The photochemical cycle of bacteriorhodopsin has no refractory period

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Double-pulse experiments were used to probe the existence of a refractory period in the photochemical cycle of bacteriorhodopsin. We demonstrate using three different approaches that no refractory period exists in purple membranes, cell vesicles or intact cells. First, we showed by applying different time delays between two flashes that every molecule which regained the initial optical state could immediately recycle again. Second, we found no difference in the effectiveness of the first and second flash when the intensity was varied. Third, different steady-state levels of the intermediates were produced with different intensities of continuous background illumination. No difference in effectivity of the first and second flash could be detected.

Bacteriorhodopsin *Flash absorption spectroscopy* *Photocycle* *Catalytic cycle* *Double-pulse excitation*

1. INTRODUCTION

Proton translocation through bacteriorhodopsin (BR) is initiated by the absorption of a photon by its chromophore (review [1]). Successive thermal reaction steps bring the molecule back to its original state, thereby transporting one (or two) protons across the membrane. This catalytic cycle has been analyzed by several biophysical methods, either detecting changes in the chromophore structure or changes in the protein part. Optical absorption spectroscopy in the time range from femto- to milliseconds revealed color changes of the chromophore ([2,3], for review see [4]) and resonance Raman spectroscopy allowed interpretation of these color changes as configurational and conformational changes in the retinal moiety [5–7]. Intermediates drastically different by their absorption maxima were arranged according to their rise and decay times into a cyclic sequence which was called the photochemical cycle, with the slowest reaction having a half time of about 10 ms at room temperature [3].

Infrared spectroscopy [8], photoacoustic calorimetry [9,10] and electric measurements [11,12] allowed one to derive rate constants for changes not seen by optical spectroscopy, indicating that the catalytic cycle is composed of more reaction steps than analyzed optically. Moreover, the cycle is slowed down by the protonmotive force created by BR catalysis [13] and under these conditions a branching of the cycle into a proton pumping and a non-proton pumping pathway becomes measureable [14].

For a detailed understanding of the catalytic cycle and its branches it is of interest to determine if a refractory period exists. This question can be addressed at two levels: (i) is there a lag phase between the two photocycles; (ii) after what time can proton translocation (catalytic cycle) be triggered again? These two questions must be separated conceptually because it cannot be excluded that a molecule which has regained its initial spectroscopic properties has not yet finished the proton translocation cycle.

Here we report on experimental results which answer the first question. By double-flash excitation of BR we demonstrate that no lag phase in the photocycle of BR exists.

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2. MATERIALS AND METHODS

Purple membrane (PM) was isolated from *Halobacterium halobium* strain JW-3 according to standard procedures [15]. Cell envelope vesicles were prepared as described elsewhere [16].

Flash-photometric measurements were carried out with a homemade set up. A monitoring beam from a 250 W tungsten lamp was passed through a 425 nm interference filter and split into two fractions. The main fraction was focused onto a 1-mm optical pathlength cell containing the sample (50 μM bacteriorhodopsin) and was detected by a photodiode (KLC P1110, USA) connected to a preamplifier (0.1 ms rise time). The reference beam was focused directly onto another diode (UDT, PIN-5 UV, USA) combined with a preamplifier. The amplifier signals from the two photodiodes were fed into a differential amplifier (Keithley 604, USA). By this method the stability of the monitoring beam could be further increased. The amplified signals were averaged (usually 100 curves, repetition rate 0.5 Hz) and stored by a CAMAC data acquisition system (KFKI, Hungary) [17]. The cuvette holder was kept by a cooling water bath at a constant temperature of 20 $\pm$ 0.2°C or at 5 $\pm$ 0.5°C as indicated.

The double-light pulses were obtained either from the same or from different light sources. The delays between the two flashes were adjusted by a programmable pulse generator (homemade).

In experiments where the delay time between the two flashes was varied (fig.1) the following light sources were used:

(i) The first flash came from a homemade Nd-YAG laser (with a frequency doubling crystal, wavelength: 530 nm, duration 20 ns; energy: ~100 mJ). The laser spot reached the sample under 45° and was defocused to excite an area of ~0.5 cm^2. It was necessary to guarantee the maximal overlap of the excited areas for the first and the second flashes.

(ii) The second flash was provided by a flash lamp pumped dye laser (Carl Zeiss, rhodamine 6G dye, wavelength: 590 nm, duration ~2 μs; energy: ~30 mJ). This flash was focused onto the centre of the first flash and reached the sample under 90° compared to the first flash.

In another series of experiments where flash intensities were varied only the dye laser was used as an actinic light source for both flashes. In this way we could guarantee excitation of precisely the same part of the sample by both flashes. The minimal interval between two flashes was 110 ms in our set up and a delay of 2 s was always set after the two flashes before repeating the flash sequence. At an interval of 110 ms the intensities of the two flashes were almost the same and were measured by a photodiode independently and simultaneously with the absorption change measurements. The amplitudes of the absorbance changes were plotted according to the actual intensities of the first or second flash.

The minimal delay time of 110 ms does not allow a large proportion of molecules to be in the M state when the second flash excites the population at room temperature. Therefore the samples were cooled to 5°C.

3. RESULTS AND DISCUSSION

The two most important conditions to be met to ensure the validity of the experiments described are the following: (i) the flash intensity of at least the first flash must have a saturating effect and (ii) the second flash must hit the same population of molecules as the first. The first condition was checked by measuring the absorbance change at 420 nm in relation to increasing flash intensity (not shown) and saturating intensities were ensured.

To fulfill the second condition the area of the second laser beam was adjusted colinear to the first laser beam which had a larger area by defocusing the laser beam. Alternatively, the same laser was used for the second flash thereby guaranteeing identical geometry.

In the experiment of fig.1, the first flash excited the molecules and at various time intervals a second flash of constant intensity produced an additional absorption change as measured at 420 nm. The amplitudes obtained by the second flash increased until they approached the control value shown by the sample which was not subjected to the first laser flash. The absolute absorbance change upon the second flash is shown in fig.2 together with the recovery of the absorbance change after the first flash as a function of the indicated delay time. Both sets of data points fit the same curve indicating that the number of

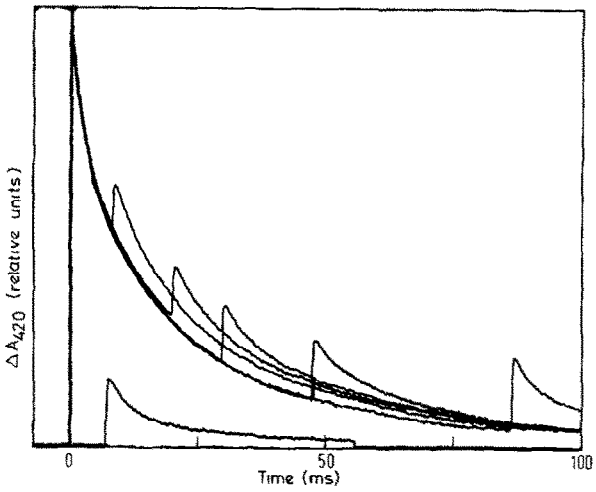

Fig.1. Double-laser pulse-induced absorbance changes of *H. halobium* cells. The first saturating flash was followed by a second flash with different delay times (repetition rate of the double pulses was 0.5 Hz). In the figure the curves of 5 independent double-pulse measurements are superimposed on each other to show the increase of the amplitude of the second flash induced ΔA with the increasing delay time. In the case of the bottom curve the 'first' flash was omitted and only the 'second' was applied. Cells were suspended in basal salt solution at pH 7.5 in 10 mM Hepes. The sample was thermostatted at 20°C.

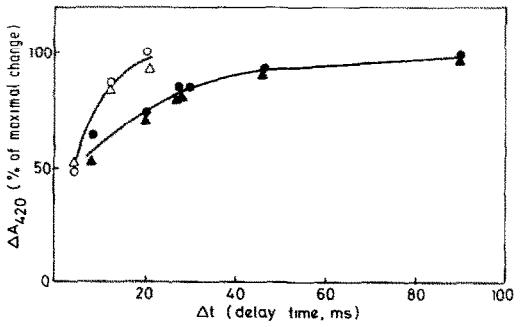

Fig.2. Correlation of the yield of intermediate M upon a second flash given at different delay times with the time course of recovery of BR. The data from the curves in fig.1 were used for the cells (closed symbols) and the same experiment was carried out with PM in polyacrylamide gels [18] soaked with the same solution as used for cells. Evaluation of the data is represented by open symbols. Recovery of BR (100 − % residual M) is estimated from the time course of the M decay measured at 420 nm. The initial absorbance change at 420 nm upon the first flash is taken as 100% M. The initial absorbance changes upon the second flash are expressed as percent of the control shown in fig.1 as the bottom trace of absorbance changes following excitation by the 'second' flash alone.

molecules which can be excited by the second flash is proportional to the number of molecules which regained the initial optical state after excitation by the first flash. In other words molecules can be reexcited as soon as they return back to the initial state. Fig.2 also shows the data obtained from the same experiment using purple membranes instead of intact cells. The analogous result is obtained with respect to the values of the amplitudes but not with respect to the decay times. The difference is explained by the fact that the generation of a proton-motive force in vesicles or in cells slows down the photocycle.

The second approach to answer our question is demonstrated with the experimental result shown in figs 3 and 4. Instead of two lasers as in the previous experiment only one laser was used to avoid possible mismatch of areas of excitation. The delay time between the two laser shots could be minimized to 110 ms; from then on intensity of the second flash started to drop compared to the first flash. Fig.3A,B shows the original traces of double-flash experiments at 110 ms delay time (fig.3A) and 250 ms delay time (fig.3B). Note that the experiments were carried out at 5°C. At a delay time of 110 ms about 10% of the BR molecules were still in the cycle when the second flash hit the population while at 250 ms less than 1% of the molecules were still cycling. The intensities of both flashes were increased in parallel and the initial amplitudes of the absorption changes at 420 nm are shown. These values are replotted in fig.4 and the resulting curve which connects all the data points demonstrates unequivocally the fact that molecules immediately after returning from the cycle can be reexcited with the same efficiency in these vesicles. Again they have kinetics slower than those of purple membranes due to the fact that they produce a proton-motive force in the light which slows down the photocycle. The control experiment with purple membrane again gave qualitatively the same result.

The third and final approach to demonstrate the absence of a lag phase in the photocycle was to use vesicles in the double-flash experiments which were

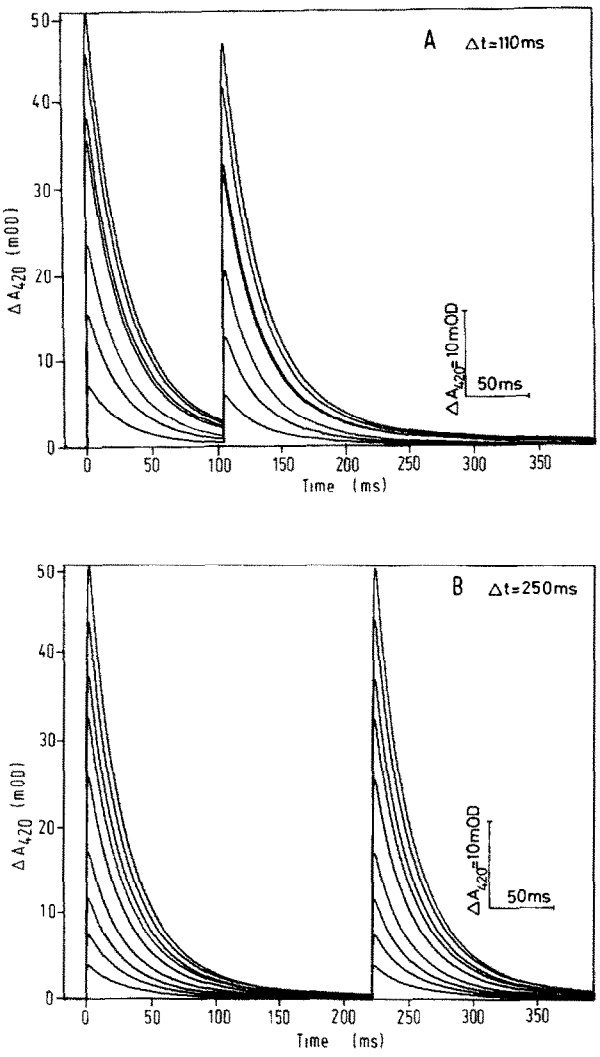

Fig.3. Dependence of the yield of M intermediate in double-flash experiments on the flash intensity in cell envelope vesicles of *H. halobium*. The time interval between the 2 flashes (Δt) was 110 ms (A) and 250 ms (B); the temperature was 5°C. The intensities for both flashes were simultaneously changed by neutral density filters. The maximal relative intensities in A and B are slightly different.

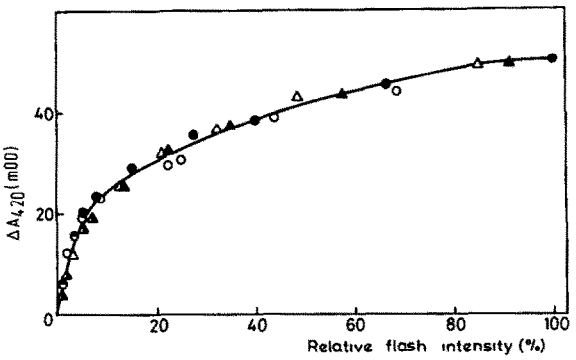

Fig.4. Initial amplitudes of ΔA_{420} traces from the experiment illustrated in fig.3 plotted against the flash intensities. Intensities were measured for each flash and are given as percent of maximal intensity which was about 10 mJ at an area of illumination of 4 mm^2: first flash, Δt 110 ms; second flash, Δt 110 ms; first flash, Δt 250 ms; second flash, Δt 250 ms.

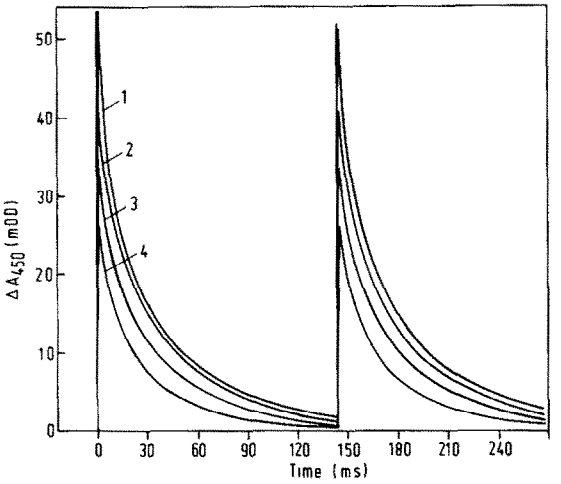

Fig.5. Influence of continuous green background illumination on the yields of the M intermediate in double-flash experiments. The experiment was carried out as in fig.3 at constant flash intensities (maximal) and background illumination varied in intensity: 1, 0; 2, 10 mW/cm^2; 3, 20 mW/cm^2; 4, 50 mW/cm^2; background light was obtained from an argon gas laser (Zeiss, GDR).

kept in background green light of increasing intensity to produce three fixed steady-state levels of the M intermediate. Fig.5 shows that regardless of the intensity of the background light the efficiency of the first and the second flash is identical indicated by the identical amplitudes of the absorbance change as measured at 450 nm.

In summary we have shown that bacteriorhodopsin in the isolated purple membrane, in cell envelope vesicles and in intact cells can be excited by light immediately after the return to the initial optical state. This finding must be considered together with the fact that the cycle itself is influenced in its kinetics by a proton-motive force

created by the pump. This well established fact proves the tight coupling between proton translocation and the photochemical cycle. Nevertheless the possibility cannot be excluded that the photocycle can be restarted even if the catalytic cycle does not regain its initial state. In this case the photocycle need not necessarily translocate a proton, leading to a partial decoupling of the photocycle and the catalytic cycle. The effect would be a decreasing stoichiometry of translocated protons compared to the fraction of cycling BR. Experiments to clarify this question are now underway.

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