PRODUCTS OF LIMITED PROTEOLYSIS OF BACTERIORHODOPSIN GENERATE A MEMBRANE POTENTIAL

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1. Introduction

In recent years, one of the most interesting events in biochemistry was the discovery of a new type of photosynthesis mediated by bacteriorhodopsin, a light-dependent proton pump in the membrane of halophilic bacteria.

The goal of the present work was to elucidate whether the water-exposed sites of the polypeptide chain of bacteriorhodopsin are involved in the generation of a transmembrane electric potential difference. We have studied the products of partial proteolysis of bacteriorhodopsin by papain and thermolysin. The experiments revealed that the products of hydrolysis of bacteriorhodopsin that retain the $A_{570\,nm}$ max are still competent in generating a transmembrane electric potential difference if illuminated with continuous light or with a flash inducing a single bacteriorhodopsin turnover. The kinetic parameters of bacteriorhodopsin as a photoelectric generator do not deteriorate in spite of removal of 17 amino acids from the C-end, 3 amino acids from the N-end and 5 amino acids from the middle part of the polypeptide chain.

2. Materials and methods

Isolation of bacteriorhodopsin membranes and treatment of the membranes by proteolytic enzymes was carried out as described in [1].

Bacteriorhodopsin proteoliposomes were prepared essentially according to [3] (for details, [4]).

Incorporation of bacteriorhodopsin into proteoliposomes was followed using centrifugation in a continuous sucrose density gradient. In the preparations studied, the band of bacteriorhodopsin sheets was absent whereas the proteoliposomes gave the characteristic band.

The PCB^- (phenyldicarbaundecaborane anion) probe [5] was used for measuring the electrogenic activity of the proteoliposomes. The dynamics of the PCB^- concentration in the incubation medium was followed with the help of a Synpore membrane filter, the pore diametre being 2.5 μm. The filter was impregnated with a solution of azolectin in decane (50 mg azolectin/ml). The incubation mixture contained 50 mM Tris–HCl (pH 7.0), 1.2×10^{-6} M PCB^- and proteoliposomes (0.5–5 μg protein/ml). The relative electrogenic activity of proteoliposomes was determined by the ratio of the amplitudes of the light-dependent PCB^- responses, when the proteoliposomes with the proteinase-treated and untreated bacteriorhodopsins were compared. In both cases the incubation mixture contained equal amounts of protein. All the measurements were taken in the region where the dependence of the photoeffect amplitude on the protein concentration was linear. That the method is adequate was demonstrated by special experiments with proteoliposomes obtained from the following preparations of bacteriorhodopsin:

(1) The sheets were bleached by hydroxylamine in the light and then reconstituted to various degrees by addition of retinal [6].
(2) The sheets were treated with sodium borohydride [7] and mixed with the intact preparations in different proportions.

In both cases the PCB^- response magnitude correlated fairly well with A_{570} values.

Direct measurements of the potential difference generated by the bacteriorhodopsin proteoliposomes was performed using the procedure developed in this group [4] and modified as described [8]. A Synpore filter (pore diam. 0.17 μm) was impregnated with azolectin and fixed in an aperture of a partition separating 2 solutions of identical composition. To one of the compartments, bacteriorhodopsin proteoliposomes were added and $CaCl_2$ to both. Incorporation of the proteoliposomes into the filter took several hours. Ag/AgCl electrodes were used to measure the light-dependent potential difference between the two compartments. It was noted that lower concentration of $CaCl_2$ (5 mM instead of 30 mM as in the previous studies) are more advantageous for incorporation of liposomes with proteinase-treated bacteriorhodopsin. Higher concentrations of $CaCl_2$ entail agglutination of such proteoliposomes. In the experiments with continuous illumination and in the flash experiments, a halogen 150 W lamp and an ISSH lamp (duration of illumination 5 μs) were used, respectively.

For measuring the fast kinetics of the photoelectric responses, a collodion film instead of a Synpore filter was applied (procedure in [8]).

3. Results and discussion

The control or the proteinase-treated sheets were mixed with a phospholipid and cholate and sonicated; then the detergent was removed by dialysis to obtain proteoliposomes. We studied the proteoliposomes that had the following bacteriorhodopsin preparations as a protein component:

(i) A control that had not been subjected to proteolysis.
(ii) A preparation treated with a low concentration of papain (ratio of papain to bacteriorhodopsin membranes 1 : 200); as a result of the treatment, the native protein converted into a peptide that had a somewhat higher mobility in SDS electrophoresis than intact bacteriorhodopsin.
(iii) A preparation treated with a higher papain concentration (papain to membranes ratio 1 : 20), contained a mixture of 3 peptides, a heavy one, identical to that obtained with the more dilute papain solution, and 2 others having higher

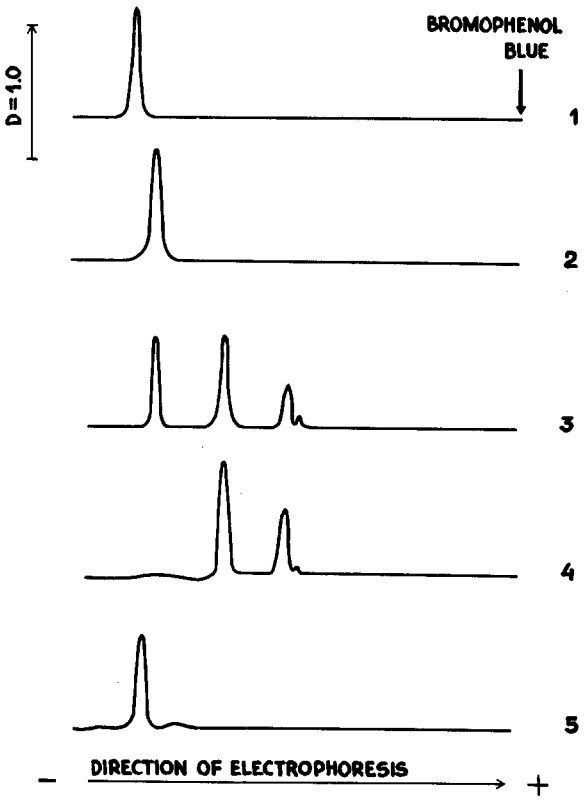

Fig.1. SDS electrophoresis of bacteriorhodopsin preparations treated by proteinases in a gradient (4–30%) polyacrylamide gel. The gel was stained with Coomassi brilliant blue R-250. The scanning was performed at 550 nm in a Gilford spectrophotometer. (1) Native membranes (app. mol. wt 22 500). (2) After papain treatment (papain : membrane ratio, 1 : 200 (mg/ml)). Duration of treatment 2 h, 37°C; here and below, the reaction mixture contained 10 mM NaCN, 2 mM EDTA, pH in the experiments with papain was 7.2. (3) After papain treatment (papain : membrane ratio, 1 : 20 (mg/ml)). Duration of treatment 20 h. (4) After papain treatment (papain : membrane ratio, 1 : 2 (mg/ml)). Duration of treatment 20 h. (5) After thermolysin treatment (thermolysin : membrane ratio, 1 : 70 (mg/ml)). Duration of treatment 20 h, 37°C, pH 8.3.

Table 1
Generation of the electric potential difference by bacteriorhodopsin treated by papain and thermolysin

Preparation of bacteriorhodopsin	Light-dependent accumulation of PCB^- anions by proteoliposomes (arbitrary units referred to the absorption at 570 nm)	Formation of light-dependent potential difference in the 'proteoliposomes–membrane filter' system (mV)	
		Continuous light	Flash
Control	1.0	230	70
Papain-treated: heavy peptides	1.2	240	65
Papain-treated: heavy, medium weight and light peptides	0.9	150	50
Papain-treated: medium-weight and light peptides	1.0	145	40
Thermolysin-treated	1.1	220	70

electrophoretic mobilities (the medium-weight and the light peptides).

(iv) A preparation treated with the highest papain concentration (the papain to membranes ratio 1 : 2), containing only medium-weight and light peptides.

(v) A preparation treated with thermolysin and containing a peptide with an electrophoretic mobility that was lower than that of the native protein and higher than that of the heavy papain peptide (fig.1).

The results of the study of the photopotential generation by proteoliposomes are presented in table 1. It is seen that photoelectric activity is present in all the preparations. The electrogenic activity assay by the PCB^- probe showed that all the preparations treated with proteinases are almost as active as the control ones if the photoeffect is referred to A_{570} value.

The spectral measurements of the proteoliposomes revealed a certain decrease of the maximum at 570 nm compared to that at 280 nm in all the preparations treated with proteinases. This decrease was especially noticeable in the 'papain' proteoliposomes that contained only the light and the medium-weight peptides: in this preparation the $A_{570} : A_{280}$ ratio proved to be just over half that in the undigested bacteriorhodopsin. Respectively, the photoeffect in the PCB^- experiments was halved if referred to the A_{280} max and was unchanged if referred to the A_{570} max. As to the experiments on direct measurement of photopotentials in the proteoliposomes–membrane filter system, the reference of the effect to the optical density of bacteriorhodopsin proves impossible because the absorption of the proteoliposomes attached to the filter surface defies measurement. On the other hand, proteoliposomes attached to membrane filter or collodion film can be used to measure fast kinetics of bacteriorhodopsin electrogenesis, which cannot be followed with PCB^- probe.

All proteinase-treated preparations demonstrate a photoeffect in the 'proteoliposome–membrane filter' system. Even in the 'papain' proteoliposomes containing only the medium-weight and the light peptides, the photoeffect amplitude amounted to more than 50% of that with undigested protein (see table 1). The photoelectric response produced by a single turnover of bacteriorhodopsin was also retained. The measurement of the flash-induced kinetics of

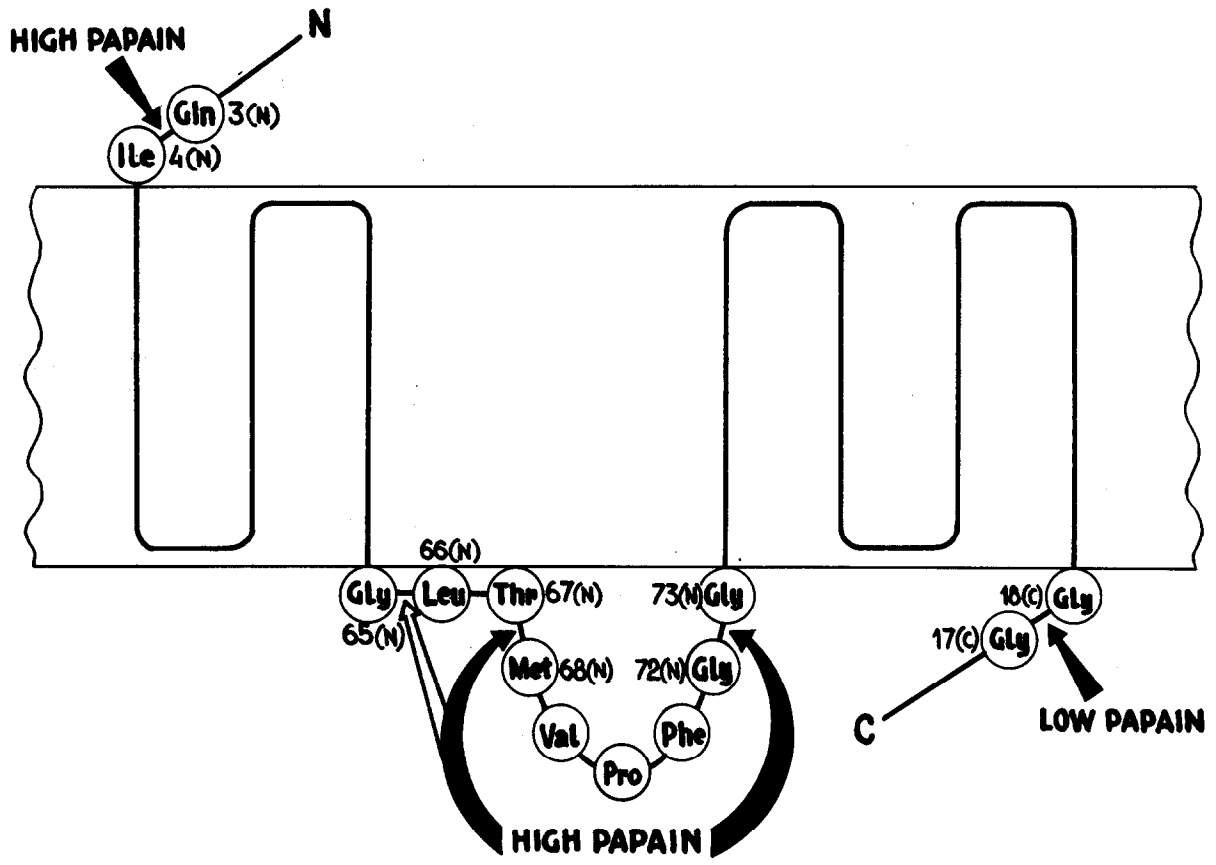

Fig.2. The sites of attack of bacteriorhodopsin by papain. The black arrows indicate the bonds that break in all the rhodopsin molecules, and the white arrow in part of the molecules after 20 h treatment with papain.

membrane potential formation with the method developed in this group [8] did not reveal any differences between the controls and the preparations treated with proteinases. In all the cases a 3-phase photoeffect was observed, the τ of the phases being (1) 0.3 μs, (2) 40 μs and (3) 10 ms; the fast phase had a direction opposite to that of the slower ones.

The photoelectric activity of the bacteriorhodopsin preparations subjected to limited proteolysis cannot be ascribed to contamination by undigested molecules of the protein. The contamination, judging by SDS electrophoresis in polyacrylamide gel, should be $< 5\%$. The more than 20-fold decrease in the amount of the active molecules of bacteriorhodopsin could not have escaped our attention, first of all in the experiments on quantitative measurements of photoelectric effects by the PCB^- probe.

Thus the data obtained are sufficient for us to conclude that the products of limited proteolysis of bacteriorhodopsin by papain and thermolysin that retain the A_{570} max, are competent in photocurrent generation.

As demonstrated [1,2], treatment of bacteriorhodopsin by a diluted solution of papain results in 17 amino acids being cleaved off from the C-end of the polypeptide chain of bacteriorhodopsin. Higher concentrations of papain cause also the removal of 3 amino acids from the N-end and cleavage of the bonds between threonine 67 and methionine 68 and between glycines 72 and 73 from the N-end. In a certain part of the bacteriorhodopsin molecules there is also the rupture of the bond between glycine 65 and leucine 66. The peptides and amino acids resulting from the proteolysis are released from the membrane.

As the ratio between the apparent molecular weights of the medium-weight and light 'papain' fragments is 4 : 3, one may suppose that the point of attack of the high concentrations of papain is localized between the third and the fourth α-helical 'columns' of bacteriorhodopsin (fig.2).

The site that is attacked by thermolysin was found to be localized closer to the C-end than the bond attacked by the diluted solution of papain.

The results obtained should be interpreted as indicating that the regions of the polypeptide chain of bacteriorhodopsin in positions 1–3, 68–72 from the N-end and 1–17 from the C-end contain no functional groups responsible for the activity of bacteriorhodopsin as a photoelectric generator. We can also infer that the structure of bacteriorhodopsin is so effectively stabilized by noncovalent intra- and intermolecular interactions that removal of the regions of the polypeptide chain that are localized outside the hydrophobic layer of the membrane hardly ever affects the functional activity of this protein.

Acknowledgements

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