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Actinic Light-Energy Dependence of Proton Release from Bacteriorhodopsin

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ABSTRACT Measuring the light-density (fluence) dependence of proton release from flash excited bacteriorhodopsin with two independent methods we found that the lifetime of proton release increases and the proton pumping activity, defined as a number of protons per number of photocycle, decreases with increasing fluence. An interpretation of these results, based on bending of purple membrane and electrical interaction among the proton release groups of bacteriorhodopsin trimer, is presented.

INTRODUCTION

The number of photons per square centimeter in the exciting flash (actinic light-energy, or as it is used in this article, fluence) influences the photocycle of bacteriorhodopsin (BR) from *Halobacterium salinarum:* with increasing fluence the apparent lifetime of the decay of absorbance of M intermediate of the photocycle increases (1,2). More accurately, the ratio of amplitudes of the two components (fast and slow) of M decay changes with fluence. These effects were explained by interactions, i.e., ''cooperativity'' between the BR molecules forming triplets in purple membrane (pm) (3,4). The main function of BR is to pump protons from the cytoplasm of the bacterium to the medium and this way to transform light energy into electrochemical energy (5). Question arises whether the cooperativity appears also in the proton pumping activity defined as number of released protons per number of photocycle of BR.

We set out to study the fluence dependence of proton release from pm. Two methods were applied: the usual proton indicator dye pyranine and the ''buffer method'' developed in our laboratory (6,7). We found by both methods that the lifetimes of proton release and uptake increase with increasing fluence, whereas the proton pumping activity decreases. The results are attributed to cooperativity at the extracellular side of BR. An explanation, based on intermolecular interaction between the proton release groups (PRG defined in Balashov et al. (8)) of BR trimers, is presented. Rather small structural changes in PRG during the transition from ground state BR to M intermediate were found by x-ray diffraction (9,10) though large bending of pm was reported and assigned to M formation (11–14). We hypothesize that the changes are due to electrical interaction in the triplet supported by bending.

MATERIALS AND METHODS

Purple membrane containing wild-type BR was isolated from *Halobacterium salinarum* strain R_1M_1. For the measurements with the proton indicator dye a batch solution (30 μM BR, 100 mM NaCl without or with 100 μM pyranine, pH 7.5) was prepared and a 3 × 3 mm cuvette was freshly filled for each measurements.

Orientation and immobilization of pm in gel was performed according to Dér et al. (15). Slabs of 1 × 1 × 0.16 cm were cut and immersed into solutions (50 μM $CaCl_2$ without or with 5 mM glycil-glycine (GG) at pH 7.5) at least overnight. The samples were put into the measuring cuvettes with the same solutions.

The fluence of a frequency-doubled NdYAG laser with wavelength 530 nm (Surelite I-10, Continuum, Santa Clara, CA) or a dye laser with rhodamin 6G excited by an excimer laser (Lambda Physik EMG 101 MSC, Göttingen, Germany) was changed using neutral absorbers and applied to start the photocycle. Data were related to values of fraction cycling (FC) determined by measuring absorbance at 410 nm and using the known absorption coefficient of M intermediate. Absorbance changes were recorded with photomultiplier measuring the transmitted light intensity at 410 nm (for FC) or at 450 nm (for pyranine) selected with heat and interference filters from a 200 W tungsten lamp.

The electric signals from the oriented samples were picked up with platinized Pt electrodes immersed into the solution and were amplified by a homemade current amplifier based on a Burr-Brown 3554 operational amplifier (Texas Instruments, Dallas, TX).

The data, optical and electric, were digitized by a computer-controlled transient recorder with 10,000 channels (LeCroy, Geneva, Switzerland).

The usual method to measure the time dependence of proton release and uptake for flash excitation is to register absorbance changes in presence and absence of proton indicator dye in BR solution and calculate their difference. The absorbance change of the indicator dye is due to protonation and is proportional to the number of protons present in the solution. With other words, this method registers the time integral of the proton release and uptake processes.

On the contrary, the ''buffer effect'' responds to the protons as released. The protein electric response signals (PERS) of light excited BR measured with and without GG differ in the microsecond time range (6,7,16,17). Detailed study of the difference between these signals showed that they originated from the protons that were released at the extracellular surface by PRG and moved on the buffer gradient (6). The hypothesis has been confirmed by data taken with different BR mutants (7). Thus the ''buffer signals'' reflect the appearance of protons in solution. We may consider these signals as a differential response to proton release. Their time integral is proportional to the number of released protons. The buffer effect is not sensitive to proton uptake because this process is rather slow and produces

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PERS not measurable in the case of wild-type BR. The lifetimes of the exponentials fitted to the integral (proton indicator dye) or to the differential (PERS) responses reveal the same process.

RESULTS

Measurements with indicator dye

First the indicator dye method was applied to measure the FC dependence of proton release and uptake. In Fig. 1, the absorbencies in absence of dye and the pyranine absorbencies (differences measured with and without pyranine) depending on FC are shown. The data were fitted with two exponentials. The lifetimes of absorbance rise in absence of pyranine (BR signals) do not change, whereas those of decays increase with increasing FC in agreement with data in Dancsházy et al. (1). It is evident, however, that the maxima of pyranine absorbencies are shifted to longer time with increasing FC. The two-exponential fits of proton release and uptake data, i.e., of the pyranine signals, show an increase of the lifetimes of rise and also a decrease of the amplitudes related to those from absorbencies in absence of dye. The amplitudes of absorbencies of pyranine signals are considered proportional to the number of released protons, whereas those of BR signals are proportional to the number of photocycle, thus their ratio provides the proton pumping activity (PPA). We emphasize that the fitted amplitudes of proton uptake in the case of pyranine signal and those of the decay of absorbance of BR signals were used in calculating PPA. To be sure the following control process was applied, curves were calculated for rise and decay with constant amplitudes using the fitted lifetimes for both pyranine and BR signals in the FC range and their maxima determined. The maxima of the pyranine and BR signals were read from the absorbencies in Fig. 1 and corrected with the calculated data. The corrected data coincided with those obtained from the fitted amplitudes.

PPA and lifetime data are depicted both normalized to one at 28% FC in Fig. 2. The lifetimes of proton release increase ≈30%, whereas PPA values decrease ≈17% with increase of FC from zero to 28%. We do not present the lifetimes of proton uptake that increase too with FC. The FC dependencies were fitted with linear functions (Fig. 2). The average change of lifetimes of proton release is (30 ± 4)% and of PPA (−16 ± 1)% from zero to 30% FC increase from four independent determinations.

Measurements based on ''buffer effect''

Fig. 3 shows PERS with and without buffer, and the difference as the ''buffer signal'' (*dotted line*). In the given time range PERS without buffer has two characteristic components: a fast, large negative component assigned to BR → K and a slower positive component assigned to L → M transition (18). The buffer signal has rising and decaying components with lifetimes of ~50 and 250 μs. The interpretation of these two components is given in Tóth-Boconádi et al. (6). Shortly, protons move from the Schiff-base to Asp-85 during L → M transition; the change of electric field rearranges the proton release group (rise of buffer signal), which then releases the proton (decay of buffer signal). Fig. 4 contains the buffer signals registered at different FC values and normalized to the maximum FC. It is well seen that the decay times increase with increasing FC. The rise times are not constant either their changes are small, not visible in Fig. 4, but the two-exponential fits show that they decrease with increasing FC. Data are collected in Fig. 5. The decay times of the BR signal do not change with FC (not shown).

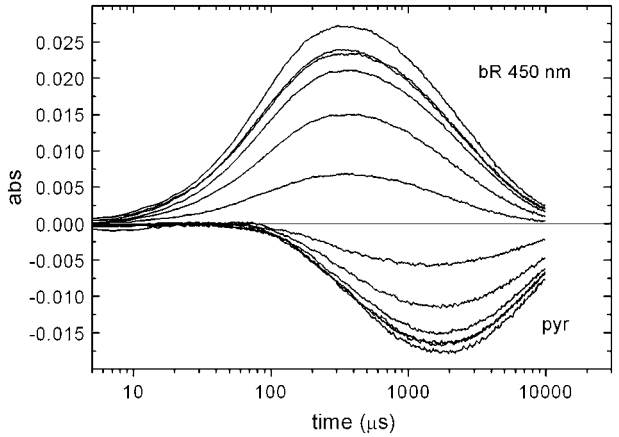

FIGURE 1 Absorbance changes as a function of fraction cycling measured at 450 nm. Traces signed BR are for bacteriorhodopsin without pyranine, pyr for differences with and without pyranine. Exciting light 580 nm, fraction cycling values for up to down (BR) or down to up (pyr), respectively, are 28, 24.5, 24.2, 21.8, 15.9, and 7.3%. Solution: 30 μM BR, 100 μM pyranine, 100 mM NaCl, pH 7.5. Temperature 24°C.

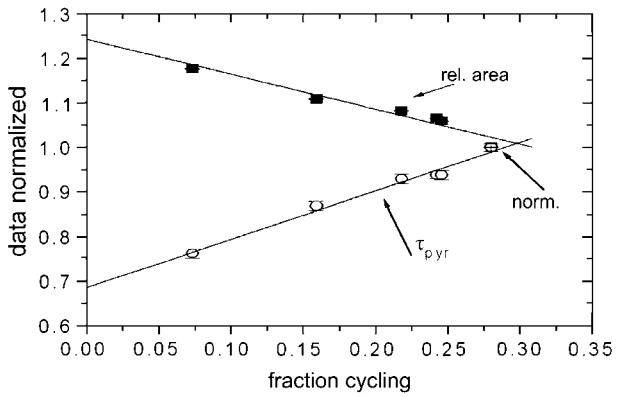

FIGURE 2 Fraction cycling dependence of lifetime (τ_1) and ratio of the amplitudes of pyr and absorbencies (BR) considered as proton pumping activity. Data taken from two-exponential fits of traces in Fig. 1. Data are normalized at 28% fraction cycling. Lines are linear fits with slopes (28 ± 3)% and (−19 ± 3)%. The actual lifetime of proton release at FC 28% is 541 ± 5 μs.

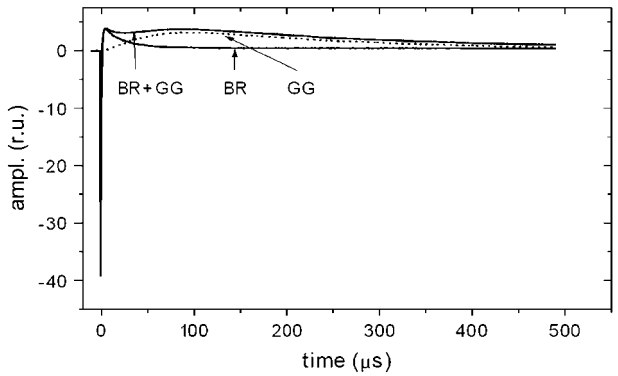

FIGURE 3 Protein electric response signals for excitation with laser flash of 530 nm. BR + GG signals with glycyl-glycine buffer and BR without it, GG their difference, i.e., the buffer signal. Bacteriorhodopsin, 30 μM, in purple membrane oriented and immobilized in gel. Solution 50 μM $CaCl_2$, pH 7.5, and 5 mM glycyl-glycine for the buffer effect. Temperature 24°C.

The time integrals of the ''buffer signals'' related to the time integrals of the positive PERS of BR considered as PPA decrease with increasing FC (Fig. 6). The average changes of the parameters for FC from zero to 30% are the rise times (-15 ± 1)%, the decay times (24 ± 4)%, and the PPA values (-5 ± 2)%, from four independent determinations.

Although the dye signals apparently have only one component for rise, i.e., for proton release, the buffer signals have two components. It is easy to reconcile the contradiction: the dye signals are the time integrals of the time dependence of the release process characterized by the differential buffer signals. In integration the contribution of the short living component is small. More detailed measurements are necessary to resolve it as found in Porschke (19).

DISCUSSION

Both methods the well-known indicator dye and the ''buffer method'' reveal the same phenomena: the lifetimes of proton release increase and the proton pumping activities decrease

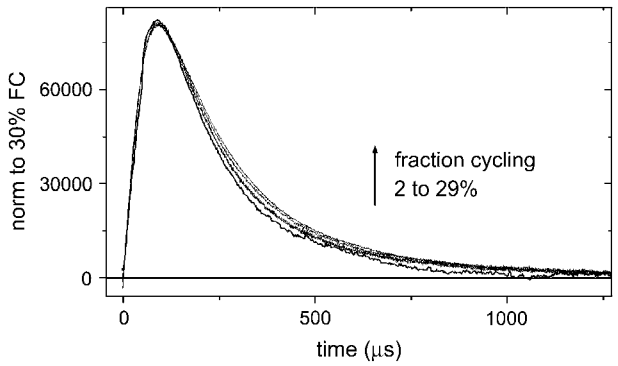

FIGURE 4 Fraction cycling dependence of ''buffer signals'' normalized to the trace at 29%. Fraction cycling values from traces with the longest to the shortest lifetime 29, 21, 13, 8.3, and 2%.

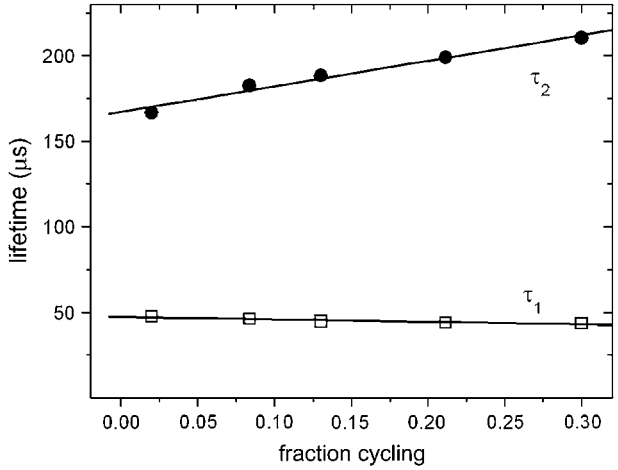

FIGURE 5 Fraction cycling dependence of lifetimes (τ_1 and τ_2) of buffer signals. Data are taken from two-exponential fits of traces in Fig. 4. Errors are smaller than the sizes of the points. Lines are linear fits with slopes (-14 ± 2)% and (26 ± 2)%, respectively.

with increasing FC, whereas the lifetimes of M rise do not change. This different behavior confirms that L→M transition and proton release are separate processes as recognized in (6–8,19). The measured lifetime values are somewhat different. These differences are, however, not surprising considering the different solutions used in the two experiments. We note that the pyranine method needs high salt solution, the buffer effect as low as possible (6).

Protons are released by PRG that contain amino acids Arg-82, Glu-196, and Glu-204, and a network of water molecules as understood in (6,8,10,20). The protonation of Asp-85 during L→M transition transforms PRG to such a conformation that enables proton release. The structures of the PRG region of L and M intermediates are already known (9,10).

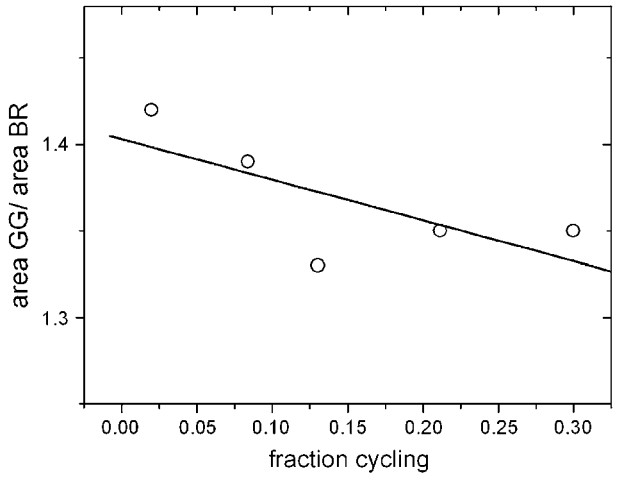

FIGURE 6 Fraction cycling dependence of proton pumping activities determined as the ratio of the areas of ''buffer'' signals and the corresponding bacteriorhodopsin signal from data in Fig. 4. The line is linear fit with slope (-5 ± 2)%.

They indicate movement of the side chain of the positively charged Arg-82 in direction to the extracellular surface approaching the side chains of Glu-194 and Glu-204 this way increasing their distance. These motions are caused by the protonation of Asp-85 that changes the electric field in the region. Thus, we may assume rightly that the alteration of the electric field induces the proton emission from PRG as formulated in Tóth-Boconádi et al. (6).

We have to contemplate how could these intramolecular changes in one member of the BR triplet influence the behavior of the excited neighboring molecules with increasing FC. A possibility could be the interaction via the hypothetical dwelling, diffusing protons in the interfacial layer of pm (21,22). According to those studies, the protons released during L→M transition dwell for ≈700 μs at the extracellular surfaces of pm before appearing in the solution. This hypothesis was criticized in Tóth-Boconádi et al. (6) and Porschke (19). Shortly, the activation entropies calculated from the surface bound fluoresceine data (21,22) and from the buffer effect (6) are the same within error and negative indicating ordering processes that are not characteristic for diffusion. A recent elaborate study of proton release affirms that there is no evidence of a diffusion barrier, the data indicate an inside cavity from where the protons are transferred to the periphery of the protein (19). Another problem is that protonation dynamics measurements show that protons dwell on the surface layer of the pm only for 40–50 μs (23), thus ~20 times shorter than the assumed dwell time for protons from excited BR. We are not sure that the offered explanation, i.e., the different boundary conditions in the two cases can explain the large factor.

Even in the case if the hypothesis were correct, the buffers, like pyranine and glycil-glycine, would conduct the protons out of the surface layer (24). Thus, we may rule out the ''dwelling protons'' as a cause of the observed FC dependence of proton release.

According to the structure data of the intermediates the motions in PRG seem to be small. However, large bending of pm associated with the photocycle was found by two methods: light scattering (11–13) and electrooptics (14). The time-resolved light scattering data show that the bending appears already during M formation (11–13), whereas the published electrooptical measurements that deal with pm containing the mutant BR D96N assign it in general to the M intermediate resolving only its decay. Careful analysis of light scattering data (11–13) indicates that the pm at pH>5 (as in our case) bends toward the extracellular side suggesting that PRGs in triplet come somewhat nearer to each other.

We now hypothesize that the arising intramolecular electric field caused by the deprotonation of Schiff-base during L→M transition and assisted by bending extends also to the neighboring molecules in the BR triplet. This electric field-based intermolecular interaction could somehow influence the parameters of PRG. We note that in case of the ''buffer effect'' the pm is immobilized in the gel, consequently the membrane bending does not occur. That explains the smaller changes in lifetimes (24% vs. 30%) and in pumping activities (5% vs. 16%). Calculations to substantiate this hypothesis are in progress in our laboratory.

Our data involve that ~16% of protons are not pumped at high FC though they are transported from the Schiff-base to Asp-85 as manifested by M absorption. Such phenomena in PPA (called slips) have already been observed in closed systems (vesicles and cells (25)) and in electric field applied against pumping in BR expressed in oocite and on planar lipid film (26). In both articles branching of the photocycle into pumping and non pumping pathways is appointed as a cause with ratio depending on backpressure of the electrochemical potential (25) or the oppositely oriented electric field (26). These fields influence the ratio of M_1 and M_2 intermediates this way influence PPA. It is well demonstrated that also the actinic light-energy influences the ratio of M_1 and M_2 intermediates (decreasing M_1 and increasing M_2 with increasing fluence (1–4)). Our data on the fluence dependence of PPA point to the same phenomena from another point of view.

We may question whether the strong bending of pm has any physiological role. It may function as a mechanical signal for mechanosensitive ion channels according to Porschke (14). Our data hint at other possible role: the increasing lifetime and the decreasing pumping activity with increasing illumination protect the cells from over energized circumstances.

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