Eur Biophys J (1990) 19:31–39

European Biophysics Journal

Quantum efficiency of native and mutant bacteriorhodopsin obtained from blue light induced relaxation experiments

H.-J. Butt *

Max-Planck-Institut für Biophysik, Kennedy-Allee 70, D-6000 Frankfurt, Federal Republic of Germany

Received March 12, 1990/Accepted in revised from May 24, 1990

Abstract. Bacteriorhodopsin was continuously excited with green background light. In this way a steady state distribution of all intermediates of the photocycle was obtained. Then a perturbation of the system was induced by a blue laser flash and the resulting absorption changes were measured. The experiments were done with native bacteriorhodopsin and with the point mutant $BR_{Asp96\rightarrow Asn}$, in which aspartate 96 is changed to asparagine. Blue light induced relaxation experiments revealed a rate constant belonging to the excitation of bacteriorhodopsin by the green background. With this rate constant the quantum efficiency of native bacteriorhodopsin and of $BR_{Asp96\rightarrow Asn}$ was determined to be 0.60 ± 0.10. Signals obtained with native bacteriorhodopsin could be explained with a simple model of the photocycle consisting of three consecutive intermediates BR_{568}, L_{550} and M_{412}. To describe the behavior of $BR_{Asp96\rightarrow Asn}$, a further photoactive intermediate after the M_{412} state had to be postulated. Properties of this intermediate are similar to those of the N_{550} state.

Key words: Bacteriorhodopsin – Blue light effect – Photocycle – Purple membrane – Quantum efficiency

Introduction

Bacteriorhodopsin is the only protein found in the purple membrane, a specialized region of the plasma membrane of Halobacterium halobium (Stoeckenius and Bogomolni 1982). Illumination of bacteriorhodopsin with green light initiates a photocycle that consists of at least four distinct spectroscopic intermediates – K_{590}, L_{550}, M_{412}, and O_{640} – before the system returns to its original state BR_{568}. These intermediates are characterized by the wavelength of maximal absorption (indicated as a subscript) and by their decay time constants. A simple scheme to describe the photocycle has been proposed by Lozier et al. (1975):

$$BR_{568} \rightsquigarrow K_{590} \rightarrow L_{550} \rightarrow M_{412} \rightarrow O_{640} \rightarrow BR_{568}$$

During the photocycle protons are pumped out of the bacterial cell. Information about the photocycle of bacteriorhodopsin comes mainly from flash photolysis experiments in which a green laser flash excites bacteriorhodopsin and subsequent absorption changes, charge transfers or other properties are measures (Stoeckenius and Bogomolni 1982).

An important parameter of the photocycle is the quantum efficiency. It is of fundamental interest because it determines the efficiency of the pumping process and it provides information about molecular behavior during the photocycle. The quantum efficiency has been measured by many groups (for review see Birge et al. (1989)) but no agreement about its value has been obtained. In contrast to the well known quantum efficiency of rhodopsin (Dartnall 1968), where the effect of light can be measured unambiguously by observing the disintegration of rhodopsin into retinal and opsin, a major problem exists for bacteriorhodopsin. Here light causes the protein to enter its photocycle and the occurrence of a certain intermediate could be used as a measure for the light effect. Unfortunately the absorption spectra of some intermediates overlap with the spectrum of the ground state.

Oesterhelt and Hess (1973) overcame this problem by using conditions where the M_{412} intermediate has a lifetime of about 15 s. The absorption spectrum of the M_{412} state is so far blue shifted that it does not overlap with other intermediates. Oesterhelt and Hess elongated the M_{412} decay time constant by suspending purple membrane in high salt and saturated ether concentration. Then they measured the accumulation of M_{412} as a function of the number of absorbed photons. They obtained a quantum efficiency of 0.79 and confirmed it later (Oesterhelt et al. 1985). Becher and Ebrey (1977) slowed the M_{412} lifetime down by decreasing the temperature to $-40\,^{\circ}C$ and suspending purple membranes in 25% NaCl

* *Present address:* Department of Physics, University of California, Santa Barbara, CA 93106, USA

at pH 10. They found a value of 0.3 for the quantum efficiency.

Goldschmidt et al. (1977) chose a different approach. They studied bacteriorhodopsin under normal conditions, where the decay time constants of all intermediates were not changed. The purple membranes were excited by a laser pulse at 530 nm with 30 ns duration. They monitored the formation of M_{412} by measuring the absorption decrease at 530 nm. A quantum efficiency of 0.25 was reported. Hurley and Ebrey (1978) found a comparable value. At $-196\,^{\circ}C$ they measured the accumulation of K_{590} and found a quantum efficiency of 0.33. This value was confirmed by the calorimetric studies of Birge and Cooper (1983) who reported a quantum efficiency of 0.28–0.33.

A value around 0.3 for the quantum efficiency of bacteriorhodopsin was accepted for several years (Stoeckenius and Bogomolni 1982). Only recently was this value questioned. Grossjean and Tavan (1988) favored, for theoretical reasons, a quantum efficiency of 0.6. In resonance Raman scattering experiments Schneider et al. (1989) used the ethylenic band at 1529 cm^{-1}, which is indicative of the ground state, to determine the number of molecules entering the photocycle after a laser flash. They found a quantum efficiency of 0.67.

Based on the observation that all measurements of the quantum efficiency are either 0.33 or below or 0.6 and above Birge et al. (1989) suggested a model of the bacteriorhodopsin photocycle with two ground states. These states are supposed to have quantum efficiencies of 0.33 and greater than or equal to 0.6.

One intermediate of the photocycle, the so called M_{412} state, is relatively long-lived (decay time 10 ms). If M_{412} is illuminated by blue light, it is photoconverted back to its initial state BR_{568} (Oesterhelt and Hess 1973). This process is not coupled to a proton transport. Thus, blue light does not enhance the pump cycle but short circuits it (Karvaly and Dancsházy 1977; Ormos et al. 1978; Dancsházy et al. 1978; Hwang et al. 1978; Ormos et al. 1980; Ohno et al. 1983). Absorption measurements suggested that blue light photoconverts M_{412} to another intermediate M' with a similar absorption spectrum. M' then decays with a time constant of 150 ns back to BR_{568} (Hess and Kuschmitz 1977; Hurley et al. 1978; Kalisky et al. 1981).

I used the blue light effect to trigger a relaxation experiment which gave information complementary to flash photolysis experiments. Bacteriorhodopsin was continuously excited with green background light. In this way a stationary distribution of all intermediates was obtained. Then a blue laser pulse transferred a fraction of the molecules in the M_{412} state back to the initial state BR_{568}. This resulted in a concentration jump of the intermediates. During the relaxation back to the steady state, absorption changes were monitored. In addition to experiments with native bacteriorhodopsin, measurements with a mutant were performed. In this mutant, $BR_{Asp96\rightarrow Asn}$, aspartate 96 is changed to asparagine. $BR_{Asp96\rightarrow Asn}$ has an interesting feature related to the blue light effect measurements: With its slow, monophasic M_{412} decay (Marinetti et al. 1989; Holz et al. 1989; Butt et al. 1989a) almost the whole population of $BR_{Asp96\rightarrow Asn}$ molecules can be driven into the M_{412} state, even at low background light intensities.

A simple model of the photocycle was sufficient to describe the experiments with native bacteriorhodopsin. To explain results obtained with $BR_{Asp96\rightarrow Asn}$ a photoactive intermediate N_{550} after M_{412} had to be introduced.

Blue light induced relaxation experiments allowed measurement of the rate of photoconversion of the ground state BR_{568}. This rate is only determined by the absorption cross section of the ground state, the light intensity of the green background light and the quantum efficiency. By determining the quantum efficiency from a rate constant rather than from absorption changes, I avoided the problem of overlapping absorption spectra of different intermediates. A value of 0.60 ± 0.10 was found for the quantum efficiency. Native bacteriorhodopsin and the mutant $BR_{Asp96\rightarrow Asn}$ had the same quantum yields.

Materials and methods

Experimental

Sample preparation. Native bacteriorhodopsin was isolated from Halobacterium halobium strain S9 (Oesterhelt and Stoeckenius 1974). Purple membranes of the mutant $BR_{Asp96\rightarrow Asn}$, where aspartate 96 is changed to asparagine, were prepared from Halobacterium sp. GRB as described in Soppa and Oesterhelt (1989). The purple membranes from wild-type and mutant cells were immobilized by polymerization of a solution containing acrylamide together with the membrane sheets according to Eisenbach et al. (1977). The gel prevented aggregation of purple membranes. In addition, it allowed use of exactly the same sample for many different measurements and under different conditions. Experiments with native bacteriorhodopsin were done at pH 7.6 to avoid complications due to the presence of the O_{640} intermediate. To slow down the photocycle the temperature was kept at 15 °C. Measurements with the mutant were done at pH 6.8. At higher pH the M_{412} decay becomes so slow that the rate of photoconversion by the green background light would exceed its thermal decay rate.

Blue light induced relaxation experiments. Gel pieces of $3 \cdot 10 \cdot 20$ mm were cut and bathed for three days in electrolyte solution. The gels were then put in a thermostatted cuvette with two windows of $2 \cdot 8$ mm for the probe light and windows for the background light and the laser pulse (Fig. 1). The relaxation was initiated by a dye laser pulse with a wavelength of 400 nm, 10 ns duration and an energy of 150 μJ per pulse. Absorption changes in the sample were monitored with light from a 250 W halogen lamp which passed a monochromator, the sample (10 mm), a cut-off filter (410 nm for measurements at 419 nm, 550 nm for measurements at 568 nm) and two interference filters, before its intensity was measured with a photomultiplier. Owing to a laser artifact at the beginning of the signal, the first 10 μs were not considered. Absorption signals were recorded in a transient recorder, transferred to a computer and there analyzed.

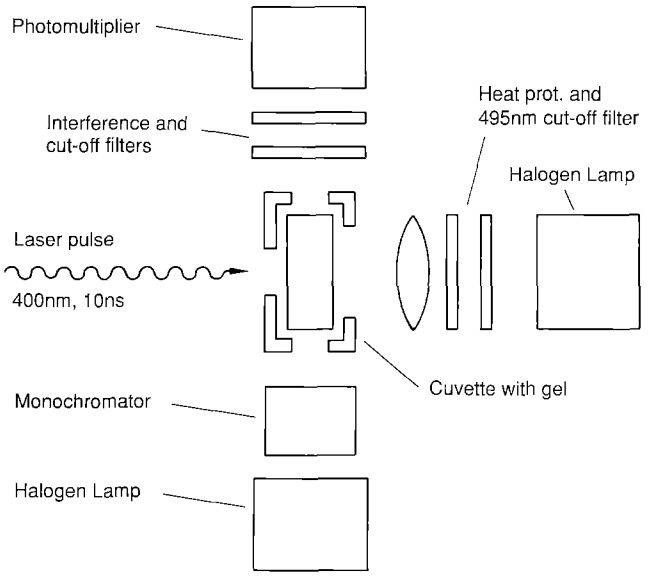

Fig. 1. Experimental set-up for the blue light induced absorption measurements

To obtain a steady state distribution of all bacteriorhodopsin intermediates a 250 W halogen lamp continuously excited the purple membranes. The light passed a heat protection filter, a 495 nm cut-off filter and was focused on the sample. In order to have a defined and homogeneous background light intensity over that part of the sample which was exposed to the laser flash, the following steps were taken. The area excited by the blue laser pulse was kept small (4 mm^2) compared to the area illuminated by the background light (40 mm^2). The focus of the background light was situated a few millimetres behind the sample to obtain a uniform illumination of the sample. The bacteriorhodopsin concentration in the gel was low (7 μM), so that the light intensity only slightly decreased with increasing penetration depth.

The intensity of the background light was measured with a calibrated photodiode (United Detectors 351A, California). Using narrow band interference filters of known absorption, the spectral irradiance at various wavelengths between 500 nm and 650 nm was determined. At 568 nm the spectral irradiance was 4.5 mW/cm^2/nm. The light intensity was varied by neutral density filters.

Flash photolysis experiments. In addition to measurements with green background light and a blue laser pulse, experiments without background light were done. In this case the sample was excited by a laser flash at 575 nm, 10 ns duration, and an energy of 300 μJ. The bacteriorhodopsin was light adapted. With these measurements the rate constants of the photocycle were obtained.

Kinetic analysis

Rate constant of photoconversion. Bacteriorhodopsin was excited by green background light and entered the photocycle with the rate of photoconversion of the ground state BR_{568}:

$$k_1 = \phi \int \frac{J(\lambda)}{h\nu}\,\sigma(\lambda)\,\mathrm{d}\lambda\,, \tag{1}$$

ϕ is the quantum efficiency. J represents the spectral irradiance, $h\nu$ is the energy of a photon with frequency ν. σ is the absorption cross section of BR_{568}. The absorption cross section was calculated from the extinction coefficient $\varepsilon_{BR}(568)$ of the ground state at 568 nm: $\sigma(568) = \varepsilon_{BR}(568)2303/N_A$ (ε in $M^{-1}\,cm^{-1}$, then σ is given in cm^2). N_A is Avogadro's constant. At 568 nm σ is $2.4 \cdot 10^{-16}$ cm^2 assuming an extinction coefficient of 62 700 $M^{-1}\,cm^{-1}$ (Rehorek and Heyn 1979). Knowing the absorption cross section at 568 nm, its dependence on wavelength was measured with a spectrophotometer. The integral goes over wavelengths between 500 nm and 650 nm; at other wavelengths the absorption of BR_{568} is negligible. As all factors in the integral are known or were measured the integration could be done numerically.

Simple model of the photocycle. To interpret blue light induced relaxation signals they were compared to results predicted by the basic, tentative model

$$BR_{568} \overset{k_1}{\rightsquigarrow} K_{590} \overset{k_{KL}}{\rightarrow} L_{550} \overset{k_2}{\rightarrow} M_{412} \overset{k_3}{\rightarrow} BR_{568}\,,$$

k_{KL}, k_2, and k_3 are the rate constants of the thermal K_{590}/L_{550} and L_{550}/M_{412} transitions and the M_{412} decay, respectively.

The K_{590} state was neglected in the further analysis for two reasons. First, the rate limiting step between BR_{568} and L_{550} is the rate of photoconversion. Second, no relaxation process correlated with the K_{590}/L_{550} transition could possibly be detected, as the time resolution was limited to 10 μs owing to the laser artifact. The remaining scheme for the photocycle is then:

$$BR_{568} \overset{k_1}{\rightsquigarrow} L_{550} \overset{k_2}{\rightarrow} M_{412} \overset{k_3}{\rightarrow} BR_{568}\,. \tag{A}$$

To calculate the measurable rate constants and concentration changes, first the appropriate set of rate equations had to be noted (Bernasconi 1976):

$$\begin{aligned}
\frac{\mathrm{d}c_{BR}}{\mathrm{d}t} &= -k_1\,c_{BR}(t) + k_3\,c_M(t)\,,\\
\frac{\mathrm{d}c_L}{\mathrm{d}t} &= k_1\,c_{BR}(t) - k_2\,c_L(t)\,,\\
\frac{\mathrm{d}c_M}{\mathrm{d}t} &= k_2\,c_L(t) - k_3\,c_M(t)\,.
\end{aligned} \tag{2}$$

c_{BR}, c_L, and c_M are the time-dependent concentrations of the intermediates BR_{568}, L_{550}, and M_{412}, respectively. The measurable rate constants were calculated from the intrinsic rate constants by solving the characteristic equation of this set of rate equations. In model A the measurable rate constants (K_1, K_2) are related to the intrinsic

rate constants (k_1, k_2, k_3) by:

$$K_{1/2} = \frac{k_1+k_2+k_3}{2} \mp \tfrac{1}{2}\sqrt{k_1^2+k_2^2+k_3^2-2k_1k_2-2k_1k_3-2k_2k_3}\,. \quad (3)$$

K_1 refers to the minus, K_2 to the plus sign.

Solving the set of rate equations gave the time-dependent concentrations of the intermediates BR_{568}, L_{550} and M_{412} after a concentration jump:

$$\begin{aligned} c_{BR}(t) &= \frac{k_2k_3C_0}{K_1K_2} + \frac{B(k_2-K_1)}{k_1}e^{-K_1t} + \frac{D(k_2-K_2)}{k_1}e^{-K_2t}, \\ c_L(t) &= \frac{k_1k_3C_0}{K_1K_2} + Be^{-K_1t} + De^{-K_2t}, \\ c_M(t) &= c_0 - c_{BR}(t) - c_L(t)\,. \end{aligned} \quad (4)$$

c_0 is the total concentration of bacteriorhodopsin. B and D are constants which depend on the initial concentrations:

$$\begin{aligned} B &= \frac{k_1c_{BR}(0) - k_1k_3c_0/K_1 - c_L(0)(k_2-K_2)}{K_2-K_1}, \\ D &= \frac{k_1c_{BR}(0) - k_1k_3c_0/K_2 - c_L(0)(k_2-K_1)}{K_1-K_2}\,. \end{aligned} \quad (5)$$

Knowing the concentrations of the intermediates, the absorption at a particular wavelength λ could be evaluated:

$$A(\lambda, t) = \varepsilon_{BR}(\lambda)\,c_{BR}(t) + \varepsilon_L(\lambda)\,c_L(t) + \varepsilon_M(\lambda)\,c_M(t)\,. \quad (6)$$

$\varepsilon_i(\lambda)$ are the extinction coefficients of intermediate i at wavelength λ. The extinction coefficients were taken from the literature (Lozier et al. 1975; Nagle et al. 1982; Maurer et al. 1987) and are given in Table 2.

To facilitate the interpretation, the measurable rate constants (3) could be approximated in the following way. The experiments were carried out under conditions where the inequality

$$k_2 \gg k_1, k_3\,, \quad (7)$$

was valid. Then all terms in the root of (3) not containing k_2 could be neglected:

$$K_{1/2} \approx \frac{k_1+k_2+k_3}{2} \mp \frac{1}{2}\sqrt{k_2^2-2k_1k_2-2k_2k_3}\,.$$

k_2^2 is the dominating term in the root. Expanding the root in a Taylor series around k_2^2 and neglecting all second order terms yielded:

$$K_{1/2} \approx \frac{k_1+k_2+k_3}{2} \mp \frac{1}{2}(k_2-k_1-k_3)$$

and

$$K_1 = k_1+k_3\,, \quad K_2 = k_2\,. \quad (8)$$

In order to test if model A described the experiments, the following procedure was carried out. First, the steady state concentrations of all three intermediates were calculated with (4) for $t \to \infty$. Second, the blue light effect was simulated by shifting a small fraction of the molecules present as M_{412} to the ground state BR_{568}. In addition, a small fraction of molecules initially in the ground state BR_{568} were assumed to be excited by the blue laser pulse, thus entering L_{550}. The blue light induced photoreactions were assumed to be faster than 10 µs (Lozier et al. 1975; Kalisky et al. 1981), the time resolution of the absorption measurements. Finally, the absorption relaxation back to the steady state was fitted with (6) using the concentrations of the intermediates after the blue light flash as starting values. The fit was done with three parameters: k_1, a scaling factor which was proportional to the total bacteriorhodopsin concentration, and the percentage of BR_{568} being excited by the blue light.

Test of other models of the photocycle. In addition to model A other models of the bacteriorhodopsin photocycle were tested. The predicted measurable rate constants were calculated in the following way. First the appropriate set of rate equations was noted. Then the characteristic equation was solved numerically and the dependence of the measurable rate constants on the light intensity was plotted. This dependence was compared to measured results.

Results

Native bacteriorhodopsin

When native bacteriorhodopsin was excited by a green laser flash without background light, the absorption at 419 nm first increased. This initial increase had to be fitted with two rate constants of 7100 s^{-1} and 2400 s^{-1} (Table 1) at 15 °C. Then the absorption decreased. The decrease had to be fitted with two rate constants of 31 s^{-1} and 83 s^{-1}. The opposite behavior was observed at 568 nm: A decrease with rate constants of 7100 s^{-1} and 2400 s^{-1} was followed by an absorption increase in the millisecond time range. These absorption changes reflect the formation and decay of M_{412}.

In Fig. 2 absorption signals obtained after a blue laser pulse with different background light intensities are

Table 1. Rate constants obtained by fitting flash photolysis (without background light) and blue light induced relaxation signals (50% and 100% background light intensity) with sums of exponentials. The rate constants are given in s^{-1}. 100% light intensity corresponds to spectral irradiance of 4.5 mW/cm²/nm at 568 nm. 30 m*M* KCl, 5 m*M* Hepes, pH 7.6 (native bacteriorhodopsin); 30 m*M* KCl, 20 m*M* *Tris*, pH 6.8 ($BR_{Asp96\to Asn}$); 15 °C

	Without background light	50% background light	100% background light
Native BR	2400/7100 s^{-1} 31/83 s^{-1}	4600 s^{-1} 150 s^{-1}	– 246 s^{-1}
$BR_{Asp96\to Asn}$	1400/5000 s^{-1} 0.6 s^{-1} –	4600 s^{-1} 85 s^{-1} 15 s^{-1}	– 165 s^{-1} 29 s^{-1}

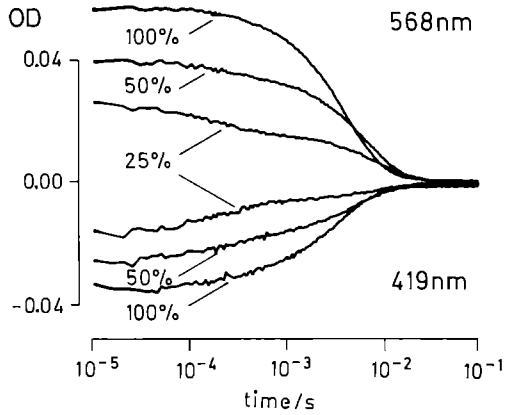

Fig. 2. Absorption signals of native bacteriorhodopsin after applying a blue laser pulse at various background light intensities. Absorption changes were measured at 568 nm and 419 nm. 100% light intensity corresponds to a spectral irradiance of 4.5 mW/cm^2/nm at 568 nm. 30 m*M* KCl, pH 7.6, 5 m*M* Hepes, 15 °C

shown. The blue light pulse transfers molecules from the M_{412} state back to the ground state BR_{568}. Consequently for $t = 10$ µs the absorption at 419 nm was lowered while at 568 nm an increased absorption was observed. From the absorptions at $t = 10$ µs it was estimated that the steady state concentration of the M_{412} state was 77%; 22% of the molecules were in the ground state BR_{568} and about 1% in the L_{550} state (100% background light intensity).

About 40 ± 20% of the molecules in the M_{412} state were photoconverted to BR_{568} by the blue laser flash. To estimate this rate of photoconversion, the absorption at $t = 10$ µs was measured at various laser light intensities. From the saturation behaviour the conversion rate was calculated (as in Tóth-Boconádi et al. (1988) or Butt et al. (1989a)).

During the relaxation process the steady state absorption was re-established. With decreasing light intensity the relaxation process slowed down and the amplitudes became smaller. The variation in amplitudes can be explained in the following way. With increasing light intensity more molecules were shifted into the M_{412} state. Then the blue laser flash transfered more molecules back to the ground state and absorption changes caused by blue light increased.

Fitting the blue light induced relaxation signals with a sum of exponentials revealed two rate constants (Table 1). One of 4600 s^{-1} coincided with the L_{550}/M_{412} transition. The other, slower rate increased linearly with the background light intensity, reaching a value of 246 s^{-1} at 100% light intensity (Fig. 4). The slope of this linear increase did not depend on the salt concentration or on pH: Measurements at pH 7.6 (5 m*M* Hepes) with salt concentrations ranging from 10 m*M* KCl to 300 m*M* KCl and experiments at pH 6.3 (5 m*M* Mes, 30 m*M* KCl) yielded the same slope.

In contrast to my flash photolysis experiments, only two rate constants and not four were observed in blue light induced relaxation measurements. It can not be decided if this was due to the lower signal to noise ratio or if there are actually only two relaxation processes.

Table 2. Extinction coefficients used for the fitting procedure and resulting rate constants for the rate of photoconversion k_1. Extinction coefficients were taken from the literature cited and are given in M^{-1} cm^{-1}. The rate constants were obtained by fitting blue light induced relaxation signals obtained at 100% background light intensity with curves predicted by the simple model A. Conditions as in Fig. 2

	$\varepsilon_L(420)$	$\varepsilon_L(568)$	$\varepsilon_M(420)$	$\varepsilon_M(568)$	k_1
Lozier et al. 1975	17 000	39 000	39 000	9 000	181 s^{-1}
Nagle et al. 1982	16 000	43 000	47 000	11 000	182 s^{-1}
Maurer et al. 1987	20 000	42 000	48 000	–	175 s^{-1}

In addition a slow component with a time constant of few seconds but low amplitude (about 3% of the maximal absorption change) was observed in the blue light induced relaxation experiments. This component could not be correlated with a transition in the photocycle. It was not considered in the further analysis. To avoid artifacts the following tests were done. An experiment with background light but without probe light was done, to check if background light scattered from the sample affected the measurements. Stray light changes were visible, but the amplitudes remained below 3% of the absorption amplitudes. A blue laser pulse without green background light resulted in a signal with only 9% of the amplitude of a signal caused by a green laser flash. Both signals had the same shape.

To find out if blue light induced relaxation measurements contained new information about the photocycle, I tried to fit measured absorption signals with curves predicted by model A (see Materials and methods). Certain parameters had to be set. The extinction coefficients $\varepsilon_i(\lambda)$ of intermediates i at wavelength λ were taken from the literature (Table 2). Thermal rate constants for the L_{550}/M_{412} (4300 s^{-1}) and the M_{412}/BR_{568} (70 s^{-1}) transition were derived from flash photolysis experiments, fitting measured signals with only one rate constant for M_{412} formation and one rate constant for M_{412} decay.

Results of the fit are given in Table 2. For all sets of extinction coefficients blue light induced absorption changes could be fitted with the calculated curves. The resulting rate constants of photoconversion varied only by 3%. Thus model A adequately described the blue light induced relaxation experiments.

$BR_{Asp96 \rightarrow Asn}$

Fitting absorption signals obtained in flash photolysis experiments with a sum of exponentials revealed two rate constants of 5000 s^{-1} and 1400 s^{-1} for M_{412} formation. To describe the M_{412} decay one rate constant of 0.6 s^{-1} was necessary.

Absorption signals obtained with $BR_{Asp96 \rightarrow Asn}$ after a blue light flash with green background light are shown in Fig. 3. In this case the amplitudes did not depend on the light intensity. Only at spectral irradiances below 0.4 mW/cm^2/nm (at 568 nm) did the absorption amplitudes became smaller.

In the case of $BR_{Asp96 \rightarrow Asn}$ the fit with a sum of exponentials led to three time constants (Table 1). Again one

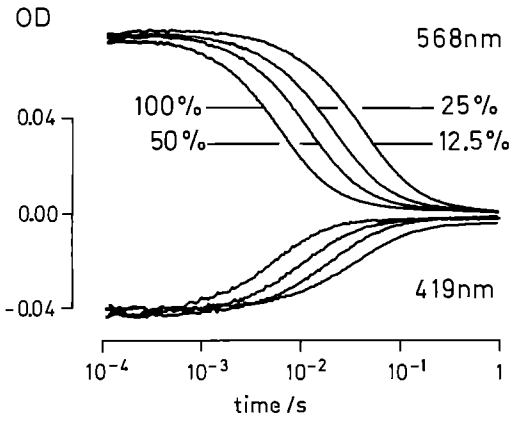

Fig. 3. Absorption signals of $BR_{Asp96 \rightarrow Asn}$ after applying a blue laser pulse at various light intensities. Absorption changes were measured at 568 nm and 419 nm. The displayed signals start at 100 µs to show slow processes in more detail. 100% light intensity corresponds to a spectral irradiance of 4.5 mW/cm²/nm at 568 nm. 30 m*M* KCl, pH 6.8, 20 m*M* *Tris*, 15 °C

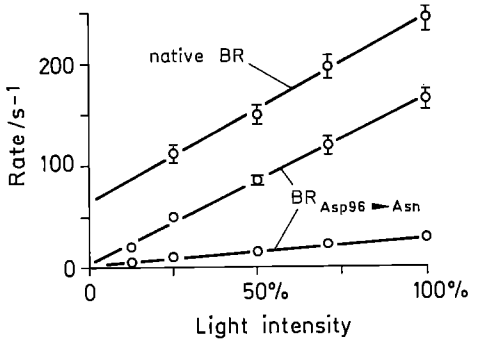

Fig. 4. Variation of rate constants obtained from absorption measurements with different background light intensities at 15 °C. 100% light intensity corresponds to a spectral irradiance of 4.5 mW/cm²/nm at 568 nm. 30 m*M* KCl, 5 m*M* Hepes, pH 7.6 (native bacteriorhodopsin); 30 m*M* KCl, 20 m*M* *Tris*, pH 6.8 ($BR_{Asp96 \rightarrow Asn}$)

component (4600 s^{-1}) was indepent of light intensity and could be correlated with the L_{550}/M_{412} transition. The second rate constant increased linearly with light intensity, reaching a value of 165 s^{-1} at 100% background light intensity (Fig. 4). In addition, a third component was necessary to fit the blue light induced absorption changes. The rate constant increased linearly with the light intensity but with a smaller slope. At 100% background light intensity a rate constant of 29 s^{-1} was measured. Again a slow component with a time constant of several seconds and low amplitude (about 4% of the maximal absorption change) was observed. As with native bacteriorhodopsin the component could not be associated with transitions in the photocycle; it was neglected in the further analysis.

Discussion

Native bacteriorhodopsin

In any model of the bacteriorhodopsin photocycle transitions between two states are characterized by intrinsic rate constants. This set of intrinsic rate constants determines the measurable rate constants which are observed in relaxation experiments. To deduce the intrinsic rate constant of photoconversion k_1 from the blue light experiments, first the relation between intrinsic and measurable rate constants had to be evaluated. Having determined the rate constant of photoconversion k_1, the quantum efficiency could be calculated with (1).

First model A was considered. It was shown, that for $k_2 \gg k_1$, k_3 the measurable rate constants are given by $K_1 = k_1 + k_3$ and $K_2 = k_2$ (8). As k_1 increases linearly with the background light intensity, K_1 does too, while K_2 is independent of the background light intensity. This behaviour was indeed observed: One measurable rate constant equalled the M_{412} formation rate constant and did not change with light intensity. The other measurable rate constant increased linearly with the background light intensity. When extrapolated to zero light intensity a rate similar to the M_{412} decay rate was obtained (70 s^{-1}). Identifying this measured rate constant with K_1 yielded the intrinsic rate constants k_1 and k_3. At 100% background light k_1 was 176 s^{-1} and k_3 was 70 s^{-1}. From the slope of increase of the rate constant versus background light intensity the quantum efficiency was calculated. A value of 0.60 was found.

Other models of the bacteriorhodopsin photocycle were tested to see if they reproduced the linear dependence of one measurable rate constant or if they could be ruled out. A back reaction from L_{550} to M_{412} was introduced. An additional intermediate between M_{412} and BR_{568} (N_{550} or O_{640}) was included in the scheme and a back reaction was allowed. A splitting of the photocycle after L_{550}, leading to different, parallel M_{412} states, was considered. For light intensities above 15% in all cases one measurable rate constant increased linearly with the background light intensity and its slope was determined by (1). The linear dependence on the rate of photoconversion k_1 seems to be a general feature of the photocycle. So the blue light induced relaxation experiments could not rule out any of the models.

The following model, originally proposed by Diller and Stockburger (1988), needs special consideration:

$$\begin{array}{ccccccccc} & k_1 & & 1\,\mu s & & 60\,\mu s & & 5\,ms & \\ BR(\alpha) & \rightsquigarrow & K(\alpha) & \rightarrow & L(\alpha) & \rightarrow & M(\alpha) & \rightarrow & BR(\alpha) \\ \downarrow\uparrow & & & & & & & & \\ BR(\beta) & \rightsquigarrow & K(\beta) & \rightarrow & L(\beta) & \rightarrow & L' & \rightarrow & BR(\beta) \\ & k_1 & & 1\,\mu s & & 220\,\mu s & & 5\,ms & \end{array} \qquad \text{(B)}$$

The time constant related to the thermal equilibrium between $BR(\alpha)$ and $BR(\beta)$ was suggested to be slower than the cycling time. Based on model B, Schneider et al. (1989) derived a quantum efficiency of 0.67 for both ground states from resonance Raman experiments done under similar conditions (pH 7.1) as in my experiments. Model B reproduces the blue light induced relaxation measurements, if molecules in the $M(\alpha)$ state are photoconverted back to $BR(\alpha)$. If a major part of the molecules was transferred from $M(\alpha)$ to $BR(\beta)$ by the blue laser pulse, the steady state absorption at 419 nm should not be re-established with the same speed as the absorption at 568 nm. However, the experiments showed the same rate

constant at 419 nm and 568 nm. Thus, only the quantum efficiency for $BR(\alpha)$ could be measured with the blue light induced relaxation experiments.

Different random and systematic errors need to be considered. From (1) it can be seen that the spectral irradiance of the green background light J, the absorption coefficient $\sigma(\lambda)$ of the ground state BR_{560}, and the rate constant k_1 must be determined. J was measured with an accuracy of 14% given by the calibration error of the light measuring device (10%) and uncertainty in the correct alignment of the background light (about 10%). Rehorek and Heyn (1979) report a value of $62\,700 \pm 700\ M^{-1}\ cm^{-1}$ for the extinction coefficient of the bacteriorhodopsin ground state at 568 nm. Knowing the extinction coefficient, $\sigma(\lambda)$ could be measured without significant error using a spectrophotometer. From repetitive measurements the random error of the rate constants was determined to be 7%. Considering all these errors, the random error of the quantum efficiency was 16%.

A big part of the random error resulted from the light intensity measurement. As long as quantum efficiencies of different samples (e.g. native bacteriorhodopsin under different conditions or native bacteriorhodopsin and $BR_{Asp96\rightarrow Asn}$) are compared, this error can be neglected.

A possible source for systematic errors are background light induced photoreactions of intermediates other than the ground state BR_{568}. It is known that L_{550}, N_{550}, and O_{640} are light sensitive (Hwang et al. 1978). To avoid photoreactions of O_{640} all measurements were done at pH 7.6. Flash photolysis experiments showed that absorption amplitudes caused by O_{640} decrease at high pH and at low temperature (Lozier et al. 1975). At pH 7.6 and at 15 °C the O_{640} concentration in the blue light experiments should be negligible. Moreover, measurements at pH 6.3, where the influence of O_{640} should be higher, yielded the same quantum efficiency. L_{550} and N_{550} both have lifetime less than 1 ms at pH 7.6. The relative concentration of an intermediate can be estimated by dividing its decay time by the cycle time. As the cycle time is about 15 ms the relative concentrations of L_{550} and N_{550} are both below 7%. To estimate possible photoreactions, extinction coefficients and the quantum efficiencies have to be considered. Extinction coefficients given for L_{550} and N_{550} in the literature are always lower than those give for the ground state BR_{568} (Lozier et al. 1975; Nagle et al. 1982; Maurer et al. 1987; Kouyama et al. 1988). As quantum efficiencies can not exceed a value of 1.0, the contribution of possible photoreactions of L_{550} and N_{550} is negligible.

Xie (1990) pointed out that the quantum efficiency depends on the wavelength. In contrast, Schneider et al. (1989) found no dependence of the quantum efficiency on the wavelength. Measurements where the action spectrum is found to be similar to the absorption spectrum (Hwang et al. 1978; Bamberg et al. 1979; Bogomolni et al. 1980) showed that the wavelength dependence must be small. However, it should be kept in mind that the quantum efficiency reported here is an average over wavelengths between 500 nm and 650 nm.

To get information about the pumping mechanism it is interesting to compare the number of protons transported per absorbed photon ϕ_H and the quantum efficiency ϕ. In this way the number of protons pumped per bacteriorhodopsin entering the photocycle n_H can be calculated: $n_H = \phi_H/\phi$. ϕ_H has been measured in whole cells (Hartmann et al. 1977; Bogomolni et al. 1980; Renard and Delmelle 1980), purple membrane sheets (Ort and Parson 1979; Govindjee et al. 1980; Marinetti and Mauzerall 1983), and bacteriorhodopsin incorporated in a lipid bilayer (Korenbrot and Hwang 1980). At high salt concentrations values reported for ϕ_H are around 0.5. Together with a quantum efficiency of 0.3 this led to the conclusion that up to 2 protons are transported per molecule entering the photocycle (Stoeckenius 1985). Direct measurements of n_H by Govindjee et al. (1980) and Kuschmitz and Hess (1981) confirmed this. Different values for n_H were measured later. Drachev et al. (1984), Grzesiek and Dencher (1986), and Grzesiek and Dencher (1988) report values of 0.7, 1.1 and 0.8–1.2, respectively. These measurements are consistent with a quantum efficiency of 0.6. A quantum efficiency of 0.6 together with a ϕ_H of 0.5 leads to about 1 proton transported per bacteriorhodopsin entering the photocycle. A more detailed discussion is found in Schneider et al. (1989).

$BR_{Asp96\rightarrow Asn}$

Model A was not sufficient to describe the behavior of $BR_{Asp96\rightarrow Asn}$. Only two measureable rate constants are predicted by model A but the absorption measurements revealed three rate constants. This additional rate constant can only be explained by an additional intermediate in the $BR_{Asp96\rightarrow Asn}$ photocycle.

Several properties of this intermediate could be deduced from the blue light experiments. It is photoactive with an absorption maximum near 568 nm (± 30 nm); otherwise the dependence on the background light intensity cannot be explained. Its thermal decay rate is slower than $10\ s^{-1}$ (at 15 °C); Fig. 4 shows that the graph corresponding to this intermediate points to such low rate constants when extrapolated to zero light intensity.

A candidate for this intermediate is N_{550} with a maximal absorption around 550 nm (Lozier et al. 1975; Kouyama et al. 1989). Including this intermediate, a possible model for the $BR_{Asp96\rightarrow Asn}$ photocycle is:

$$\begin{array}{l} \qquad\quad k_1 \quad 4600\ s^{-1}\ \ 0.6\ s^{-1}\ \ 5\ s^{-1} \\ BR_{568} \rightsquigarrow L_{550} \rightarrow M_{410} \rightarrow N_{550} \rightarrow BR_{568} \end{array} \qquad \text{(C)}$$

Information about N_{550} in the photocycle of $BR_{Asp96\rightarrow Asn}$ comes from experiments with azide. Azide (N_3^-) accelerates the M_{412} decay in the mutant up to $1000\ s^{-1}$ (Tittor et al. 1989). At these high M_{412} decay rates the N_{550} state becomes visible. It decays with a rate constant of about $5\ s^{-1}$ (J. Tittor, personal communication). If N_{550} is photoactivated, it returns to the initial state BR_{568}.

Based on the blue light induced relaxation experiments it can not be decided if photoconversion of N_{550} leads directly back to BR_{568} or if another intermediate M' is involved, as originally proposed by Kouyama et al. (1988). Both reaction schemes led to the same dependence of rate constants on background light intensities.

To calculate the quantum efficiency from the measured rate constants, first the relation between measurable and intrinsic rate constants had to be evaluated. In model C the L_{550}/M_{412} transition is much faster than any other transition. As in the simple model for native bacteriorhodopsin the fastest measurable rate constant then equals the rate for the L_{550}/M_{412} transition (Fahr et al. 1981). It remains a scheme of three consecutive intermediates BR_{568}, M_{412} and N_{550}. The measurable rate constants of such a cycle have already been calculated in the Materials and methods section. Only the intrinsic rate constants need to be redefined. In the case of $BR_{Asp96\rightarrow Asn}$, k_1 is the rate for the BR_{568}/M_{412} transition, k_2 belongs to the M_{412}/N_{550} transition and k_3 to the N_{550} decay. k_1 and k_3 are supposed to be light sensitive. For light intensities higher than 10% of maximum, k_1 is much bigger than k_2 and k_3 and the approximation $K_1=k_1$, $K_2=k_2+k_3$ is valid. Hence the measurable rate constants K_1 and K_2 increase linearly with light intensity.

Again the quantum efficiency was determined from the slope of the graph of rate constant versus light intensity. A value of 0.60 was found for $BR_{Asp96\rightarrow Asn}$, which is the same quantum efficiency as measured with native bacteriorhodopsin. This agrees with the results of Marinetti et al. (1989). As the extinction coefficient of N_{550} is not known, no quantum efficiency for N_{550} could be calculated. The measurements only indicate that the product of extinction coefficient and quantum efficiency is bigger for the ground state BR_{568} than for N_{550}.

Conclusion

Blue light induced relaxation experiments are an additional method for the study of the bacteriorhodopsin photocycle. In contrast to non-equilibrium techniques such as flash photolysis, these measurements probe the behavior of purple membranes in or near the steady state (see also Butt et al. 1989b).

Experiments with native bacteriorhodopsin revealed one relaxation process which can be attributed to the photoconversion of the ground state. This rate constant depends only on the background light intensity, the absorption cross section of the ground state and the quantum efficiency. Knowing the absorption cross section and the light intensity, the quantum efficiency would be calculated from measured rate constants. A value of 0.60 ± 0.10 was found.

In contrast to experiments with native bacteriorhodopsin which could be explained by a simple model of three consecutive intermediates BR_{568}, L_{550} and M_{412}, experiments with the mutant $BR_{Asp96\rightarrow Asn}$ revealed an additional photoactive intermediate, probably N_{550} after M_{412}. $BR_{Asp96\rightarrow Asn}$ had the same quantum efficiency as native bacteriorhodopsin.

Acknowledgement. I thank Drs. Ernst Bamberg, Klaus Fendler, and Jörg Tittor for many helpful discussions and Dr. Dieter Oesterhelt for providing the native and mutant bacteriorhodopsin used in this study. This study was supported by the Deutsche Forschungsgemeinschaft, SFB 169.

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