The water-exposed C-terminal sequence of bacteriorhodopsin does not affect H^+ pumping

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The fast kinetics of photocycle and H^+-pumping activity of papain-treated bacteriorhodopsin deprived of 17 C-terminal amino acid residues has been investigated. As demonstrated by a single-turnover study, the formation and decomposition of the M412 intermediate as well as the generation of the photoelectric potential are similar in the native and in the papain-treated protein. On the other hand, acidification of the medium caused by the deprotonation of bacteriorhodopsin due to M412 formation is much smaller in the C-tail-deprived protein. Short-term sonication or addition of a small amount of detergent completely abolishes this effect. As a result, papain-treated bacteriorhodopsin exhibits the same acidification as the native one. It is concluded that a decrease in the light-induced pH response of the C-tail-deprived bacteriorhodopsin is caused by the aggregation of purple sheets rather than by a special role of the C-terminal sequence in H^+ pumping.

H^+ pumping *Bacteriorhodopsin* *Kinetics*

1. INTRODUCTION

Bacteriorhodopsin functions as a light-driven H^+ pump localized in the cytoplasmic membrane of *Halobacterium halobium*. The primary structure of this protein composed of 248 amino acid residues was described in [1,2]. A limited proteolysis made it possible to reveal the water-exposed parts of the protein [3,4]. In 1978 we studied the photoelectric activity of papain-treated bacteriorhodopsin. It was shown that the partially digested bacteriorhodopsin generated a membrane potential of the same magnitude as the native one, although papain eliminated 17 amino acids from the C-terminus, 3 from the N-terminus and 5 from the middle part of the polypeptide chain [3]. This fact was confirmed in 1984 by Liao and Khorana [5] who measured continuous light-induced H^+ uptake by bacteriorhodopsin proteoliposomes.

In 1982 Govindjee et al. [6] reported that papain or trypsin treatment results in an about 50% decrease in the short-flash induced acidification of the medium accompanying M412 formation. The authors suggested that the removal of the C-tail induced a 2-fold decrease in the efficiency of bacteriorhodopsin as an H^+ pump.

To resolve the obvious contradiction between our and Khorana's results on the one hand and the data by Govindjee et al. on the other, we reinvestigated this problem by using fast measurement of optical, pH and electric responses of bacteriorhodopsin. It was found that the C-tail is not essential for H^+ pumping. As to the results by Govindjee et al., they are due to the aggregation of the papain-treated bacteriorhodopsin membranes. Some of these results were reported at the 16th FEBS Meeting [7,8].

2. MATERIALS AND METHODS

Purple membrane sheets were isolated from *H.*

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halobium 353-P, according to [9]. Specific removal of the C-terminal sequence composed of 17 amino acid residues was carried out with papain as described in [3]; see the procedure for low papain treatment (for details, see also [10,11]).

The photoelectric activity of bacteriorhodopsin was studied in the purple sheets attached to a collodion film impregnated with a decane solution of egg lecithin and octadecylamine (70 and 0.5 mg per ml, respectively). Before treatment of the film with the purple sheets, they were sonicated by a USDN-1 U4.2 sonicator (0.5 A current) in a Teflon tube for 30 s. Electric potential generation was directly measured by a voltmeter as described in [12–15].

Spectral measurements were carried out as in [14,15] by using a suspension of the purple sheets.

Light-induced pH changes in the purple sheet suspension were monitored according to [16] with the aid of *p*-nitrophenol as pH-sensitive dye. The response of *p*-nitrophenol was measured as the difference between the light absorbance changes at 400 nm with and without this dye. Usually 100–200 responses were summed up. Calibration of the response was done by addition of a small amount of HCl.

A Quantel YG-481 Nd-YAG Q-switched laser with frequency doublers (532 nm, 15 ns, 50 mJ) was used as a light source.

All the measurements were taken at 23°C. Light-adapted sheets were used.

3. RESULTS

Fig.1 shows the formation and decomposition kinetics of the M412 intermediate of the bacteriorhodopsin photocycle. It is seen that a 15 ns laser flash (vertical arrow) induces an absorbance increase at 412 nm. The rates of the process occurring within the microsecond time scale are the same in native and papain-treated bacteriorhodopsins. As to the millisecond 412 nm absorbance decrease, it is slightly faster in the papain-treated preparation: $t_{1/2}$ is 3 ms instead of 4 ms in native bacteriorhodopsin (fig.1). The magnitude of the 412 nm absorbance changes was similar in both preparations (not shown).

These data are in agreement with those obtained by Govindjee et al. [6] who did not observe that the

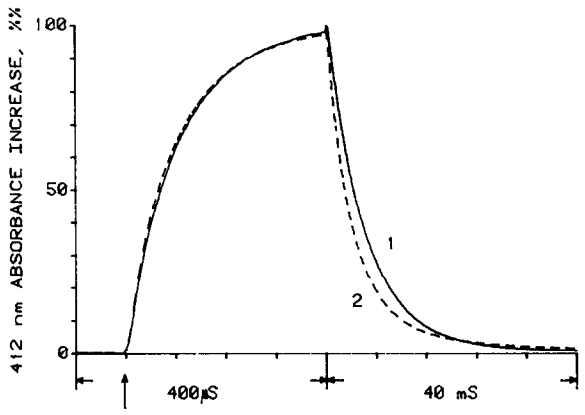

Fig.1. Formation and decomposition of the bacteriorhodopsin M412 intermediate. Incubation mixture: 20 mM NaCl, 5 mM Mes, pH 6.8, and purple sheets (8×10^{-6} M bacteriorhodopsin). (1) Native sheets, (2) papain-treated sheets. Here and in the other figures, the vertical arrow denotes the laser flash.

partial digestion of bacteriorhodopsin produced any significant effect upon the photocycle.

Adsorption of the native or papain-treated purple sheets onto one surface of the phospholipid-impregnated collodion film produced a system which generated an electric potential difference in response to the illumination. In both preparations, similar magnitudes of the photoelectric generation were obtained, namely, 130–150 or 40–50 mV in the case of continuous light or laser flash, respectively. The response showed three electrogenic phases: very small negative (shorter than 50 ns), small positive (on the microsecond time scale) and large positive (on the millisecond time scale) (fig.2). As previously shown in this group [12,13], the negative phase is associated with the formation of the K intermediate, while the micro- and millisecond positive phases are due to M412 formation and decay. Fig.2 shows that the only difference between the native and partially digested bacteriorhodopsins consists in a slightly faster kinetics of the millisecond electrogenic phase, which is in agreement with some acceleration of the decomposition of M412 (cf. fig.1).

In the next series of measurements, H^+ release and uptake accompanying the bacteriorhodopsin photocycle were studied. In agreement with Govindjee et al. [6], it was found that papain digestion caused (i) an almost 3-fold decrease in the

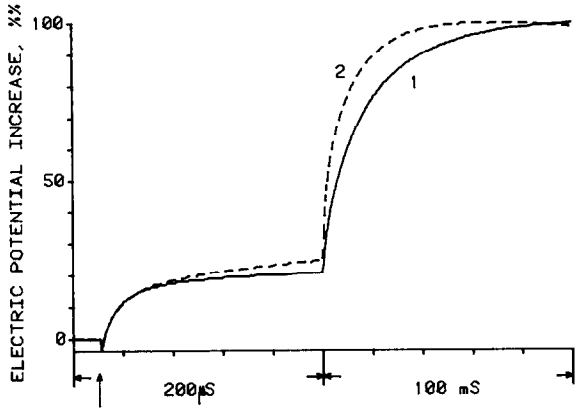

Fig.2. Generation of the electric potential difference by bacteriorhodopsin incorporated into the phospholipid-impregnated collodion films. Conditions and designations as in fig.1.

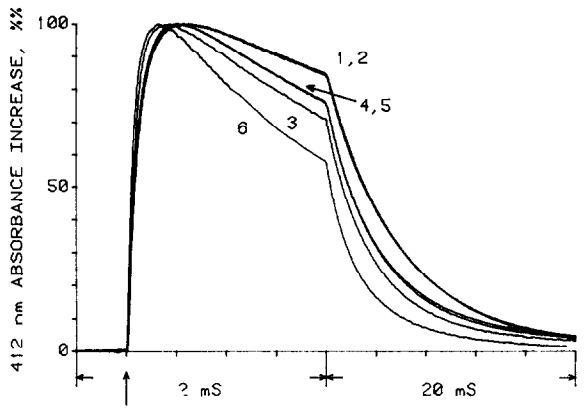

Fig.4. Comparison of the effects of papain, sonication and Triton X-100 upon M412 formation and decay. Incubation mixture: 20 mM NaCl, pH 6.8, and purple sheets (8×10^{-6} M bacteriorhodopsin). 1–3, native sheets; 4–6, papain-treated sheets; 1,4, without sonication and without Triton X-100; 2,5, 30 s sonication; 3,6, in the presence of 0.01% Triton X-100.

magnitude of flash-induced H^+ release and (ii) some deceleration of the subsequent reabsorption of H^+ ($t_{1/2}$ of H^+ uptake was 5 ms in native and 7 ms in papain-digested bacteriorhodopsin) (fig.3A,B, curves 1). However, both these differences disappeared if the purple sheets were sonicated for a short time or treated with a small amount (0.01%) of the detergent, Triton X-100 (fig.3A,B, curves 2 and 3). After sonication or Triton X-100 treatments, both the magnitude and the decay rates of the pH responses of digested bacteriorhodopsin proved to be as high as in the native preparation. Sonication did not affect the pH and optical responses of the native purple sheets whereas Triton X-100 slightly accelerated M412 decay (fig.4).

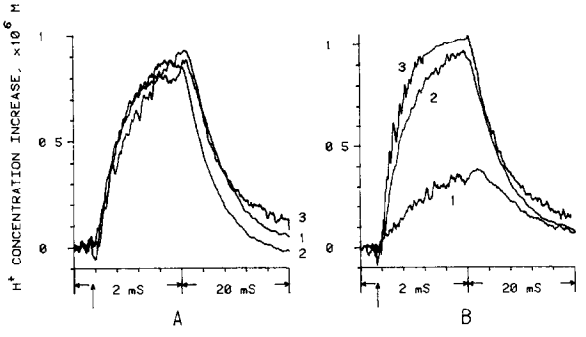

Fig.3. Bacteriorhodopsin pH responses. Incubation mixture: 20 mM NaCl, 0.1 mM *p*-nitrophenol, pH 6.8, and purple sheets (8×10^{-6} M bacteriorhodopsin). (A) Native sheets, (B) papain-treated sheets. 1, without sonication and without Triton X-100; 2, sheets sonicated for 30 min; 3, mixture supplemented with 0.01% Triton X-100. The *p*-nitrophenol absorbance changes were measured at 400 nm. In all cases, maximal absorbance changes in the absence of *p*-nitrophenol were 0.05 unit.

4. DISCUSSION

One of the approaches to understanding the mechanism of the bacteriorhodopsin H^+ pump may be elucidation of the functional role of various parts of the protein molecule. A possible function of the rather long water-exposed C-terminal sequence was first studied in this group, and it was concluded that it is not necessary for bacteriorhodopsin photoelectric activity [3]. This conclusion was later confirmed by Liao and Khorana [5]. On the other hand, Govindjee et al. [6], by measuring the flash-induced fast pH response of bacteriorhodopsin, obtained an indication that the C-tail doubles the efficiency of the H^+ pump. According to Liao and Khorana, the difference between their data and those of Govindjee et al. may be due to the experimental systems used, since they studied bacteriorhodopsin proteoliposomes in the second time range. Govindjee et al. investigated purple sheets in the millisecond

range. In proteoliposomes, bacteriorhodopsin was in the monomeric form, and in the sheet it was in the crystalline trimeric form. Moreover, as Liao and Khorana specified, the absence of C-tail-linked negative charges from the sheet surface may cause adherence of the sheets preventing the bacteriorhodopsin-released H^+ from fast equilibration with the bulk water phase.

The latter explanation seems to be most plausible, if we take into account the above-described data. (i) Neither optical nor electric responses were affected by the removal of the C-tail so that the pH response proved to be the only parameter which changed after papain digestion. (ii) The pH response of 'tailless' bacteriorhodopsin could be completely normalized by treatments preventing aggregation of the sheets, such as sonication and addition of a small concentration of Triton X-100. It should be noted that the concentration of Triton X-100 was as low as 0.01%, i.e. more than 2 orders of magnitude lower than those inducing disruption of the sheet per se and solubilization of bacteriorhodopsin.

Aggregation of the purple sheets after removal of the C-tail was observed by Wallace and Henderson [17] with the aid of electron microscopy. Kenitry et al. [18] noted that the surface amino acid residues are immobilized to some degree in the tailless bacteriorhodopsin, an effect which may also be the result of sheet aggregation.

A sheet aggregation increase apparently results from a loss of the 4 residues of dicarboxylic amino acids localized close to the C-terminus. A decrease in the surface charge should facilitate aggregation.

Significantly, a change in the surface charge per se may in principle influence the amplitude of the bacteriorhodopsin photoresponse [16,19,20]. However, had this been the case, it would have been hardly possible to eliminate the effect of the papain treatment by sonication or the non-ionic detergent, Triton X-100.

The effects of sonication and Triton X-100 are particularly apparent at low ionic strength. Thus, in fig.3, 20 mM NaCl was present. An increase in the salt concentration up to 100 mM was found to cause the formation of visually recognized aggregates of the purple sheets pre-treated with papain. In this case, sonication and low Triton X-100 concentrations were less effective.

An increase in ionic strength resulted in a greater magnitude of the pH response of the native purple sheets [16,19,20] (apparently due to the effect of the salt on the membrane surface potential). In the sheets stored in bidistilled water at 4°C for 2–3 weeks, the dependence of the pH response magnitude upon salt concentration showed an optimum at 100 mM. An increase in the NaCl level above this value resulted in a lower pH response. As suggested by Govindjee et al. [6], the lower magnitude of long-stored bacteriorhodopsin preparations is caused by the removal of the C-terminal sequence by endogenous proteinases. According to our data, it might be possible for the sheets stored at least for a year. However, as shown by a special chemical analysis, a month-long storage does not lead to a loss of the C-tail. Thus the tendency of the sheet to aggregate during storage needs some other explanation.

Special attention should be given to the question how many H^+ are transported across the membrane per photocycle. The H^+/M412 ratio, according to Govindjee et al. [6,16], is 1.7–1.8 and 0.5–0.8 in native and C-tail-deprived bacteriorhodopsin at high ionic strength. In our experiments involving both the native sheets immediately after their isolation and the papain-digested ones, this ratio was 0.7 ± 0.1 (100–500 mM NaCl present). Perhaps some differences in calibrating the pH response by adding HCl [21] might be involved. It was found that the addition of HCl resulted in a biphasic response of pH dye absorbance: a fast absorbance decrease changed into a slow increase, $t_{1/2}$ of the slow phase being 4 s at room temperature. The magnitude of the slow phase was approx. 2-fold lower than that of the fast one. Apparently, it was due to the buffer effect of CO_2 in solution. It seems obvious that the fast (micro- and millisecond) flash-induced bacteriorhodopsin-mediated pH changes must be calibrated by the fast phase of the HCl response. Its slow phase would give an approx. 2-fold overestimation of the bacteriorhodopsin pH response and hence, of the H^+/M412 ratio.

Our conclusion that the C-tail of bacteriorhodopsin is not essential for H^+ pumping is in agreement with the data of Wallace and Henderson [17], who showed by using electron and X-ray diffraction that this part of the polypeptide chain does not hold any fixed position in the bacteriorhodopsin molecule. Its removal does not induce

any changes either in the conformation of the rest of the polypeptide or in the crystalline arrangement of the sheet.

The question arises of whether the water-exposed C-terminal sequence of bacteriorhodopsin performs any biological function. One of the possibilities could be that it is necessary to stabilize the membrane organization of the bacteriorhodopsin-containing sheets.

A membrane exists only if it is composed of components forming a hydrophobic layer and hydrophilic interfaces. In phospholipids, these functions are carried out by hydrocarbon chains of fatty acids and by charged phosphate-containing head groups, respectively. However, the amount of phospholipids in the bacteriorhodopsin sheets is too small to organize a bilayer continuum since bacteriorhodopsin is estimated to comprise 75% of the membrane substances. It is obvious that 7 α-helical rods of bacteriorhodopsin are responsible for formation of the hydrophobic membrane core. As to the hydrophilic part, it may be organized by water-exposed sequences removed by papain, i.e. (i) by the C-terminal segment at the cytoplasmic membrane surface and (ii) by the N-terminal sequence and a loop between the 65th and 73rd amino acid residues at the outer membrane surface.

The fact that both bacteriorhodopsin sheets (see above) and visual rhodopsin-containing photoreceptor discs [22] tend to aggregate as a result of papain treatment speaks in favor of this suggestion.

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