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The effect of trypsin treatment on the incorporation and energy-transducing properties of bacteriorhodopsin in liposomes

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Bacteriorhodopsin and trypsin-modified bacteriorhodopsin have been reconstituted into liposomes by means of a low pH-sonication procedure. The incorporation of bacteriorhodopsin in these proteoliposomes is predominantly in the same direction as in vivo and the direction of proton pumping is from inside to outside the liposomes. The direction of proton translocation and electrical potential generation was studied as a function of the reconstitution pH. Light-dependent proton extrusion and generation of a Δp, interior negative and alkaline was observed at a reconstitution pH below 3.0 using bacteriorhodopsin, and at a pH below 3.5 using trypsin-modified bacteriorhodopsin. The shift in inflection point is explained in terms of differences between bacteriorhodopsin and trypsin-modified bacteriorhodopsin in a specific protein-phospholipid interaction which depends on the surface charge density of the cytoplasmic side of bacteriorhodopsin. The magnitude of the protonmotive force (Δp) generated by trypsin-modified bacteriorhodopsin in liposomes was quantitated. Illumination of the proteoliposomes resulted in the generation of a high Δp (135 mV, inside negative and alkaline), with a major contribution of the pH gradient. The ionophores nigericin and valinomycin induced, respectively, a compensatory interconversion of ΔpH into $\Delta\psi$ and vice versa. If no endogenous proton permeability of the membrane would exist, a protonmotive force could be generated of -143 mV as electrical potential alone or -162 mV as pH gradient alone.

Introduction

Bacteriorhodopsin contains one single polypeptide and catalyzes light-driven proton translocation across the cytoplasmic membrane in *Halobacterium halobium* [1]. Bacteriorhodopsin has been isolated and purified to homogeneity and various techniques have been described for the reconstitution of this protein into phospholipid vesicles (liposomes). Most procedures yield bacteriorhodopsin proteoliposomes which show light-dependent proton uptake [2–5]. Consequently a protonmotive force (Δp), inside acid and positive, will be generated by bacteriorhodopsin upon illumination of these liposomes. Two different methods have been described for the reconstitution of bacteriorhodopsin into liposomes in the in vivo orientation, e.g., bacteriorhodopsin proteoliposomes that show light-dependent proton extrusion [6,7]. A direct incorporation of bacteriorhodopsin into liposomes, facilitated by short-

Abbreviations: $\Delta\psi$, transmembrane electrical potential; ΔpH, transmembrane pH gradient; Δp, protonmotive force, $\Delta\tilde{\mu}_{H^+}$; Ph_4P^+, tetraphenylphosphonium; Ph_4B^-, tetraphenylboron; S-13, 5-chloro-3-*tert*-butyl-2′-chloro-4′-nitrosalicylanilide; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid.

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chain lecithines, has been described [6]. On the other hand, 'right-side out' bacteriorhodopsin proteoliposomes can also be obtained when purple membranes are cosonicated with liposomes containing acidic phospholipids at low pH (2.4–3.0) for a short period of time [7]. During the sonication step a considerable part of bacteriorhodopsin becomes denaturated. Although these bacteriorhodopsin proteoliposomes show light-dependent proton extrusion, part of the bacteriorhodopsin proteoliposomes have the 'inside-out' orientation. Furthermore, the extent of proton extrusion varies considerably between different batches of purple membranes.

It has been suggested that the distribution of charges over the two sides, e.g., cytoplasmic and extracellular side in bacteriorhodopsin is an important factor in determining the orientation of this protein in the reconstituted membrane [8]. Under native conditions bacteriorhodopsin is highly resistant against proteolytic treatment. Only the C-terminal part of the peptide chain is sensitive to trypsin treatment [9] and this treatment results in the removal of 21 amino acids. This polypeptide fragment contains 5 carboxylic acid groups (3 glutamate, 1 aspartate and the C-terminal serine) which are negatively charged at neutral pH. Removal of this peptide will therefore influence the surface-charge density of the cytoplasmic side of bacteriorhodopsin. In an attempt to optimalize the right-side out reconstitution of bacteriorhodopsin into liposomes, we studied the effect of trypsin treatment of bacteriorhodopsin in purple membranes on the direction of proton pumping and electrical potential generation. In addition, the protonmotive force generated by bacteriorhodopsin in these proteoliposomes was quantitated by the simultaneous measurement of the electrical potential and pH gradient using ion-selective electrodes.

Materials and Methods

Growth of cells and isolation of purple membranes. *H. halobium* NRL (strain R_1) [10] was grown according to Danon and Stoeckenius [11]. Purple membranes were isolated by extensive washing as described [12], except that during all steps 5 μg/ml phenylmethylsulfonylfluoride (PMSF) and 1 mM EDTA were present. Purple membranes resuspended in distilled water (12 mg protein/ml) were stored in liquid nitrogen.

Trypsin treatment of purple membranes. Purple membranes (5 mg protein/ml) were treated with trypsin (0.5 mg protein/ml) for 2 h at 37 °C in 20 mM Tris-HCl (pH 7.0) supplemented with 5 mM $CaCl_2$. Proteolysis was terminated by the addition of a 4-fold (w/w) excess of trypsin-inhibitor (i.e., 2 mg protein/ml). Control experiments were carried out at 4 °C in the presence of trypsin-inhibitor. Trypsin-treated bacteriorhodopsin and native (bacteriorhodopsin) purple membranes were washed twice in a 20-fold volume of 150 mM NaCl (KCl) (pH 6.5) (centrifugation: 30 min; 48 200 × *g*; 4 °C).

Preparation of bacteriorhodopsin proteoliposomes. Bacteriorhodopsin was incorporated into liposomes in the inside-out mode by the sonication method as described [12]. Purple membranes (2.5 mg protein/ml) and cardiolipin (6.25 mg/ml) were cosonicated in 150 mM NaCl (pH 6.5).

Bacteriorhodopsin was reconstituted into liposomes in the rightside out mode as described [7,8]. Purple membranes (2.5 mg protein/ml) were mixed with sonicated cardiolipin liposomes (6.25 mg phospholipid/ml) in 150 mM NaCl (or 150 mM KCl) (pH 6.5). The mixture was acidified to the desired pH, using 1 M HCl, sonicated with a probe-type sonicator (MSE Scientific Instruments, West Sussex, U.K) two times 10 s (interrupted for 10 s) at an amplitude of 2 μm (peak to peak), and the pH was rapidly readjusted to pH 6.5, using 1 M NaOH (KOH).

Measurement of the protonmotive force. $\Delta\psi$, interior negative, was measured with a tetraphenylphosphonium (Ph_4P^+) selective electrode [13] which was inserted in a thermostatted, magnetically stirred vessel. A final concentration of 2 μM Ph_4P^+ was used.

$\Delta\psi$, interior positive, was measured with the same electrode using tetraphenylboron (Ph_4B^-) as the indicator ion [14]. A final concentration of 1 μM Ph_4B^- was used. Measurements were performed in the presence of 0.1 μM Ph_4P^+ to increase the permeability of the negatively charged membranes for Ph_4B^-. This also improved the stability of the electrode and enhanced its response to Ph_4B^-.

ΔpH, interior alkaline, was measured with an ion-selective salicylate electrode as described [15]. A final salicylic acid concentration of 200 μM was used.

Extravesicular pH changes were measured as described [12]. All measurements were performed in a final volume of 2.0 ml. A bacteriorhodopsin concentration of 0.25 mg protein/ml in 150 mM NaCl (pH 6.5) or 150 mM KCl (pH 6.5) was used at 25°C, unless stated otherwise. For pH measurements 150 mM KCl (pH 6.5) was used and experiments were performed in the presence of valinomycin (0.8 nmol/mg phospholipid).

For determination of the maximal Δp generated by bacteriorhodopsin in liposomes, $\Delta\psi$ and ΔpH were measured simultaneously in a 5 ml thermostated polyvinylchloride vessel, in which both an ion-selective Ph_4P^+ electrode and an ion-selective salicylate electrode were inserted [16]. Measurements were performed using a bacteriorhodopsin concentration of 0.98 mg protein/ml (ΔpH) or 0.25 mg protein/ml ($\Delta\psi$) in 10 mM potassium Hepes (pH 7.0), supplemented with 45 mM KCl and 10 mM $MgSO_4$, 2 ml final volume. Valinomycin (400 nM) or nigericin (20 nM) were added as indicated.

Liposomes were illuminated with a slide projector (150 Watt) equipped with fiber optics. The maximal light intensity was 2000 W/m^2.

Calculations. The magnitude of the membrane potential was calculated with the Nernst equation. A correction for Ph_4P^+ and Ph_4B^- binding to the liposomes as described in Ref. 17 was applied. A binding constant of 15–22 was used for Ph_4P^+, and of 3–7 for Ph_4B^- (see Refs. 17 and 18). The magnitude of the pH gradient was calculated with the Nernst equation from the distribution of salicylate between bulk phase and intraliposomal space. A correction for non-linearity of the salicylate-selective electrode response was applied based on a polynomal fit of the calibration curve of the electrode [15]. The sensitivity of the electrodes is expressed as their *Z*-value: the electrode response to a 10-fold increase in probe concentration. A value of 2.0 μl/mg phospholipid was used as internal volume of the bacteriorhodopsin proteoliposomes [19]. The concentration of native bacteriorhodopsin was determined using a molar extinction coefficient at 560 nm of 63000 $M^{-1} \cdot cm^{-1}$ [20]. Denaturation of bacteriorhodopsin was estimated from the decrease in absorption at 560 nm as described [8].

Other analytical methods. Protein [21] was assayed as described. SDS polyacrylamide gel electrophoresis was performed as described [22]. Sucrose gradient density centrifugation was performed as described elsewhere [23].

Materials. Bovine heart cardiolipin, trypsin and trypsin-inhibitor were obtained from Sigma Chemical Co. All other materials were of analytial grade.

Results

Incorporation of trypsin-modified bacteriorhodopsin into liposomes

Cosonication of purple membranes and cardiolipin liposomes at pH 2.7 yielded bacteriorhodopsin proteoliposomes which acidify their suspending medium upon illumination (Fig. 1A). Only a short period of sonication was used, since under the conditions employed bacteriorhodopsin molecules become dissolved from their two-dimensional crystal lattice and diffuse laterally in the plane of the membrane [8]. In its monomeric configuration, bacteriorhodopsin is highly susceptible to low pH [8]. Interestingly prior to sonication, purple membrane fragments can be stored on ice at pH 2.7 for several hours without any significant denaturation (not shown; Ref. 8). Upon trypsin treatment, purple membranes become aggregated as indicated by the turbidity of the suspension. Trypsin treatment resulted in a complete removal of the C-terminal peptide fragment as could be deduced from SDS (16%) gel electrophoresis. One single large product could be identified with a molecular weight of approx. 24500, e.g., 1500 less than the molecular weight of bacteriorhodopsin (not shown). Flocculation of trypsin-treated bacteriorhodopsin was observed upon mixing with cardiolipin liposomes at pH 2.7. However, after a short burst of sonication and pH readjustment a typical blue shift in the visible absorption was observed and a homogeneous suspension was obtained. Illuminated trypsin-treated bacteriorhodopsin proteoliposomes showed a maximal extent of proton extrusion which was considerably higher than observed for native bacterio-

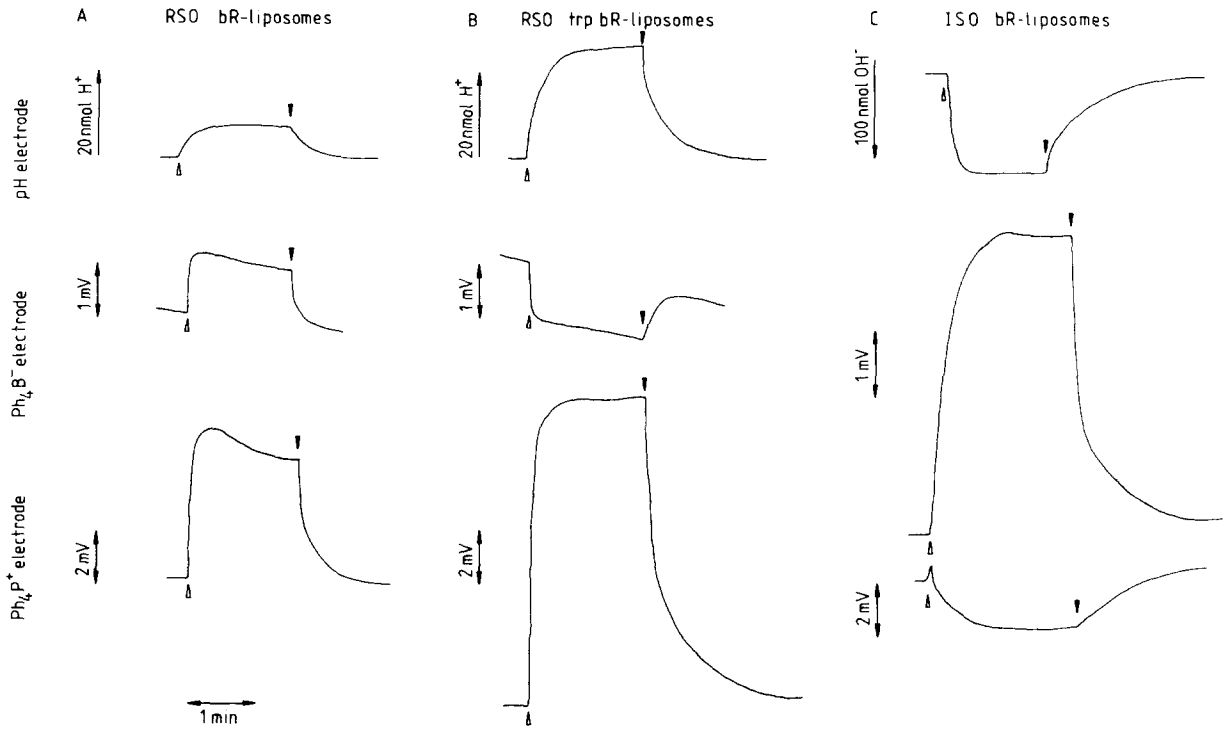


Fig. 1. Light-dependent pH changes, Ph_4B^- and Ph_4P^+ electrode responses in the presence of proteoliposomes reconstituted from cardiolipin and purple membranes. (A) Purple membranes reconstituted at pH 2.7 by the low pH-sonication procedure. (B) As (A), except that trypsin-modified purple membranes were used. (C) Purple membranes reconstituted at pH 6.5 by the sonication procedure. pH changes were recorded in the presence of 0.8 nmol valinomycin per mg of phospholipid. Ph_4P^+ and Ph_4B^- electrode responses were recorded in the presence of 20 pmol triphenyltin per mg phospholipid. Light was switched on and off at maximal light intensity as indicated by the arrows.

rhodopsin (Fig. 1B). Light-induced proton extrusion by trypsin-treated bacteriorhodopsin proteoliposomes was stimulated 1.5- to 2-fold by the addition of the $\Delta\psi$ dissipating ionophore valinomycin. This was not observed with bacteriorhodopsin proteoliposomes reconstituted with native bacteriorhodopsin.

Removal of the C-terminal peptide fragment did not significantly alter the capacity of bacteriorhodopsin to pump protons, as was shown by others [24]. Reconstitution of bacteriorhodopsin and trypsin-treated bacteriorhodopsin into cardiolipin liposomes in the inside-out mode by sonication at neutral pH, yielded comparable values of proton uptake of 4.5–5.3 H^+/bacteriorhodopsin (Fig. 1C).

The right-side-out bacteriorhodopsin proteoliposomes were further characterized for their ability to generate a $\Delta\psi$. Upon illumination a transient uptake of Ph_4P^+ was observed in both types of liposome reconstituted at low pH. In the presence of triphenyltin, which collapses the ΔpH by an electroneutral exchange of Cl^- for OH^- [25], a steady-state $\Delta\psi$ was generated by the trypsin-treated bacteriorhodopsin proteoliposomes (Fig. 1B). Illuminated bacteriorhodopsin proteoliposomes showed an overshoot in Ph_4P^+ accumulation, while the steady-state level was low compared to the trypsin-treated bacteriorhodopsin proteoliposomes (Fig. 1A). This overshoot is most likely explained by the presence of inside-out bacteriorhodopsin proteoliposomes, since low levels of Ph_4B^- accumulation were observed in the same preparation upon illumination (Fig. 1A). The trypsin-treated bacteriorhodopsin proteoliposomes on the other hand extruded Ph_4B^- upon

illumination (Fig. 1B) which must imply that the majority of trypsin-treated bacteriorhodopsin particles generate a $\Delta\psi$, interior negative, upon illumination.

Inside-out bacteriorhodopsin proteoliposomes showed light-induced extrusion of Ph_4P^+ and accumulation of Ph_4B^- (Fig. 1C).

In agreement with previous studies [7,8] an inversion of the direction of proton translocation upon illumination could be observed when the pH of the acid stage of the reconstitution procedure was varied (Fig. 2A). The inflection point was found around pH 3.0 for bacteriorhodopsin proteoliposomes. When trypsin-treated bacteriorhodopsin was used instead of native bacteriorhodopsin, the extent of light-dependent proton extrusion over the whole pH range tested was found to be larger (Fig. 2A). Furthermore, a shift of the inflection point to a higher pH (approx. 3.4) was observed. To substantiate the improved homogeneity of the transmembranous incorporation of trypsin-treated bacteriorhodopsin in the right-side-out mode, light-dependent Ph_4P^+ and Ph_4B^- uptake was investigated, while the pH of the acidic phase of the reconstitution was varied, trypsin-treated bacteriorhodopsin proteoliposomes were found to accumulate Ph_4P^+ over about the whole reconstitution pH range with an apparent inflection point around pH 3.5 (Fig. 2B). Bacteriorhodopsin proteoliposomes already started to extrude Ph_4P^+ at a reconstitution pH of about 3.2, while Ph_4B^- accumulation was already found at pH 2.6 (Fig. 2B and C). Trypsin-treated bacteriorhodopsin proteoliposomes extruded Ph_4B^- at a reconstitution pH below 3.2 (Fig. 2C). Uptake of both Ph_4P^+ and Ph_4B^- as found for bacteriorhodopsin proteoliposomes implies that a large degree of heterogeneity with respect to the orientation of bacteriorhodopsin exists in this preparation (pH < 3.3). On the other hand, uptake of Ph_4P^+ and extrusion of Ph_4B^- as found for trypsin-treated bacteriorhodopsin proteoliposomes reconstituted at pH < 3.3 suggests that these preparations are virtually completely devoid of bacteriorhodopsin proteoliposomes that generate a Δp, interior acid and positive, upon illumination. When trypsin-treated bacteriorhodopsin proteoliposomes and bacteriorhodopsin proteoliposomes, reconstituted

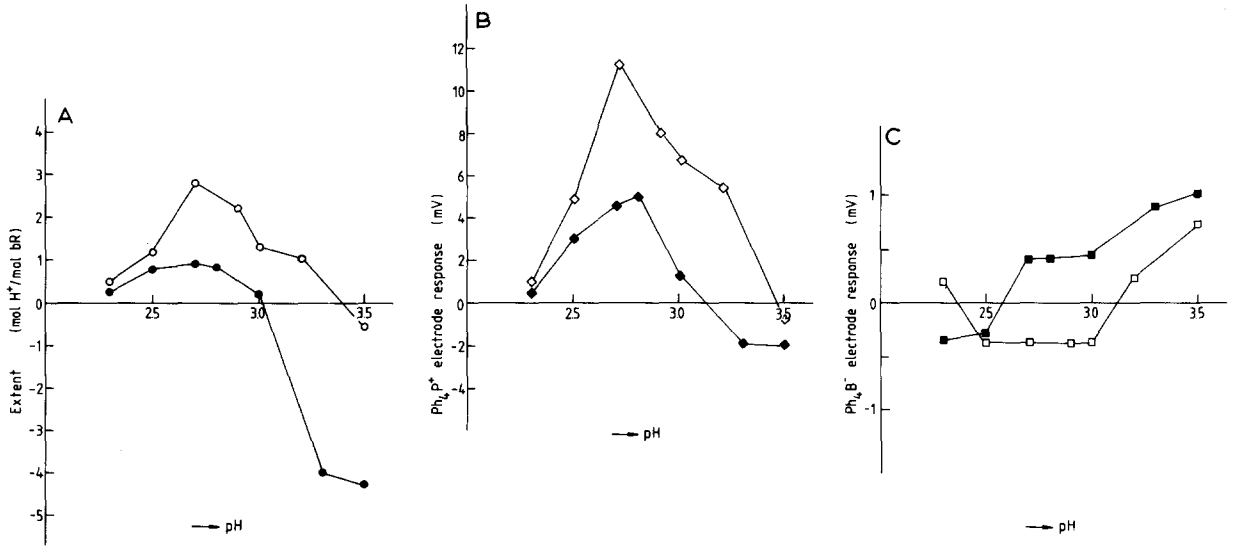


Fig. 2. Dependence of the direction of proton-translocation and $\Delta\psi$ generation by proteoliposomes on the pH during reconstitution with cardiolipin. (A) Extent of proton extrusion expressed in mol H^+ per mol native bacteriorhodopsin. A negative extent indicates proton uptake. (B) Generation of a $\Delta\psi$, interior negative, expressed as Ph_4P^+ electrode response in mV. A positive response indicates Ph_4P^+ accumulation. The z of the electrode was 52 mV. (C) Generation of a $\Delta\psi$, interior positive, expressed as Ph_4B^- electrode response in mV. The z of the electrode was 12.4 mV. Filled symbols represent the responses found for bacteriorhodopsin proteoliposomes and the open symbols for trypsin-treated bacteriorhodopsin proteoliposomes. Experimental conditions were as described in the legend to Fig. 1 and Materials and Methods.

at pH 2.7 are compared by sucrose-density gradient centrifugation no difference was observed (not shown). A small fraction of protein-free liposomes was visible. On the other hand, virtually no free bacteriorhodopsin was detectable, indicating that the differences observed between native bacteriorhodopsin and trypsin-treated bacteriorhodopsin are not due to different amounts of bacteriorhodopsin reconstituted. The degree of denaturation increased linearly with the pH of the acid stage from 55% at pH 3.0 to 70% at pH 2.3. In this respect no difference was found between bacteriorhodopsin and trypsin-treated bacteriorhodopsin (not shown).

Quantitation of Δp in trypsin-treated bacteriorhodopsin proteoliposomes

The homogeneous, in vivo incorporation of bacteriorhodopsin into liposomes with respect to Δp generation, allows a quantitation of the magnitude of the protonmotive force (Δp), generated upon illumination. In addition to measurements of the $\Delta\psi$, interior negative, by the use of a Ph_4P^+-selective electrode [13], the ΔpH, interior alkaline, can most conveniently be measured by the use of an anion-selective electrode [15]. This electrode senses the external concentration of the dissociated form of the weak acid, salicylic acid. The use of ion-selective electrodes offers the possibility to measure the external concentration of the probes continuously with a high sensitivity without the need to separate the liposomes from their external medium. Furthermore, both components of the Δp can be measured simultaneously.

Illumination of trypsin-treated bacteriorhodopsin proteoliposomes (reconstituted at pH 2.7) results in a transient generation of a $\Delta\psi$, as demonstrated by the rapid accumulation of Ph_4P^+, which is followed by a slow release (Fig. 3). Simultaneously, a ΔpH was generated, as demonstrated by the uptake of salicylate (Fig. 3). Both $\Delta\psi$ and ΔpH collapsed when the light was turned off.

Upon addition of the trypsin-treated bacteriorhodopsin proteoliposomes to medium, the external Ph_4P^+ concentration not only decreased as a result of dilution of the suspending medium, but also as a result of energy independent binding of Ph_4P^+ to the membranes. As reported earlier

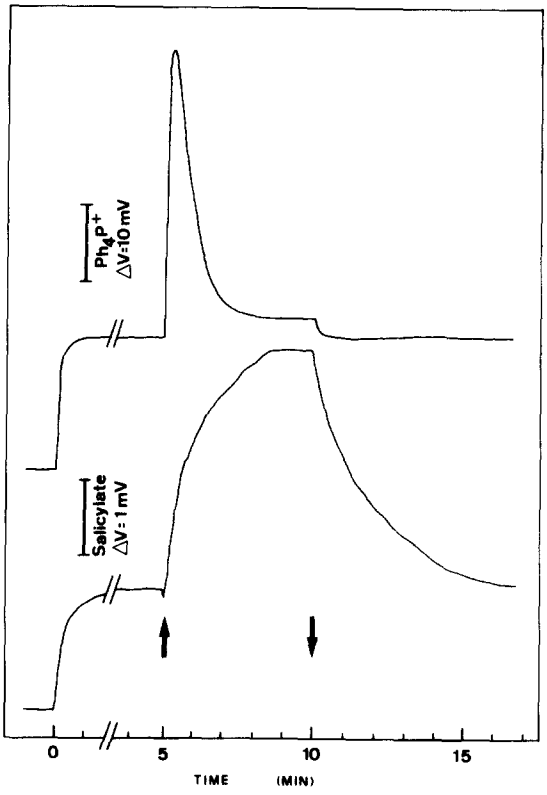


Fig. 3. Simultaneous registration of the external Ph_4P^+ and salicylate concentration in trypsin-treated bacteriorhodopsin proteoliposomes. Light was switched on and off at maximal intensity as indicated by the arrows 37.7 μM bacteriorhodopsin (about 70 pmol bacteriorhodopsin per mg phospholipid) was used. Experimental details were as described in Materials and Methods.

[18,26], binding of Ph_4P^+ was largely diminished by the addition of Mg^{2+}. Energy-independent binding of Ph_4P^+ increased linearly with increasing external Ph_4P^+ concentration. Even in the presence of 100 μM Ph_4P^+ no saturation of binding was observed (not shown). Binding of Ph_4P^+ appears to be equally distributed over the internal and external surface of the membrane. Imposition of a $\Delta\psi$, interior positive, by the use of an inwardly directed valinomycin-mediated potassium diffusion gradient resulted in a 48% $\pm$ 8% decrease of the amount Ph_4P^+ bound to the membranes. Therefore, to quantitate the $\Delta\psi$, Ph_4P^+ accumulation was corrected for concentration-dependent binding of Ph_4P^+, according to the model of Lolkema et al. [17]. Recently, we showed that a

good correlation exists between the imposed and calculated potentials in liposomes, when a correction was made for binding of Ph_4P^+ [18]. The external salicylate concentration decreased only by dilution of the medium upon addition of the membrane suspension. No binding of salicylate was observed, therefore no correction for probe binding was made.

At pH 7.0, the main component of the Δp is the ΔpH (Table I). The transient generation of the $\Delta\psi$ and the low steady-state value of the $\Delta\psi$ (less than 20 mV) suggests that rapid charge-compensating ion movements occur. A nearly complete interconversion of the ΔpH into $\Delta\psi$ could be induced by the ionophore nigericin (Table I). Nigericin catalyzes electrically neutral exchange of protons for K^+ or Na^+, thereby collapsing ΔpH with a compensatory increase in $\Delta\psi$. On the other hand, dissipation of the $\Delta\psi$ by valinomycin resulted in a compensatory increase of ΔpH (Table I). Although further addition of valinomycin resulted in a complete dissipation of $\Delta\psi$, also a decrease of Δp (ΔpH) was observed. At high concentrations, valinomycin does not only act as an ionophore, but it also directly inhibits the turnover of bacteriorhodopsin [27]. In order to evaluate the efficiency of energy conversion in bacteriorhodopsin proteoliposomes, Westerhoff and Van Dam [28] and Hellingwerf and his coworkers [29] studied the effect of increased proton permeability on the protonmotive force. Using a mosaic non-equilibrium thermodynamic description of ion translocation in bacteriorhodopsin proteoliposomes, it was predicted that a linear relation exists between the reciprocal Δp and the proton leakage (L_H^l) of the membrane, according to Eqn. 1:

$$\frac{1}{\Delta p} = \frac{1}{n(1-2\alpha)L_\nu A_\nu} L_H^l - \frac{1}{(1-2\alpha)A_\nu} \text{ (mV)} \quad (1)$$

in which n is the number of protons pumped by bacteriorhodopsin per fully coupled photochemical cycle; α, the fraction of bacteriorhodopsin molecules in the right-side out orientation, e.g., for the in vivo orientation of bacteriorhodopsin, or α represents the fraction of inside-out oriented bacteriorhodopsin molecules; L_ν, the activity of bacteriorhodopsin, which is proportional to the light intensity; and A_ν, the effective thermodynamic force on bacteriorhodopsin exerted by illumination. Measurement of the Δp with different amounts of uncoupler present (to vary L_H^l) at different light intensities (to vary L_ν), resulted in a series of lines, intersecting in one point. The reciprocal Δp at this point equals:

$$\frac{n}{(1-2\alpha)A_\nu} \quad (2)$$

In general terms, from this type of experiment an estimate can be made of the Δp generated under conditions that the endogenous ion leakage of the membrane would equal zero. Under those conditions, the Δp generated would only depend on the properties of the proton pump. This maximal Δp can only be estimated if the proton permeability of the membranes increases linearly with the amount of added uncoupler. The linearity of the proton permeability with uncoupler concentration has been demonstrated by Arents et al. [29] for the uncoupler S-13. Eqn. 1 has been verified using bacteriorhodopsin proteoliposomes with an inside-out orientation [28,29].

The effect of S-13 on steady-state ΔpH (Fig. 4) and $\Delta\psi$ (Fig. 5) at different light intensities (obtained by using neutral density filters) by trypsin-treated bacteriorhodopsin proteoliposomes has been evaluated. ΔpH was measured in the presence of valinomycin and a high concentration of K^+ (55 mM). Under these conditions the $\Delta\psi$ was found to be smaller than -5 mV. The reciprocal of the ΔpH plotted against the uncoupler con-

TABLE I

QUANTITATION OF $\Delta\psi$ (INTERIOR NEGATIVE) AND ΔpH (INTERIOR ALKALINE) IN TRYPSIN-TREATED BACTERIORHODOPSIN PROTEOLIPOSOMES UPON ILLUMINATION

37.7 μM bacteriorhodopsin (about 70 pmol bacteriorhodopsin per mg phospholipid) was used for the Δp measurements. Nigericin and valinomycin were used at a final concentrations of 80 nM and 400 nM, respectively.

Ionophore	$\Delta\psi$	$-Z\Delta$pH (mV)	Δp
No additions	−19	−115	−134
Nigericin	−137	0	−137
Valinomycin	−3	−125	−128

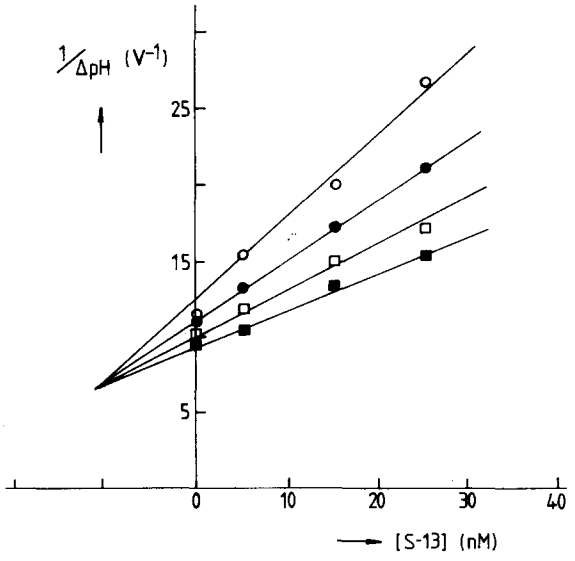
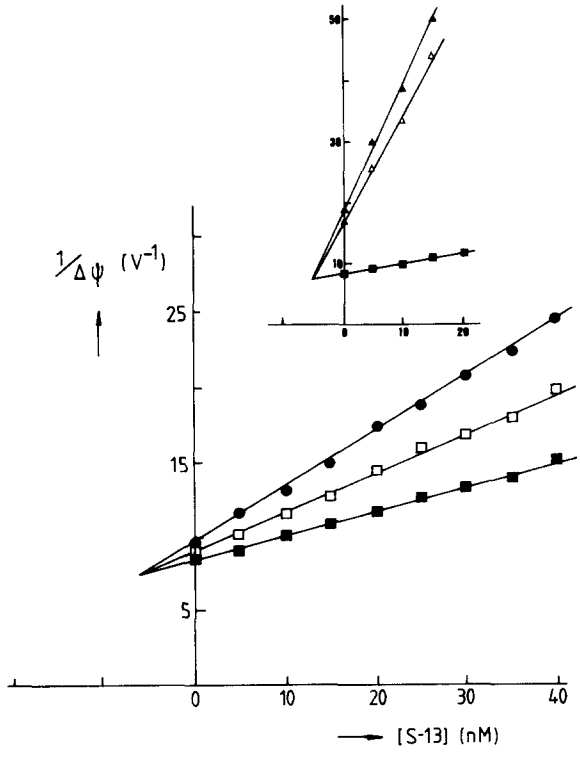


Fig. 4. Determination of the ΔpH generated by trypsin-treated bacteriorhodopsin in liposomes in the absence of endogenous proton leakage. ΔpH was measured with a salicylate selective electrode as described in Materials and Methods. Further experimental details were as described in the legend to Table I. The turnover of bacteriorhodopsin was varied by different light intensities (% of maximal light intensity): ■, 100%; □, 79%; ●, 57%; and ○, 38%.



centration at different light intensities yielded straight lines (correlation coefficient, 0.97–0.99) intersecting at the vertical axis (Fig. 4). The endogenous proton leakage of these bacteriorhodopsin proteoliposomes equals a leak created in these membranes by the addition of 4.9 pmol S-13 per mg phospholipid. The extrapolated maximal ΔpH (Δp) equals -162 mV (± 10 mV).

$\Delta\psi$ was measured in the presence of nigericin and K^+. ΔpH was found to be smaller than -5 mV under these conditions. Again, a plot of the reciprocal $\Delta\psi$ against the uncoupler concentration at different light intensities, yielded straight lines (correlation coefficient, 0.997–0.999), intersecting at the vertical axis (Fig. 5). An endogenous proton leakage equivalent to 7.4 pmol S-13 per mg of phospholipid was found, while the extrapolated maximal (Δp) equalled -143 mV (± 8 mV). Similar results were obtained when the experiments were performed at low light intensities (low bacteriorhodopsin turnover) (inset Fig. 5).

Discussion

The results presented in this paper clearly demonstrate that modification of bacteriorhodopsin by trypsin treatment affects its properties. Upon reincorporation of trypsin-treated bacteriorhodopsin into liposomes by a low pH-sonication procedure [7,8], a homogeneous right-side-out bacteriorhodopsin proteoliposome preparation, with respect to the Δp generation, can be obtained. By the use of ion-selective electrodes, Δp in both directions could be measured independently, which offers a more rigorously characterization of Δp generation by bacteriorhodopsin proteoliposomes. Bacteriorhodopsin proteoliposomes reconstituted with trypsin-treated bacterio-

Fig. 5. Determination of the $\Delta\psi$ generated by trypsin-treated bacteriorhodopsin in liposomes in the absence of endogenous proton leakage. $\Delta\psi$ was measured with a Ph_4P^+ selective electrode as described in Materials and Methods. A final concentration of 9.6 μM bacteriorhodopsin was used and the experiment was performed in the presence of 20 nM nigericin. Further experimental details are described in the legend to Table I and in Materials and Methods. The turnover of bacteriorhodopsin was varied by varying the light intensities (% of maximal light intensity): ■, 100%; □, 31.6%; ●, 10%; △, 1%; and ▲, 0.1%.

rhodopsin at a pH between 2.5 and 3.0 showed in the light: (i) valinomycin-stimulated proton extrusion, (ii) Ph_4P^+ accumulation, (iii) Ph_4B^- extrusion and (iv) salicylate accumulation. No differences in incorporation efficiency or denaturation due to the low pH treatment were observed between native and trypsin-treated bacteriorhodopsin. These observations strongly suggest that these bacteriorhodopsin proteoliposomes are virtually free from inside-out bacteriorhodopsin proteoliposomes. Bacteriorhodopsin proteoliposomes reconstituted with native purple membrane fragments had distinctly different properties. An intriguing question is why the extent of proton uptake by native bacteriorhodopsin proteoliposomes was found to vary considerably from batch to batch of purple membrane fragments, as reported by Happe et al. [7] even in one batch from experiment to experiment. Recently it was found by Arrio et al. [30] that purple membrane fragments contain protease activity which was inhibited by benzamidine indicating trypsin-like activity. They suggested that stacking of purple membranes is a result of low-level proteolysis. This stacking phenomenon is also observed upon trypsin treatment, while at acidic pH the purple membrane fragments even tend to flocculate. Slow, but uncontrolled proteolysis during storage might explain the differences in reconstitution behaviour between the different batches of purple membranes. In this respect it should be emphasized that purple membranes isolated from *H. halobium* in the presence of protease inhibitors are very poor samples for reconstitution in the right-side-out mode.

The observation that the inflection pH of reconstitution shifts to a higher pH when bacteriorhodopsin was treated with trypsin is of interest. Removal of the highly negatively charged C-terminal peptide most probably decreases the net negative surface charge density of the cytoplasmic side of bacteriorhodopsin. At neutral pH, bacteriorhodopsin is mainly negatively charged with the highest charge density at the cytoplasmic side. At low pH an inversion of the side with the highest negative charge density takes place, since at pH 3 preferentially, binding of the extracellular side to polylysine-coated glass is observed [29]. The positively charged lysine residues at the cytoplasmic side will promote binding of this side to the negatively charged liposomes. After successful reconstitution the cytoplasmic side will face the inner surface of the phospholipid bilayer. After removal of the highly negatively charged C-terminal peptide by trypsin treatment, inversion of the side with the highest negative charge density will occur at a higher pH. This implies that a specific interaction between bacteriorhodopsin and the negatively charged phospholipids can take place at a higher pH. This is exactly what has been observed.

An alternative explanation is that incorporation of bacteriorhodopsin into liposomes is the result of low pH induced fusion between purple membrane fragments and the negatively charged liposomes. Cardiolipin liposomes fuse at pH values below the pK of this phospholipid (3.5 [8]). It is, however, difficult to envision how fusion can be so specific that bacteriorhodopsin is unidirectionally right-side out incorporated into liposomes. Furthermore, it is not clear how trypsin modification of bacteriorhodopsin would affect this fusion event. The effect of trypsin treatment of bacteriorhodopsin on its orientation in liposomes reconstituted by the low pH-sonication procedure are in agreement with a previous proposition [8], that the distribution of charges over the two sides, e.g., the cytoplasmic side and external side of bacteriorhodopsin, determines the orientation.

The second part of the paper deals with the quantitation of the Δp in trypsin-treated bacteriorhodopsin proteoliposomes. The homogeneity of the direction of proton pumping by these proteoliposomes offers the possibility to quantitate the Δp generated upon illumination. Although more than 55% of the bacteriorhodopsin molecules become denaturated due to the low pH treatment, still Δp values of approx. -135 mV were found. The magnitude of Δp in these proteoliposomes is in the same order as found for the reduction of cytochrome *c* mediated by cytochrome *c* oxidase, reconstituted in liposomes [18,24]. In contrast to cytochrome-*c* oxidase, only a minor contribution of the $\Delta\psi$ to the steady state Δp was observed in bacteriorhodopsin proteoliposomes.

Ionophores are able to induce a nearly complete interconversion of $\Delta\psi$ into ΔpH, and vice versa in trypsin-treated bacteriorhodopsin proteoliposomes. In contrast, collapse of a $\Delta\psi$ of about

-100 mV by valinomycin in cytochrome-*c*-oxidase proteoliposomes, results only in a small increase (20–30 mV) in ΔpH [18,26]. Interconversion of ΔpH into $\Delta\psi$ was found to be nearly complete. The magnitude of the ΔpH will be determined by the activity of the Δp-generating system and the internal buffer capacity. The latter is most probably not very different for bacteriorhodopsin and cytochrome-*c* oxidase when reconstituted into cardiolipin liposomes. Therefore, the activity of cytochrome-*c* oxidase is most likely the limiting factor in the conversion of $\Delta\psi$ into ΔpH. In this respect, ΔpH and $\Delta\psi$ appear to affect respiratory control in a similar manner, since dissipation of the $\Delta\psi$ with a small increase in ΔpH by valinomycin decreases the coupling ratio. On the other hand, dissipation of ΔpH by nigericin with a compensatory increase of $\Delta\psi$ does not alter the coupling ratio (De Vrij, W., unpublished results).

The trypsin-treated bacteriorhodopsin proteoliposomes were analysed for the protonmotive force which can be generated maximally upon illumination (A_p). The use of ion-selective electrodes for these measurements allows a more accurate determination of A_p. A correction was made for the endogenous proton permeability of the membrane using an uncoupler/turn-over (L^1_H/L_p) tritration as described [8,28,29]. A value of -143 mV was found for the $\Delta\psi$, and a value of -162 mV (2.7 pH units) was found for the $\Delta\psi$, and a value of -162 mV (2.7 pH units) was found for the ΔpH. These values should be interpreted as lower limits, since in order to determine the A_p, the fraction of bacteriorhodopsin molecules $1-\alpha$ pumping proton in the vesicle interior should be known (see Eqns. 1 and 2). Hellingwerf and coworkers [29] made an estimate of α for bacteriorhodopsin molecules with the in vivo orientation in a population of inside-out bacteriorhodopsin proteoliposomes. The amount of the C-terminal amino-acid serine accessible to carboxypeptidase was determined. In view of the results of Arrio et al. [30], which indicate that part of the bacteriorhodopsin molecules in purple membranes are already devoid of their C-terminal peptide, the value of α determined by the carboxypeptidase assay should be considered as a lower limit, which might result in a lower A_p than reported (Table II). The maximal protonmotive force, uncorrected for bacteriorhodopsin molecules with an opposite direction of proton pumping in inside-out bacteriorhodopsin proteoliposomes, is in the same order as reported in this study for right-side-out bacteriorhodopsin proteoliposomes (Table II). The uncertain factor of bacteriorhodopsin molecules with an opposite direction not only affects A_p determinations by L^1_H/L_p titrations, but also affects the effect of an external electrical field on $\Delta\psi$ generation by bacteriorhodopsin reconstituted into planar bilayers (Table II). It should be emphasized that a two-fold increase in internal volume results in a decrease in the calculated ΔpH values of 18 mV, while a 2-fold decrease in the internal volume results in an increase of the calculated ΔpH with 18 mV. However, since A_p is determined by extrapolation, A_p is hardly affected by either a two-fold increase or decrease in the internal volume. This additional error hardly adds to the already indicated error of ± 10 mV. $\Delta\psi$ measurements are even less affected by a possible error in the internal volume due to nonspecific binding of Ph_4P^+, which largely determines the amount of accumulated probe [17]. The A_p determined with proteoliposomes absorbed or fused to planar bilayers [32,33] is relatively high compared to the A_p values listed in Table II, the method with planar bilayers has the drawback that part of the externally applied electrical field drops across the supporting bilayer phospholipid membrane [34]. In this respect, the patch pipet method [35] seems to have several advantages. This method has been applied to cytochrome-*o* oxidase from *Escherichia coli*. But also in this case, the uncertainty remains how large the contribution of opposite-oriented oxidase molecules in the Δp generation is. This problem does not exist for cytochrome-*c* oxidase when it is reduced by the membrane impermeable electron-donor cytochrome *c*. The A_p determined for bovine heart [18] and *Bacillus subtilis* cytochrome-*c* oxidase by the L^1_H/L_p titration procedure is in the same order of magnitude as found for bacteriorhodopsin with the L^1_H/L_p titration procedure or cytochrome-*o* oxidase with the patch pipet procedure (Table II).

One critical aspect of L^1_H/L_p titrations is that L^1_H should increase in a linear fashion with the amount of added uncoupler. This has been experimentally verified for inside-out bacteriorhodopsin

TABLE II

FUNCTIONAL COMPARISON OF PROTONMOTIVE-FORCE-GENERATING SYSTEMS

(±), no specified direction of Δp generation required. ( ), Protonmotive force corrected for orientation (A_ν).

Method		Procedure	Maximal Δp	Ref.
Bacteriorhodopsin				
$\Delta\psi$	planar bilayer	ext. field	(±)200 mV	38
	planar bilayer/proteoliposome	ext. field	(±)230 mV	33,34
	proteoliposomes	L^1_H/L_νtit. Ph_4P^+	−143 mV	this paper
ΔpH	proteoliposomes	L^1_H/L_νtit. CH_3NH_3	+122 mV (+200 mV)	29
		L^1_H/L_νtit. pH electrode	+160 mV (+270 mV)	29
		L^1_H/L_νtit. salicylate	−162 mV	this paper
Cytochrome-*c* oxidase				
Bovine heart				
$\Delta\psi$	planar bilayer/proteoliposome	ext. field	(±)210 mV	34
	proteoliposomes	L^1_H/L_νtit.[a] Ph_4P^+	−130 mV	18
B. subtilis				
$\Delta\psi$	proteoliposomes	L^1_H/L_νtit.[b] Ph_4P^+	−130 mV	18
Cytochrome-*o*-oxidase				
E. coli				
$\Delta\psi$	patch pipet	ext. field	(±)150 mV	35

[a] Cytochrome *c* was clamped at 20% reduction.
[b] Cytochrome *c* was clamped at 50% reduction.

proteoliposomes [29], in which it was shown that the initial proton efflux rate at fixed ΔpH increased linearly with the uncoupler concentration. On the other hand, the proton permeability of the membrane should be a linear function of the Δp. The proton conductivity of the membrane has been found to be a linear function of the ΔpH [36,37]. However, the proton conductivity increased in a non-linear fashion with the $\Delta\psi$ [36,37], although a linear relation was found as long as the $\Delta\psi$ did not exceed 140 mV. This is the situation in our experiments. Furthermore, the lines obtained from L^1_H/L_ν titrations (Fig. 5) suggest that a possible nonlinear relation between the $\Delta\psi$ and the proton conductivity does not lead to a significant deviation of the linear relationship.

In conclusion, trypsin modification of bacteriorhodopsin improves the reconstitution of bacteriorhodopsin into liposomes in the in vivo orientation, as demonstrated by Δp measurements in both directions. Bacteriorhodopsin is able to generate a large Δp in these liposomes upon illumination. This makes bacteriorhodopsin an attractive protein for generating a protonmotive force in liposomes or fused membranes for studies on energy-transducing systems [23,26].

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