BACTERIORHODOPSIN IS A POWERFUL LIGHT-DRIVEN PROTON PUMP

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ABSTRACT The activity of bacteriorhodopsin was investigated with *Halobacterium halobium* cell envelopes, which lack cytoplasmic constituents. It was found that the physiological concentration of magnesium ion greatly enhanced the light-induced pH change; under optimal conditions, the pH change of the external medium was as large as 3.5 pH units, even though the volume fraction of the envelope vesicles was as low as 0.01. This pH change is about three times larger than the largest change reported thus far. This same effect was observed with transition metal ions, but not with other alkaline divalent cations. That is, divalent cations that formed hydroxides below pH 10 were effective in enhancing the light-induced pH change. This result suggests that some divalent cations acted as buffers against a large increase in the internal pH, and that the internal pH was an important factor in determining the activity of bacteriorhodopsin. It was also shown that a high level of the proton-pump activity was maintained in a wide range of external pHs, at least between 4.5 and 9.4.

INTRODUCTION

Bacteriorhodopsin, a membrane protein of *Halobacterium halobium*, uses light energy to translocate protons across the cell membrane and thereby generates a pH gradient and an electric potential (1). The activity of the light-driven proton pump has been extensively investigated with the cell envelope vesicle (2–6) or with reconstituted vesicles (7–11). Presently, the largest change in external pH induced by light is ~0.1 pH unit. In our work, a much larger change (3.5 pH units) was observed at moderate light intensity. It was also shown that, in the presence of some divalent cations, bacteriorhodopsin can pump protons in a considerably wide range of external pHs (at least between 4.5 and 9.4). This property may be used to control any pH-dependent chemical reactions by light.

MATERIALS AND EXPERIMENTS

Envelope vesicles were prepared from *H. halobium* JW-3 (12) (kindly provided by J. H. Weber), which is a mutant over-producing bacteriorhodopsin. The cells harvested from a stationary state culture were suspended in "basal salt" solution (250 g of NaCl, 20 g of $MgCl_2 \cdot 2H_2O$, 2 g of KCl in 1 liter of water). The suspension was frozen at −70°C and thawed at room temperature, resulting in the breakage of cells (13). Cell envelope vesicles thus obtained were separated by centrifugation at 20,000 *g* for 30 min. This freeze-thaw procedure was repeated three times with basal salt solution and twice with 4 M NaCl or KCl solution. The purified envelope vesicles exhibited a single absorption band with a peak at 560 nm. The average diameter of the cell envelope was ~0.4 μm, according to results from filtration experiments. Before measuring light-induced pH changes, we treated the envelope vesicles with a weak acid (pH 3.5–4.0) at 40°C. This procedure was found to simplify the kinetics of light-induced pH change (Fig. 1).

Measurements of external pH were performed with a glass electrode (type GK2321C; Radiometer America Inc., Westlake, OH). The pH chamber was a cylindrical glass of 10-mm diam, in which ~1.2 ml of the suspension (OD_{560} ~ 1.2) was stirred; the volume fraction of the vesicles was ~0.01. The top opening of the pH chamber was sealed with silicon rubber through which reagents were added with microsyringes, and the inside of the chamber was kept under Ar gas flow. A projector lamp (300 W) was used as a light source, and its emission was passed through an infrared cut filter and a yellow optical filter. The light intensity at the pH chamber was ~40 mW/cm^2. Temperature of the suspension was kept at 40° ± 0.5°C.

RESULTS AND DISCUSSION

Fig. 2 shows light-induced pH changes in the envelope vesicle suspension in the presence of varying concentrations of Mg^{++}. When the suspension contained no free Mg^{++}, the pH change was almost saturated within a few seconds after illumination, and no more than five protons per bacteriorhodopsin were released into the medium. When more than 5 mM Mg^{++} was present, the pH changes occurred in two phases; an initial phase, in which a few protons per bacteriorhodopsin were burst into the medium, and a slow phase, in which the pH of the medium continued to decline. Although the initial rate of proton release was not affected by Mg^{++}, the rate of pH change in the slow phase increased almost linearly with increasing concentrations of Mg^{++} (up to 150 mM).

In the presence of the physiological concentration (80 mM) of Mg^{++}, a decrease in external pH as large as 3.5 pH units could be induced by moderate light intensity (40 mW/cm^2); Fig. 3, *inset* shows a typical example of such a large pH change. We failed, however, to observe a much larger pH change. It was found that whenever the pH was set between 6 and 9.4, the external pH decreased to 5.8 after a long illumination (Fig. 3). The result shown in Fig. 3, which was obtained in the absence of pH buffer, was not altered by the presence of a low concentration of pH buffer

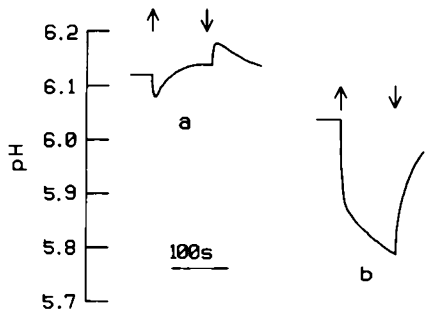

FIGURE 1 Light-induced pH changes of the envelope vesicle suspension before (*a*) and after (*b*) acid treatment. When the untreated suspension in 4 M NaCl was illuminated, quick acidification of the external medium was followed by slow alkalization. The latter component disappeared after the acid treatment (at pH 3.5) at 40°C for 1 h.

(5 mM HEPES). In this case, however, a much longer illumination must be allowed until the external pH decreased to the same value. To overcome the buffering action of external HEPES, one bacteriorhodopsin molecule must pump out several hundreds of net protons.

The following question may arise: From where did these protons originate? The only possible main proton source is water molecules that exist inside the vesicles. In this case, many OH^- ions must be produced. However, if the OH^- ions thus produced maintained their activity, the internal pH or the internal concentration of OH^- would become unbelievably high (pH > 12). To avoid this, it is possible that the OH^- ions lose their activity by reacting with Mg^{++}. This hypothesis is based on observations of the strong buffering action of Mg^{++} at an alkaline pH; when a

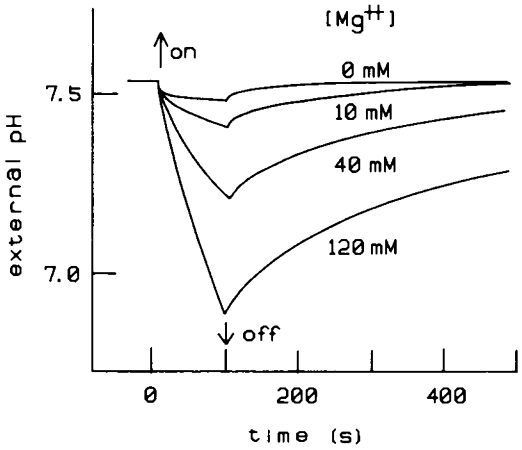

FIGURE 2 The light-induced pH changes of the medium of the envelope vesicle suspension in the presence of varying concentrations of Mg^{++}. In this experiment, a desired amount of 1 M $MgCl_2$ solution was added to the suspension in 3 M KCl and 2 mM HEPES. After addition of $MgCl_2$, the extent of light-induced pH change gradually increased with time and the full enhancement was attained 1 or 2 h later. Replacement of 3 M KCl with 4 M NaCl did not change the result essentially. The Mg effect was also observed for the envelope vesicles prepared by the sonication method (14) or those prepared from another strain R_1M_1, but not for any reconstituted vesicles in which the orientation of bacteriorhodopsin was inside out.

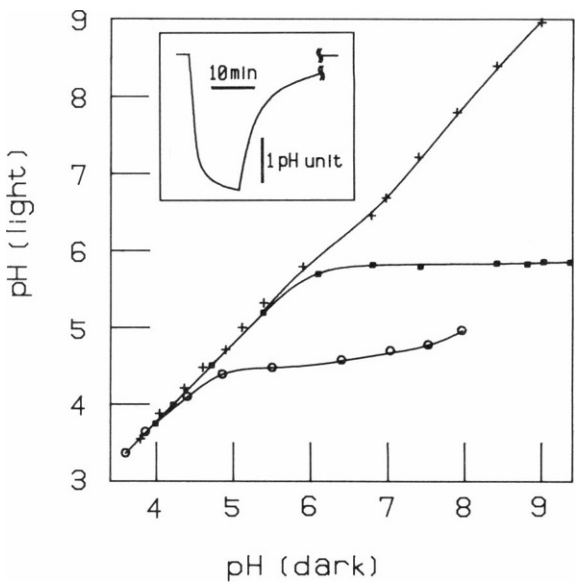

FIGURE 3 The external pH observed under stationary illumination was plotted against the pH observed before illumination. The envelope vesicles were suspended in 3 M KCl and 80 mM divalent cations; magnesium (■), manganese (○), calcium (+). The inserted curve represents the light-induced pH change observed with the envelope vesicles suspended in 3 M KCl and 80 mM $MgCl_2$.

3 M KCl solution containing 80 mM Mg^{++} was titrated with NaOH, the buffering capacity increased sharply above pH 9.4; further addition of NaOH resulted in the precipitation of magnesium hydroxide. In addition, it was found that only the divalent cations, which formed the hydroxides at a relatively low pH (<10), were effective in enhancing the light-induced pH change; transition metal ions (Mn^{++}, Ni^{++}, and Zn^{++}) were as effective as Mg^{++}, whereas other alkaline divalent cations (Ca^{++}, Sr^{++}, and Ba^{++}) did not effect the light-induced pH change at all (Fig. 3). From these results, it is suggested that the upper limit of the internal pH for a high activity of the proton pump is ~10.

Due to the low solubility (0.15 mM), it is very possible that $Mg(OH)_2$ aggregates inside vesicles that are exposed to light. This scheme is consistent with the observation that the turbidity of the envelope vesicle suspension gradually increased in the light and decreased in the dark. It was suggested that a very slow pH recovery observed after turning off the light (Fig. 2) reflected the dissolving process of solid $Mg(OH)_2$. Although the kinetics of the pH recovery was dependent on many factors (e.g., the pH gradient), a strong correlation between the half time of the pH recovery and the magnitude of the light-induced pH change was always observed. The aggregation of $Mg(OH)_2$ inside the vesicles seemed to play an important role in the enhancement of the light-induced pH change; probably the aggregation helped to restrict the outward flow of $Mg(OH)_2$, which would cause alkalization of the medium.

Fig. 4 shows the dependence of the apparent activity of the light-driven proton pump on the external pH. In this

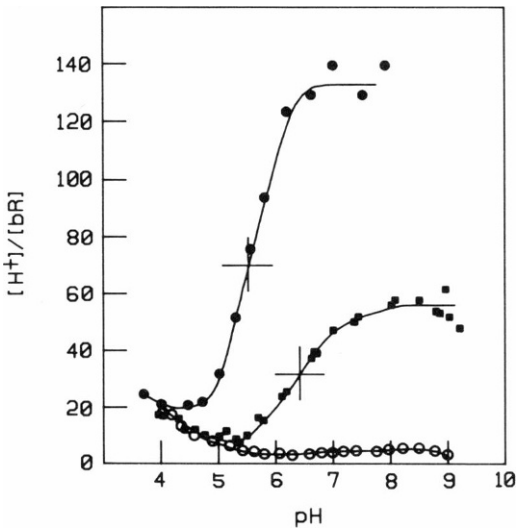

FIGURE 4 The number of protons released during the first 100-s illumination was plotted as a function of the initial pH of the medium. 2 mM (or 4 mM) HEPES and 2 mM (or 4 mM) MES were added so that the induced pH changes were restricted to within 0.5 pH units. Different symbols represent the data obtained in the absence (O) and presence of the 80 mM Mg^{++} (■) and 80 mM Mn^{++} (●). The number of released protons was calibrated from the magnitude of pH change induced by a HCl pulse.

experiment, few pH buffer molecules were added so that the external pH change induced during the first 100-s illumination was restricted to within 0.5 pH units. In the absence of Mg^{++}, more protons were pumped out at lower pH. This pH dependence is exactly the same as that reported by several workers (5). Since the buffering capacity of the envelope vesicles themselves exhibited a similar pH dependence, it is likely that the bump of the apparent activity at pH 4 came from the buffering action of carboxyl groups distributed on the internal membrane surface. In the presence of the physiological concentration of Mg^{++}, however, more protons were pumped out at higher pH. That is, the enhancement of the light-induced pH change by Mg^{++} was notable only when the external pH was higher than 5.5. When Mg^{++} was replaced with Mn^{++}, a similar pH dependence was observed, except that the effect of Mn^{++} was notable above pH 4.5. The shift of the pH dependence seemed to correspond to the shift of the internal pH maintained in the light; $Mn(OH)_2$ precipitates at a pH lower than does $Mg(OH)_2$ (by 1.2 pH units). This result suggests that the activity of the light-driven proton pump does not depend much on the external pH, and that the extent of proton release is reduced by a large pH gradient (>3 pH units). Since the extent of proton release was almost constant in the alkaline pH region (pH 7–9 in Fig. 4), it is likely that either the proton motive force or proton leak is nonlinearly regulated by the pH gradient. In addition, the observation of a more notable effect of Mn^{++} than Mg^{++} suggests that the activity of bacteriorhodopsin is coupled with the dissociation state of some amino acid residue with a pK ~9.5, which is sensitive to the internal pH.

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