BLUE LIGHT EFFECT ON PROTON PUMPING BY BACTERIORHODOPSIN

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ABSTRACT Proton pumping in closed vesicular systems containing bacteriorhodopsin that is initiated by an orange flash, is diminished by a subsequent blue flash. This blue light effect is due to light absorbed by the photocycle intermediate M412 (M), which was formed by the orange flash. A kinetic analysis of the blue-light–induced reduction of proton pumping shows that of the two components of M, only the slowly decaying component is involved in the reduction of proton movement. This may be the first correlation between a proton movement and a specific photochemical intermediate of bacteriorhodopsin. Furthermore, we report that blue light, acting on the slowly decaying intermediate, probably causes a movement of the protons in a direction opposite to that normally seen for light absorbed by bacteriorhodopsin.

INTRODUCTION

Bacteriorhodopsin (BR) is the only protein found in the purple membrane, a specialized region of plasma membrane of *Halobacterium halobium* (1). Illumination of bacteriorhodopsin initiates a photochemical cycle that passes through a series of spectroscopically distinct intermediates; during this photocycle, protons are released on the outside and taken up on the inside of the bacterial cell (1). Thus bacteriorhodopsin, acting as a light-driven proton pump, converts light energy into chemical free energy in the form of a proton gradient.

One of the intermediates of the photocycle called M412 (M) has an absorption maximum at 410 nm and is relatively long-lived. There are two kinetically distinguishable components in the decay of M (2–4). If M is illuminated by blue light during its lifetime, it is photoconverted back to bacteriorhodopsin (5–7). Oesterhelt and Hess reported that proton rebinding is enhanced by blue light (8). Dancshazy and co-workers (9–11) observed that supplementary illumination by blue light decreased a photovoltage induced by orange light in oriented purple membrane samples. The action spectrum for this effect coincided with the absorption spectrum of the M intermediate; its time dependence coincided approximately with the time course for decay of the M intermediate. Based on these observations, Dancshazy and co-workers concluded that blue light either causes rebinding of protons or inhibits proton pumping by bacteriorhodopsin, and called this phenomenon the blue light effect. Using a closed vesicular system, *H. halobium* cell envelopes, Lozier et al. (12) showed that irradiation with a blue laser flash 2 ms after an orange flash decreases the yield of H^+ released by the orange laser flash.

MATERIALS AND METHODS

H. halobium strain S-9 was grown and the purple membrane isolated as described by Becher and Cassim (13), except that no DNAase was used in the preparation of the purple membane.

Cell-envelope vesicles were prepared from the bacteria following the method of MacDonald and Lanyi (14). The cell envelopes were prepared in 4 M NaCl and finally suspended in 3 M KCl, pH 7.2, for proton pumping experiments. Incorporation of bacteriorhodopsin into egg phosphatidylcholine vesicles was performed as previously described (15).

The samples were placed in a water-jacketed cylindrical cuvette (inner diameter 12 mm) and stirred with a magnetic stirrer during the experiment. A combination glass electrode (Beckman Instruments, Inc., Fullerton CA, model 39030) was used to follow the light-induced pH change due to proton pumping by bacteriorhodopsin. The electrode was connected to an electrometer (Radiometer America Inc., Westlake, OH, model PHM 62) whose output signal was offset and fed into a chart recorder (Houston Instrument, Model 4901). Illumination was provided from opposite sides of the cuvette with two photoflash lamps. An orange cutoff filter (Toshiba V-05) was placed in front of one flash lamp (half-pulse width of 200 μs) to provide a saturating pulse of wavelengths greater than ~530 nm. A band-pass filter (Hoya B-390) was placed in front of the second photoflash lamp (half-pulse width of 150 μs) to select blue light (transmission maximum, ~390 nm). A laboratory-built delay circuit was used to vary the time between the two flashes. All experiments were done under dim red light on light-adapted samples.

A single-beam kinetic spectrophotometer was used to measure the decay of M and proton kinetics. Movement of protons was followed using a pH indicator dye, *p*-nitrophenol, as described previously (15).

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RESULTS

Flash-induced pH Changes

Fig. 1 shows flash-induced pH changes in bacteriorhodopsin-containing cell-envelope vesicles. In the case of cell-envelope vesicles, a transient decrease in the pH of the medium was observed upon excitation with the orange flash (Fig. 1*a*). In contrast, reconstituted phospholipid vesicles showed a transient pH increase (Fig. 2*a*). In reconstituted vesicles, the COOH-terminus of bacteriorhodopsin was predominantly toward the outer side of the vesicle, whereas in cell envelopes, it was toward the inner side. Thus, the polarity of pH change is correlated with the sidedness of bacteriorhodopsin in the two systems. The rise time of the pH signals ($t_{1/2}$ = 0.5 s) was almost the same as the characteristic response time of the glass electrode, measured by applying a small proton (HCl) pulse. The recovery of the pH change had a half time of ~20 s in both cell envelopes and reconstituted vesicles. These values were in a good agreement with the half time of passive proton leakage, measured by absorption changes of the pH indicator dye, *p*-nitrophenol. Because the time of the proton leakage was much longer than the response time of the electrode, we believe that the measured signal correctly reflects the amount of protons pumped. In contrast to the cell envelopes and reconstituted vesicles, purple membrane sheets gave no signal with the pH electrode. This was expected, because in this system protons are released and taken up within milliseconds, which is very short compared with the response time of the electrode. Thus, in the glass electrode system, only pH changes from a closed system (such as cell envelopes or reconstituted vesicles) can be measured. The absence of a pH signal in purple membrane sheets also rules out any significant direct response of the electrode to the excitation light flash.

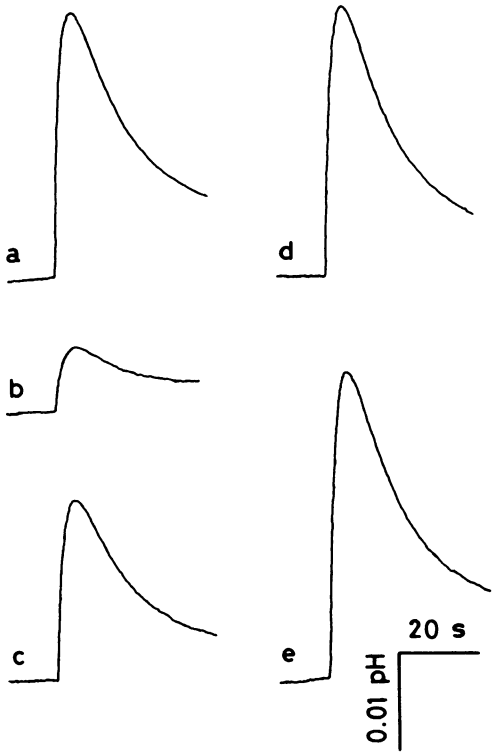

FIGURE 1 Flash-induced pH changes (acidification) in cell-envelope vesicles in 3 M KCl, 0.1 mM potassium phosphate, pH 7.2, 20°C, OD_{570} ~ 0.3. *a*, orange flash; *b*, blue flash; *c*, orange flash followed by blue flash ~1 ms later; *d*, blue flash followed by orange flash 1 ms later; and *e*, orange flash followed by blue flash 172 ms later. Orange flash ($\lambda > 530$ nm, half-pulse width 200 μs) was obtained by placing an orange cut-off filter (Toshiba V-05) between the flash lamp and the sample. Blue flash (half-pulse width 150 μs, a band-pass filter [Hoya B-390]) was used to select blue light with a peak at 390 nm.

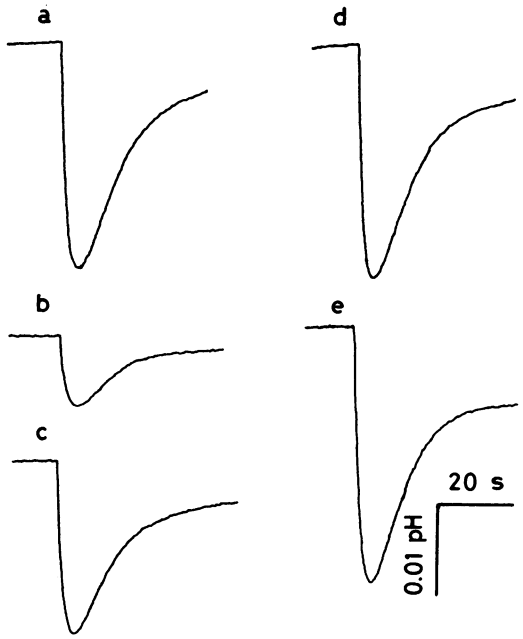

FIGURE 2 Flash-induced pH changes (alkalinization) in bacteriorhodopsin-containing egg phosphatidylcholine vesicles in 0.5 M KCl, 0.1 mM potassium phosphate, pH 6.8, 20°C, OD_{570} ~ 0.3. *a*, orange flash; *b*, blue flash; *c*, orange flash followed by blue flash 1 ms later; *d*, blue flash followed by orange flash 1 ms later; and *e*, orange flash followed by blue flash 172 ms later.

Blue Light Can Reduce Proton Pumping

We show here that light-initiated proton pumping in cell envelope vesicles is reduced by a subsequent blue flash. The pH change induced by an orange flash is shown in Fig. 1*a*. Excitation with the blue flash we used gave ~1/5 as large a pH change as that from the orange flash (Fig. 1*b*). When the orange flash was followed 1 ms later by the blue flash, the proton release was 30% lower than that from the orange flash alone (Fig. 1*c*). If we gave the blue flash 1 ms before the orange flash, the size of the pH change was almost the same as the change from the single orange flash (Fig. 1*d*). When the blue flash was given long after the orange flash, say 172 ms, the resultant signal was almost as large as the sum of the two independent signals from the orange and the blue flashes, and the size of the pH change

was not dependent on the sequence of the two flashes (Fig. 1*e*).

Similar results were observed in reconstituted phospholipid vesicles (Fig. 2, *a–e*) except the direction of the proton flow was opposite to that seen in cell-envelope vesicles. Thus, we conclude that under appropriate conditions blue light can reduce the orange-light–induced ΔpH.

Photoreversal from the Slow-decaying form of M is Responsible for Reducing the pH Response

The action spectrum measurements and the approximate time dependence of the photovoltaic effect (10) suggested that the photointermediate M is responsible for the blue light effect. Under our experimental conditions, the decay of the M intermediate was biphasic in both cell envelopes and reconstituted phospholipid vesicles. To determine if one or both of the two M's is involved in the reduction of the pH response, we compared the decay of the M absorbance change with the extent of the reduction of ΔpH as a function of the delay time between the orange and blue flashes.

Fig. 3 and 4 show the data for cell envelopes and phospholipid vesicles, respectively. There was ~30% reduction of proton pumping when the time between the two flashes was around 1 ms, but the extent of this reduction became smaller with increasing time between flashes. In the case of cell envelopes (Fig. 3), the M decay was composed of two exponentials with half times of ~2 and 16 ms; the time dependence of the reduction of proton pumping can be described by an exponential decay with a half time of ~14 ms. In various preparations, the half time of the slowly decaying form of M varied from 8 to 16 ms, and in every case the decay of the blue light effect matched this half time. Thus, it seems likely that of the two M intermediates, photoreversal from only the slowly decaying M is responsible for the reduction of the orange flash-induced pH change.

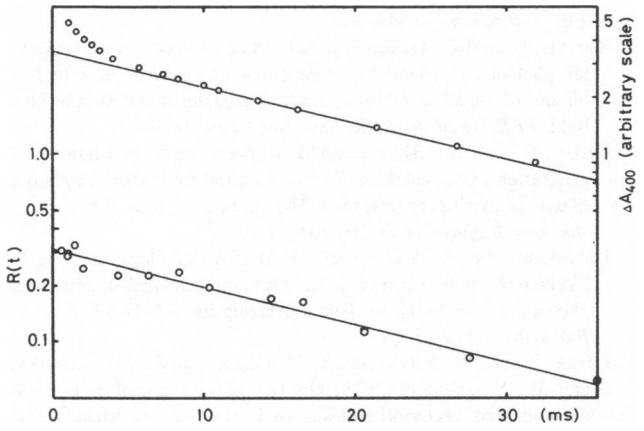

FIGURE 3 Time course of the decay of the M intermediate (*upper* curve) and of the reduction in proton pumping caused by blue light (*lower* curve) in cell envelope vesicles. The decay kinetics of M intermediate were measured as described by Govindjee et al. (15) except that the actinic light source was replaced by the 200 μs flash used for the glass electrode experiment. The delay time between the orange and the blue flashes was varied and signal from the electrode following the double excitation was recorded. The amplitude of pH change was obtained by extrapolating the pH trace on logarithmic plots. The extent of the reduction, $R(t)$, is defined as $R(t) = [\Delta pH(-t) - \Delta pH(t)]/\Delta pH(-t)$ where $\Delta pH(-t)$ is the pH change when the blue flash precedes the orange flash by time equal to t, and $\Delta pH(t)$ is the pH change when the orange flash precedes the blue flash by time equal to t.

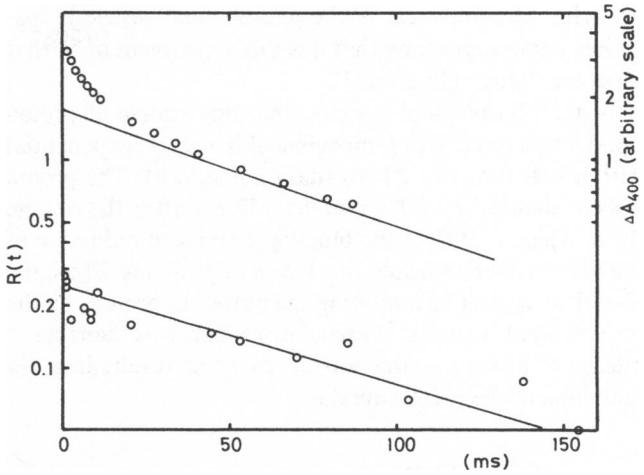

FIGURE 4 Time course of decay of the M intermediate (*upper* curve) and of the reduction in proton pumping caused by blue light (*lower* curve) in reconstituted phospholipid vesicles. Egg phosphatidylcholine vesicles, the characteristics of which are described in Fig. 2 were used here. The pH of the sample was maintained at 6.8 ± 0.1. Other conditions and definitions are the same as in the legend of Fig. 3.

In cell-envelope vesicles, protons are translocated from inside to the outside medium; the half time of proton release from the outside of the cell envelopes at 20°C, pH 6.8, 3 M KCl, was ~0.7 ms, as measured by *p*-nitrophenol (data not shown). Therefore, proton release to the outside should be >99% complete by, say, 14 ms after the orange flash excitation in the experiments presented above. But the results shown in Fig. 3 suggest that proton pumping can still be decreased by as much as 50% of the maximum reduction if the blue flash is given 14 ms after the orange flash. Thus we conclude that the effect of the blue light is not an inhibition of proton release, but rather a reuptake of protons from the suspending medium on the outer surface of the cell envelope vesicles (from the NH_2-terminal side of bacteriorhodopsin), which can take place long after the protons have been released; Lozier et al. (12) and Kalisky et al. (7) come to a similar conclusion.

In reconstituted phospholipid vesicles, bacteriorhodopsin molecules are incorporated predominantly with their originally cytoplasmic sides facing the outside of the vesicles (1). The decay of the M intermediate is again biphasic with half-times of ~8 and 58 ms (Fig. 4). The time dependence of the reduction of the ΔpH by blue light is an exponential with a half time of ~60 ms (Fig. 4). This is in good agreement with the half time of the slow

decaying M component in the phospholipid vesicle preparation, again suggesting that it is this component of M that gives the "blue light effect."

In the phospholipid vesicles, the time course of proton uptake measured with *p*-nitrophenol is a single exponential with a half time of ~27 ms (data not shown). The proton uptake should be ~80% complete 60 ms after the orange flash, whereas 50% of the blue-light–induced reduction of the pH change can be obtained even after 60 ms. Thus, the blue flash cannot be inhibiting the uptake of protons by the phospholipid vesicles; rather, it probably is causing a release of protons to the outside, and this results in a net reduction of the proton uptake.

DISCUSSION

There are three quite interesting findings in our experiments. First, we have shown by direct measurements a blue light effect on the pH changes in bacteriorhodopsin-containing closed systems (cell-envelope vesicles and phospholipid vesicles). That is, after the blue-absorbing intermediate M is formed in response to a bright orange flash that normally leads to a pH change in the medium, a second blue flash can reduce the size of the pH change expected in its absence.

Second, we have found a direct correlation between a photochemical intermediate, the slowly decaying form of M, and a proton pumping process, the reduction of the pH change by a blue flash. Early tentative correlations between proton uptake or release and the formation or decay of the M intermediate did not hold up when experimental conditions were varied (3). We think it is likely that the correlation presented in this paper is a firm one because the lifetimes of the blue light effect and M decay can be varied by a factor of 2–3 with the correlation persisting.

Finally, the long half times for the decay of the blue light effect compared to the half times for proton release from the NH_2-terminal side of the membrane and uptake from the COOH-terminal side are unexpected. This requires that blue light be able to cause a pH change after proton release (or uptake) has taken place. Thus it seems that blue light does not inhibit proton pumping. Rather, blue light, absorbed by the slowly decaying form of M, must cause a H^+ uptake from the NH_2-terminal side and release from the COOH-terminal side of bacteriorhodopsin. This is equivalent to a blue-light–powered proton pump in the direction opposite to the proton pump of bacteriorhodopsin. A second possible, but less plausible, explanation is that blue light makes the membrane leaky to protons, thus reducing the size of the pH change. This possibility seems unlikely because the half-decay time of the ΔpH signal is not faster when orange and blue flashes are given successively 1 ms apart, compared with the $t_{1/2}$ of decay of the ΔpH from the orange flash alone. However, the explanation that blue light causes a short-lived leak in the membrane cannot be excluded.

This work was supported by National Science Foundation grant PCM 82-01924 and Department of Energy grant DE-AC02-82-ER12087.

Received for publication 18 October 1983 and in final form 16 March 1983.

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