about the pump mechanism can be obtained by analyzing the flash kinetic and T-jump experiments. For a specific model the rate constants and charge displacement coefficients were extracted from the optical and electric data obtained by flash kinetic experiments. This was done for a variety of models (Fig. 7). The procedure of evaluating the rate constants and charge displacement coefficients is described in the Appendix. In addition the experiments were performed at different temperatures to determine the activation energies of the transitions.

Based on the calculated charge displacement coefficients and rate constants, the T-jump induced relaxation current was simulated. Therefore, the rate equations were solved numerically to get the initial steady-state concentrations c_i (0) of all intermediates i. The pump current can be calculated by summing up the contributions of all reactions:

$$I_p(t) = e_o \Sigma_i \Sigma_j c_i(t) \cdot \alpha_j \cdot k_j. \quad (1)$$

e_o is the unit charge, c_i represents the concentration of intermediate i divided by the total concentration of BR molecules, and α_j is the charge displacement coefficient (see Appendix). The summation runs over all intermediates i. For a particular intermediate i the sum over j is restricted to rate constants corresponding to a decay of this intermediate. With this equation the initial steady-state current was evaluated. To simulate the T-jump, all rate constants were changed according to their activation energies and the laser-induced temperature increase. Again the rate equations were solved numerically using the initial concentrations c_i (0) as starting values. Then the electric current was calculated from Eq. 1. To improve the agreement between simulations and T-jump measurements, the rate constants, displacement coefficients, and activation energies were allowed to vary ±10, ±15, and ±15%, respectively, approximately the errors of the flash kinetic measurements.

RESULTS

Signals without background light

Excitation of a sample of oriented purple membranes with the infrared laser gave rise to an electric signal, which decayed with time constants of 100 μs–10 ms. As will be

shown some of the components cannot be attributed to charge movements during the pump cycle of BR. This was investigated with various control samples. To separate information relevant for the protein activity from artifacts, difference signals were calculated. They were obtained by subtracting the IR laser-induced signals with and without background light.

Without background illumination a 'dark signal' was observed after applying the T-jump (Fig. 2). This signal had the same sign as the light-induced current. It could be described by two exponentials with time constants of 100 μs (not resolved here) and 2 ms. At 4°C the dark signal was unchanged, indicating that it was not due to a shock wave caused by the expansion of the solute after the T-jump. The 100-μs component was an artifact that also occurred without a sample, having only electrolyte in the cuvette.

The 2-ms component was observed with BR gels, but oriented, bleached membrane samples or deionized BR, which are both considered to be electrically inactive, gave the same signal. Gels made with oriented Tween membranes or H,K-ATPase containing membrane fragments showed a similar signal. With unoriented purple membranes the 2-ms component disappeared. It was concluded that this component was a general effect of charged, oriented membrane sheets.

In general a change in the offset current was observed in the dark. This was attributed to an increase of ion mobility after the T-jump which resulted in an offset current because of a residual potential difference between the electrodes.

Control experiments

Fig. 2 shows a trace of the current with full background light. By subtracting the dark signal from signals measured with background light, difference signals were obtained (Fig. 3).

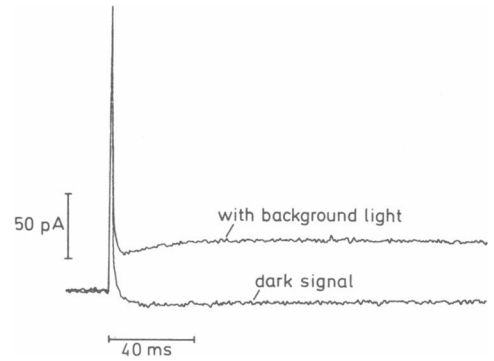

FIGURE 2 Current relaxation after applying a T-jump. One signal was obtained without, the other with green background illumination. 5°C, 10 mM NaCl, 10 mM Mops, pH 7.2.

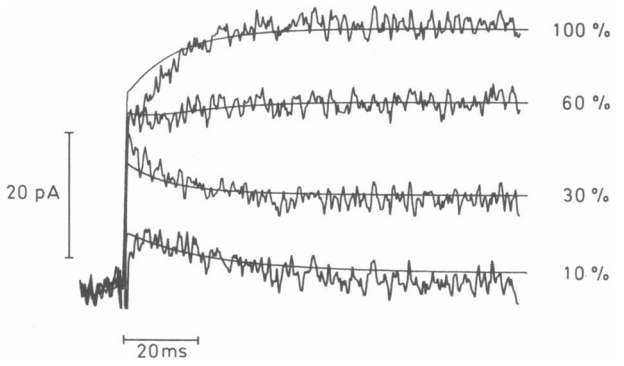

FIGURE 3. Difference signals between currents measured with and without background light at 5°C. The four signals were obtained with 100, 60, 30, and 10% light intensity. 10 mM NaCl, 10 mM Mops, pH 7.2. In addition signals resulting from simulations using model E are shown. These curves were simulated using the following relative displacement coefficients, rate constants, and activation energies: $\alpha_{Br \to X} = -4\%$, $\alpha_{X \to M} = 7\%$, $\alpha_{X \to M'} = 15\%$, $\alpha_{M \to Br} = 45\%$, $\alpha_{M' \to Br} = 37\%$, $k_1 = 135\ s^{-1}$, $k_{X \to M} + k_{X \to M'} = 4{,}000\ s^{-1}$, $k_{M \to Br} = 49\ s^{-1}$, $k_{M' \to Br} = 23\ s^{-1}$, $E_{M \to Br} = E_{M' \to Br} = 55$ KJ /mol, and a branching ratio $k_{X \to M}/k_{X \to M'}$ of 0.23 with an associated activation energy of 60 KJ/mol.

Several tests were carried out with the difference signal. Neither blue nor bleached BR gels gave a difference signal. Experiments with unoriented purple membranes showed no difference signal, indicating that the signal was not caused by straylight effects. A disadvantage of platinized platinum electrodes are polarization effects. To show that electrode polarization did not affect the difference signal, we applied an external voltage to the electrodes which induced an offset current. After the T-jump the dark current changed instantly (<4 μs) to a new value due to an increase of ion mobility, but the difference signal remained the same.

To prove that no temperature-induced pH jumps were measured, we performed experiments with different buffers. We used 10 mM Tris (ΔpH/Δ°C = -0.031) and 10 mM Mops (ΔpH/Δ°C = -0.011) plus 10 mM NaCl at pH 7.2 as buffers. The same difference signals resulted. So effects due to temperature-induced pH jumps could be excluded.

The control experiments show that all current components observed in the difference signals are due to the electrogenic activity of BR during its pump cycle.

Difference signals of oriented purple membranes

Fig. 3 shows difference signals measured at various background light intensities at 5°C. With increasing light

intensity the new steady-state current increased too. At low light intensities we observed an initial peak current that declined to a steady-state current. At high intensities the current rose until it saturated at the steady-state current. At a certain light intensity in between, a step current was observed. This effect was measured at all pH values tested (4.1, 6.3, 7.2, 8.5).

At 9°C (data not shown) the light intensity that caused a step current after the T-jump was shifted to higher values. At 15°C (Fig. 4) only difference signals with a peak were observed, but the overshoot was more pronounced at low light intensities.

Flash kinetic experiments

Current and absorption changes after a 575-nm laser pulse were measured simultaneously with the same sample at various temperatures (see Fig. 5). From multiexponential fits of the observed traces, information about different intermediates of the BR photocycle was obtained such as time constants, transported charge, and activation energies.

Results of the time-resolved experiments are summarized in Table 1. The fast components could only be measured electrically. Absorption measurements at 419 nm and current signals revealed two time constants for M formation. The electric data were not accurate enough to obtain the activation energy for both components, so the given value was calculated from results of four exponential fits instead of five. Optical measurements of 649 nm were too noisy to resolve more than one component for M formation. The M decay had to be fitted with two time constants. The temperature dependence of all time constants between 1 and 31°C could be described by a single activation energy for each relaxation time; Arrhenius plots always yielded straight lines. The results agree with time constants reported in the literature (e.g., Maurer et al., 1987*a*; Holz et al., 1988).

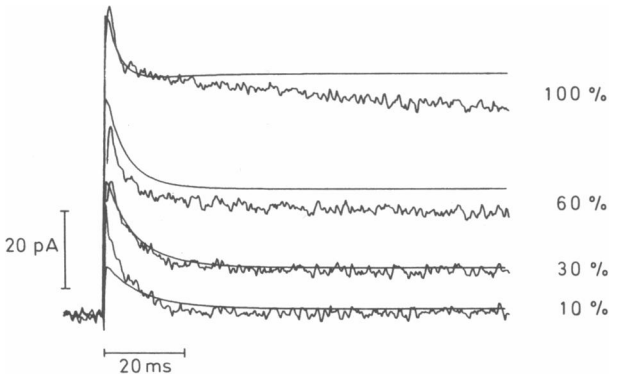

FIGURE 4. Difference signals between currents measured with and without background illumination at 15°C. The four signals were obtained with 100, 60, 30, and 10% light intensity. The slow decline of the curve at 100% is due to falling temperature. 10 mM NaCl, 10 mM Mops, pH 7.2. In addition signals resulting from simulations with model E are shown, using the following displacement coefficients, rate constants, and activation energies: $\alpha_{Br\to X} = -4\%$, $\alpha_{X\to M} = 7\%$, $\alpha_{X\to M'} = 15\%$, $\alpha_{M\to Br} = 45\%$, $\alpha_{M'\to Br} = 37\%$, $k_1 = 135\ s^{-1}$, $k_{X\to M} + k_{X\to M'} = 8{,}400\ s^{-1}$, $k_{M\to Br} = 121\ s^{-1}$, $k_{M'\to Br} = 58\ s^{-1}$, $E_{M\to Br} = E_{M'\to Br} = 55$ KJ/mol, and a branching ratio $k_{X\to M}/k_{X\to M'}$ of 0.6 with an associated activation energy of 60 KJ/mol.

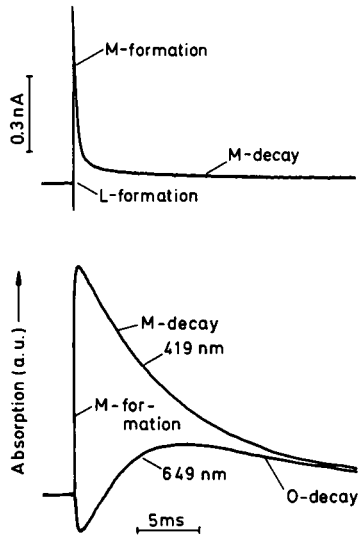

FIGURE 5 Current and absorption signals after exciting bacteriorhodopsin with a 575-nm laser pulse. Absorption was measured at 419 and 649 nm. It is indicated which parts of the signals are attributed to the formation or decay of certain intermediates. 10 mM NaCl, 10 mM Mops, pH 7.2, 20°C.

Fig. 6 shows the variation of absorption amplitudes with temperature. The amplitudes of the fast M formation (τ = 71 μs at 20°C) and the fast M decay (τ = 6.2 ms at 20°C) increased with rising temperature. In addition the absorption amplitude at 649 nm, representing the O intermediate, increased with temperature (twofold between 10 and 20°C).

The electric signal could be fitted with only one time constant in the millisecond range; biexponential fits gave no reproducible results (Müller et al., 1988). As the absorption measurements showed two time constants in the millisecond range the question arises if there is only one time constant in the electric signal or if there are actually two that could not be resolved. Both cases were tested. First it was assumed that one of the relaxation processes is not correlated with a current. Second it was assumed that the current actually contains two exponentials but they could not be distinguished. The later assumption is justified by the results of different authors, that absorption changes and charge translocation are correlated (Keszthelyi and Ormos, 1980; Dér et al.,

TABLE 1 Results of time-resolved current and absorption measurements at 419 and 649 nm

	K/L formation		M formation		M decay	
Electric						
τ	<150 ns	1.4 μs	75 μs	220 μs	10 ms	
E_a		41	43		55	
Q	−1.5	−1.1	6.0	6.0	41	
419 nm						
τ	—	—	71 μs	200 μs	6.2 ms	14 ms
E_a	—	—	39	45	57	52
649 nm						
τ	—	—	110 μs		4.0 ms	8.3 ms
E_a	—	—	48		49	68

τ are the measured time constants. The activation energies E_a are given in killojoules per mole and the product of current amplitude with the corresponding time constant Q in nanoAmperes times milliseconds. 10 mM NaCl, 10 mM Mops, pH 7.2, 20°C.

1985). To find current amplitudes belonging to the two time constants, we determined the ratio of both constants from the optical measurements. Then the current signal was fitted with two exponentials, keeping the ratio of time constants fixed. In this way reproducible amplitudes were obtained.

DISCUSSION

T-Jump simulations

The time constants and current amplitudes obtained by the flash kinetic experiments represent a set of data describing the charge translocation during the photocycle of bacteriorhodopsin. Together with the activation energies of the different components, relaxation currents after a sudden increase of temperature can be simulated. It is important to note that the results obtained by the simulation depend on the choice of the model for the photocycle. Therefore by comparing the simulated with the measured T-jump relaxation currents, conclusions about the appropriate model for the BR photocycle can be drawn.

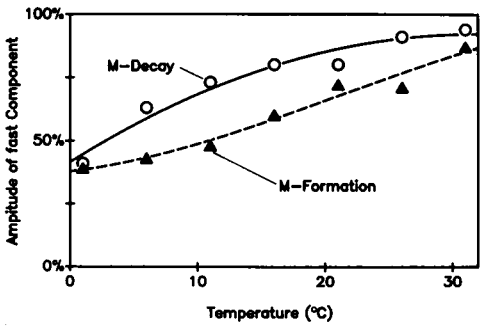

FIGURE 6 Temperature dependence of absorption amplitudes. The curves are the amplitudes of the fast M formation and the fast M decay, measured at 419 nm and divided by the total absorption amplitude.

In the simulation only the rate constants were taken to be temperature dependent and the charge displacement coefficients were kept constant. This is justified by calorimetric studies which revealed no phase transition in the considered temperature range (Jackson and Sturtevant, 1978; Tsuda et al., 1983; Marque and Eisenstein, 1984; Tristram-Nagle et al., 1986). Therefore large structural changes due to an increase in temperature which may affect the displacement coefficients can be excluded. The absence of a phase transition is confirmed by the finding that kinetic data available could always be described in terms of a single activation energy for each relaxation time (Fahr et al., 1981; Rayfield, 1985; Xie et al., 1987; Maurer et al., 1987*b*).

Fig. 7 shows all tested models of the photocycle. The early steps of the photocycle were comprised in one transition. This simplification did not modify the results on a millisecond timescale because only a small part of the molecules occupied the fast decaying intermediates, even with high background light intensities. As related displacement coefficient the sum of all early charge transitions was used. The rate constant was dominated by the time BR needed to absorb a photon from the background light and enter the photocycle. It was estimated from

$$k_1 = J_\nu/h\nu \cdot \sigma \cdot \Delta\lambda \cdot \eta, \qquad (2)$$

where J_ν is the spectral irradiance at 570 nm, $h\nu$ is the energy of a photon, σ is the absorption cross section of BR at 570 nm, $\Delta\lambda$ the corresponding halfwidth, and η the quantum efficiency. σ is well known ($2.4 \cdot 10^{-16}$ cm^2), $\Delta\lambda$ was determined from the absorption spectrum to be 110 nm, and $J_\nu/h\nu$ was measured ($1.5 \cdot 10^{16}$ cm^{-2} s^{-1} nm^{-1}). Different values for the quantum efficiency are suggested in the literature (Stoeckenius and Bogomolni, 1982; Oesterhelt et al., 1985), so in the simulations the first rate constant was varied between 120 and 240 s^{-1} for 100% background light, belonging to quantum efficiencies of 0.3 and 0.6, respectively. As σ and η depend only slightly

on temperature (e.g. Oesterhelt and Hess, 1973; Cladera et al., 1988), k_1 was kept constant in the simulation.

Test of models for the bacteriorhodopsin photocycle

In Fig. 7 all tested models of the photocycle are shown. Model A was a cycle of consecutive intermediates (Lozier et al., 1975). In this case the rate constants could directly be determined from the kinetic measurements. As the number of rate constants and relaxation times was the same, no additional parameter had to be used. With this model the behavior at 15°C could be described but at 9 and 5°C using high light intensities the model failed; an initial peak current was predicted for all conditions.

The absorption amplitude of the O intermediate increases with rising temperature (Gillbro, 1978; Maurer et al., 1987*b*), so models B (Parodi et al., 1984) and C (Li et al., 1984) were tested, where the O state played a crucial role. In both models one additional parameter was necessary; it was chosen to be $k_{O\to M}/k_{M\to O}$ and varied between 0 and 0.6. None of the models could reproduce the T-jump measurements. Again the largest discrepancies occurred at low temperature and high light intensities. The pH dependence of the experiments also suggested that the O intermediate did not contribute to the T-jump–induced current relaxation. The O intermediate can only be observed at low pH, but signals like those shown in Figs. 3 and 4 were observed at pH 4.1, 6.3, 7.2, and 8.5.

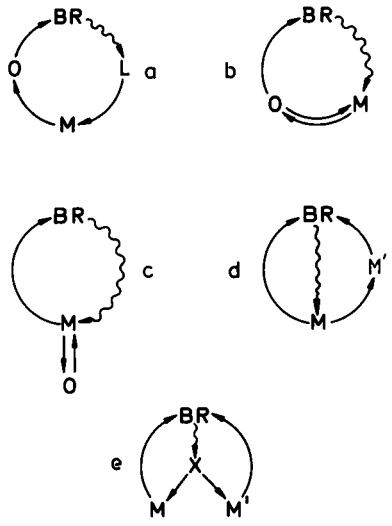

FIGURE 7 Models of the bacteriorhodopsin photocycle used to simulate the T-jump experiments. As the current was monitored the T-jump measurements do not distinguish intermediates with different absorption maxima. M, M', and O stand for intermediates which decay in few milliseconds, whereas L and X represent faster decaying states. So e.g., M' in model *d* could be identified with O.

The last two models represented branched photocycles. Splitting occurred at the M intermediate (model D, Sherman et al., 1976; Beach and Fager, 1985) or before the M intermediate, e.g., K or L (model E, Kalisky et al., 1981; Li et al., 1984). One additional parameter had to be introduced, which was chosen to be the branching ratio $k_{M\to Br}/k_{M\to M'}$, and $k_{X\to M}/k_{X\to M'}$, respectively; it was varied between 0.2 and 5. It was further assumed that the charge translocations in both branches are either equal ($\alpha_{M\to Br} = \alpha_{M\to M'} + \alpha_{M'\to Br}$ and $\alpha_{X\to M} + \alpha_{M\to Br} = \alpha_{X\to M'} + \alpha_{M'\to Br}$, respectively) or that one branch does not pump at all ($\alpha_{M\to Br} = 0$ and $\alpha_{X\to M'} + \alpha_{M'\to Br} = 0$, respectively).

Like the first three cases model D could not describe the T-jump signals at low temperature and high light intensities. However reasonable agreement between T-jump simulations and measurements was obtained using model E. Simulated curves are shown in Figs. 3 and 4 together with the measured currents. There it was assumed that both branches are involved in proton pumping. The assumption, that only one branch is active gave also a satisfying agreement. So based on these T-jump experiments it could not be decided if both branches are active or not.

To get a good agreement between measurements and simulations it was essential that the splitting ratio $k_{X\to M}/k_{X\to M'}$ increased with temperature. Otherwise the T-jump signals at low temperature and high light intensities could not be simulated. If both branches are active, more molecules are directed to the fast decaying M state. Then the photocycle is not only accelerated by faster decaying intermediates, but also because more molecules pass through the faster decaying branch. In the second case, with only one active branch, more molecules are directed from an inactive branch to a pumping branch.

The method of comparing simulations to deduce the appropriate kinetic model has to be applied with caution. The reason for this is the impossibility to test all imaginable models. In this case a model has to be accepted which is actually wrong, but yields the least discrepancies between simulation and experiment. This principal problem cannot be eliminated. It was tried to include all proposed basic models of the BR photocycle. Also the agreement between experimental data and simulations using model E is remarkable in view of the fact that only two free parameters (scaling factor and splitting ratio), which had the same value for all simulations shown in Figs. 3 and 4, were involved.

A second possible error is that a model is rejected, which can in fact explain the T-jump experiments. This can be avoided by screening the parameter space in

sufficiently narrow intervals, a very time-consuming procedure if many parameters are required. Because the simulations used here are based on only one (model A) or two parameters (other models), it is very unlikely that a successful combination of parameters for a rejected model was overlooked. In addition the parameters were varied in the largest possible range that was kinetically reasonable. Also a variation of the fixed parameters α_j and k_j, exceeding the range of 10 and 15% as given in Materials and Methods, was tried. The result was that only model E described the experiments adequately. The remaining differences between simulations using model E and experimental results could be diminished by adding further states like an O intermediate.

The bacteriorhodopsin photocycle

The success of model E to describe the T-jump experiments adequately was confirmed by absorption measurements. As pointed out before (Li et al., 1984) the ratio of the fast and slow decaying M intermediates increases with temperature. This was confirmed by our own measurements. In addition the same temperature dependence was observed for M formation, where the share of the fast component increased with rising temperature (Fig. 6).

Based on the T-jump experiments alone, it cannot be decided if splitting occurs between L and M or at an earlier stage because of the limited time resolution of the experiments. Flash photolysis measurements showed a biphasic M formation. This can be explained with the existence of two L intermediates (for a detailed discussion see Maurer et al., 1987*b*). So the simplest model of the BR photocycle that agrees with the kinetic measurements and with the T-jump experiments is a branched cycle, where branching occurs before the L state (Fig. 8 *a*). This model implies that the way M decays or how a proton is taken up is already determined in the L state.

Another model that agrees with the T-jump experiments and the time-resolved absorption measurements is shown in Fig. 8 *b*. After evaluating the displacement coefficient it turned out that the charge translocation between L and L′ was greater than the charge movement between L and M or M′. It even exceeded the total charge translocation of one cycle. Such a process should effect the absorption characteristics of the protein, leading to an absorption shift between L and L′. On the other hand L′ absorbs like L at 550 nm. Therefore model 8 *b* was rejected and model 8 *a* was regarded as the best model for the photocycle of BR.

Recently distinct forms of BR ground states leading to different photocycles have been proposed (Dancshazy et al., 1988; Diller and Stockburger, 1988). These models agree with the T-jump measurements, if the two forms

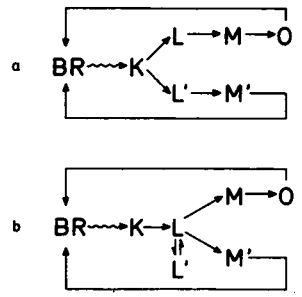

FIGURE 8 Two models of the photocycle which agree with absorption and T-jump measurements.

are in a thermal equilibrium and the form leading to a faster photocycle is favored at high temperature.

Kouyama et al. (1988) observed a photoactive intermediate N. This intermediate was located after M with an absorption maximum at 550 nm; it was only observable at pH values higher than 9. As our measurements were confined to a pH range from 4.1 to 8.5 the intermediate was too short-lived to initiate photoreactions by catching photons from the background light.

Conclusions

The charge translocation of BR was studied using the steady state T-jump technique. In contrast to nonequilibrium techniques like flash photolysis the T-jump experiments probe predominantly the behavior of the system in or near the steady state. Consequently information about the population of the main steady-state intermediates BR ground state and M can be obtained. In particular a branched model of the photocycle was confirmed, where branching occurs before the M state.

The steady-state T-jump technique can also be applied to biological systems without a chromophore because its time resolution is based on the rapid temperature change due to the infrared absorption of the solute. This offers promising possibilities for the investigation of ion pumps driven by chemical energy like ATPases.

APPENDIX

Evaluation of rate constants and charge displacement coefficients

The time constants and current amplitudes of the flash photolysis experiments were used to determine the rate constants and displacement coefficients of a particular model of the BR photocycle. In the first step the rate constants were evaluated. Therefore the coefficient matrix was calculated from the appropriate set of rate equations. After the laser flash a certain amount of BR molecules are transferred into the first

intermediate. In the case of model E for example the decay of X to BR via the intermediates M and M' is described by the rate equations:

$$\begin{aligned} dc_1/dt &= -(k_1 + k_2)c_1 \\ dc_2/dt &= +k_1c_1 - k_3c_2 \\ dc_3/dt &= +k_2c_1 - k_4c_3. \end{aligned} \quad \text{(A1)}$$

c_1, c_2, and c_3 are the time-dependent relative concentrations of intermediates X, M, and M', respectively. Solving the characteristic equation of this set of rate equations,

$$\begin{vmatrix} -k_1 - k_2 - \lambda & 0 & 0 \\ k_1 & -k_3 - \lambda & 0 \\ k_2 & 0 & -k_4 - \lambda \end{vmatrix} = 0 \quad \text{(A2)}$$

yields the eigenvalues $\lambda_{1,2,3}$, which are the negative inverse of the time constants. In addition the eigenvectors (e_{1j}, e_{2j}, e_{3j}) of the corresponding matrix were calculated. For model E three eigenvalues,

$$\lambda_1 = -k_1 - k_2, \lambda_2 = -k_3, \text{ and } \lambda_3 = -k_4,$$

and eigenvectors were evaluated. In some models of the photocycle, as in model E, the number of reactions exceeded the number of intermediates. Then one free parameter had to be introduced. This parameter was not totally undetermined because usually the absorption amplitudes provided additional information. If for instance a backreaction from O to M was considered, the absorption amplitudes at 419 nm and 649 nm provided information on the ratio of forth- and backreaction. In all cases the parameter was changed within the limits given by this additional information. For model E $p = k_1/k_2$ was defined as parameter, so that $k_2 = -\lambda_1/(p + 1)$.

Then the relative displacement coefficients α between two subsequent states were evaluated. These coefficients are defined by $\alpha = q/e_o \cdot d/D$, where d is the distance a charge q moves, e_o is the unit charge, and D the membrane thickness (Läuger et al., 1981). The measured current amplitudes are functions of the displacement coefficients and the rate constants. Using Eq. 1 in the case of model E, the following equation was obtained:

$$I_P(t) = e_0 \cdot [(\alpha_1k_1 + \alpha_2k_2)\, c_1(t) + \alpha_3k_3c_2(t) + \alpha_4k_4c_3(t)]. \quad (1')$$

The concentrations $c_i(t)$ can be described by sums of the three exponentials,

$$c_i(t) = \sum_j b_je_{ij} \exp(\lambda_jt), \quad \text{(A3)}$$

where b_j are constants only depending on the initial concentrations. As the initial concentrations after the laser flash are $c_1(0) = 1$, $c_2(0) = 0$, and $c_3(0) = 0$ the factors b_j could be determined, which was done numerically. With the last equation the pump current can be described by

$$I_P(t) = \sum_j I_j \exp(\lambda_jt). \quad \text{(A4)}$$

I_j are the measured current amplitudes belonging to the time constants $-1/\lambda_j$. Using the last three equations the charge displacement coefficients can be calculated from b_j, λ_j, and I_j. For model E the following equations were obtained:

$$I_j = (\alpha_1k_1 + \alpha_2k_2)b_je_{1j} + \alpha_3\, k_3b_je_{2j} + (\alpha_1 + \alpha_3 - \alpha_2)k_4b_je_{3j}, \quad \text{(A5)}$$

assuming that in both branches the same charge is transported and so $\alpha_1 + \alpha_3 = \alpha_2 + \alpha_4$ is valid.

Using this set of equations it was possible to calculate numerically the relative displacement coefficients from the rate constants and measured current amplitudes.

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REFERENCES

Bamberg, E., H. J. Apell, N. A. Dencher, W. Sperling, H. Stieve, and P. Läuger. 1979. Photocurrents generated by bacteriorhodopsin on planar bilayer membranes. *Biophys. Struct. Mech.* 5:277–292.

Bamberg, E., P. Hegemann, and D. Oesterhelt. 1984. The chromoprotein of halorhodopsin is the light-driven electrogenic chloride pump in *Halobacterium halobium*. *Biochemistry*. 23:6216–6221.

Beach, J. M., and R. S. Fager. 1985. Evidence for branching in the photocycle of bacteriorhodopsin and concentration changes of late intermediate forms. *Photochem. Photobiol.* 41:557–562.

Brock, W., G. Stark, and P. C. Jordan. 1981. A laser-temperature-jump method for the study of the rate of transfer of hydrophobic ions and carriers across the interface of thin lipid membranes. *Biophys. Chem.* 13:329–348.

Chernavskii, D. S., I. V. Chizhov, R. H. Lozier, T. M. Murina, A. M. Prokhorov, and B. V. Zubov. 1989. Kinetic model of bacteriorhodopsin photocycle: pathway from M state to BR. *Photochem. Photobiol.* In press.

Cladera, J., L. Galisteo, M. Dunach, P. L. Mateo, and E. Padros. 1988. Thermal denaturation of deionized and native purple membranes. *Biochim. Biophys. Acta.* 943:148–156.

Dancshazy, Z., R. Govindjee, and T. G. Ebrey. 1988. Independent photocycles of spectrally distinct forms of bacteriorhodopsin. *Proc. Natl. Acad. Sci. USA.* 85:6358–6361.

Dér, A., P. Hargittai, and J. Simon. 1985. Time-resolved photoelectric and absorption signals from oriented purple membranes immobilized in gel. *J. Biochem. Biophys. Meth.* 10:295–300.

Diller, R., and M. Stockburger. 1988. Kinetic resonance Raman studies reveal different conformational states of bacteriorhodopsin. *Biochemistry*. 27:7641–7651.

Drachev, L. A., A. D. Kaulen, S. A. Ostroumov, and V. P. Skulachev. 1974. Electrogenesis by bacteriorhodopsin incorporated in a planar phospholipid membrane. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 39:43–45.

Eisenbach, M., C. Weissmann, G. Tanny, and S. R. Caplan. 1977. Bacteriorhodopsin-loaded charged synthetic membranes. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 81:77–80.

Fahr, A., P. Läuger, and E. Bamberg. 1981. Photocurrent kinetics of purple-membrane sheets bound to planar bilayer membranes. *J. Membr. Biol.* 60:51–62.

Gillbro, T. 1978. Flash kinetic study of the last steps in the photoinduced reaction cycle of bacteriorhodopsin. *Biochim. Biophys. Acta.* 504:175–186.

Herrmann, T. R., and G. W. Rayfield. 1976. A measurement of the

proton pump current generated by bacteriorhodopsin in black lipid membranes. *Biochim. Biophys. Acta.* 443:623–628.

Holz, M., M. Lindau, and M. P. Heyn. 1988. Distributed kinetics of the charge movements in bacteriorhodopsin: evidence for conformational substates. *Biophys. J.* 53:623–633.

Jackson, M. B., and J. M. Sturtevant. 1978. Phase transitions of the purple membranes of *Halobacterium halobium*. *Biochemistry.* 17:911–914.

Kalisky, O., M. Ottolenghi, B. Honig, and R. Korenstein. 1981. Environmental effects on formation and photoreaction of M412 photoproduct of bacteriorhodopsin: implications for the mechanism of proton pumping. *Biochemistry.* 20:649–655.

Keszthelyi, L., and P. Ormos. 1980. Electric signals associated with the photocycle of bacteriorhodopsin. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 109:189–193.

Keszthelyi, L., and P. Ormos. 1983. Displacement current on purple membrane fragments oriented in a suspension. *Biophys. Chem.* 18:397–405.

Knoll, W., and G. Stark. 1977. Temperature-jump experiments on thin lipid membranes in the presence of valinomycin. *J. Membr. Biol.* 37:13–28.

Kouyama, T., A. Nasuda-Kouyama, A. Ikegami, M. K. Mathew, and W. Stoeckenius. 1988. Bacteriorhodopsin photoreaction: identification of a long-lived intermediate N (P,R350) at high pH and its M-like photoproduct. *Biochemistry.* 27:5855–5863.

Läuger, P., R. Benz, G. Stark, E. Bamberg, P. C. Jordan, A. Fahr, and W. Brock. 1981. Relaxation studies of ion transport systems in lipid bilayer membranes. *Q. Rev. Biophys.* 14:513–598.

Li, Q. Q., R. Govindjee, and T. G. Ebrey. 1984. A correlation between proton pumping and the bacteriorhodopsin photocycle. *Proc. Natl. Acad. Sci. USA.* 81:7079–7082.

Lozier, R. H., R. A. Bogomolni, and W. Stoeckenius. 1975. Bacteriorhodopsin: a light-driven proton pump in *Halobacterium halobium*. *Biophys. J.* 15:955–962.

Marque, J., and L. Eisenstein. 1984. Pressure effects on the photocycle of purple membrane. *Biochemistry.* 23:5556–5563.

Maurer, R., J. Vogel, and S. Schneider. 1987*a*. Analysis of flash photolysis data by a global fit with multiexponentials. I. Determination of the minimal number of intermediates in the photocycle of bacteriorhodopsin by the 'stability criterion'. *Photochem. Photobiol.* 46:247–253.

Maurer, R., J. Vogel, and S. Schneider. 1987*b*. Analysis of flash photolysis data by a global fit with multi-exponentials. II. Determination of consistent natural rate constants and the absorption spectra of the transient species in the bacteriorhodopsin photocycle. *Photochem. Photobiol.* 46:255–262.

Müller, K. H., H. J. Butt, M. Engelhard, B. Hess, and E. Bamberg. 1988. Simultaneous photoelectric and optical measurements of the bacteriorhodopsin photocycle. *In* Molecular Physiology of Retinal Proteins. T. Hara, editor. Yamada Science Foundation, Osaka. 341–342.

Oesterhelt, D., and B. Hess. 1973. Reversible photolysis of the purple complex in the membrane of *Halobacterium halobium*. *Eur J. Biochem.* 37:316–325.

Oesterhelt, D., and W. Stoeckenius. 1974. Isolation of the cell membrane of *Halobacterium halobium* and its fractionation into red and purple membrane. *Methods Enzymol.* 667–678.

Oesterhelt, D., P. Hegemann, and J. Tittor. 1985. The photocycle of the chloride pump halorhodopsin. II. Quantum yields and a kinetic model. *EMBO (Eur. Mol. Biochem. Organ.) J. 4:2351–2356.*

Parodi, L. A., R. H. Lozier, S. M. Bhattacharjee, and J. F. Nagle. 1984. Testing kinetic models for the bacteriorhodopsin photocycle. II. Inclusion of an 0 to *M* backreaction. *Photochem. Photobiol.* 40:501–512.

Rabon, E., R. D. Gunther, A. Soumarmon, S. Bassilian, M. Lewin, and G. Sachs. 1985. Solubilization and reconstitution of the gastric H,K-ATPase. *J. Biol. Chem.* 260:10200–10207.

Rayfield, G. W. 1985. Temperature dependence of photovoltages generated by bacteriorhodopsin. *Biophys. J.* 48:111–115.

Sherman, W. V., R. Korenstein, and S. R. Caplan. 1976. Energetics and chronology of phototransients in the light response of the purple membrane of *Halobacterium halobium*. *Biochim. Biophys. Acta.* 430:454–458.

Sinton, M. H., and T. G. Dewey. 1988. Phase-lifetime spectroscopy of photocycle processes. *Biophys. J.* 53:153–162.

Stoeckenius, W., and R. A. Bogomolni. 1982. Bacteriorhodopsin and related pigments of *Halobacteria*. *Annu. Rev. Biochem.* 52:587–616.

Trissl, H. W., and M. Montal. 1977. Electrical demonstration of rapid light-induced conformational changes in bacteriorhodopsin. *Nature (Lond.).* 266:655–657.

Tristram-Nagle, S., C. P. Yang, and J. F. Nagle. 1986. Thermodynamic studies of purple membrane. *Biochim. Biophys. Acta.* 854:58–66.

Tsuda, M., R. Govindjee, and T. G. Ebrey. 1983. Effects of pressure and temperature on the M412 Intermediate of bacteriorhodopsin photocycle. *Biophys. J.* 44:249–254.

Xie, A. H., J. F. Nagle, and R. H. Lozier. 1987. Flash spectroscopy of purple membrane. *Biophys. J.* 51:627–635.

Zubov, B. V., T. M. Murina, A. M. Prokhorov, N. A. Sulimov, N. M. Chernavskaya, D. S. Chernavsky, and I. V. Chizhov. 1983. Investigation of bacteriorhodopsin intermediate relaxation by means of temperature pulse. *Biochim. Biophys. Acta.* 725:162–167.