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Proteoliposomes with right-side-out oriented purple membrane/bacteriorhodopsin require cations inside for proton pumping

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Proteoliposomes were prepared by sonication of phospholipids and blue membranes (cation-free purple membranes carrying little activity of light-driven proton pumping) in an acidic medium of very low ionic strength. The majority of the bacteriorhodopsin population in these proteoliposomes was in the right-side-out (as in living cells) orientation as judged from the resultant polypeptides after papain digestion. By raising the pH of sonication, the population of right-side-out oriented bacteriorhodopsin decreased, and consequently that of the inversely oriented one increased. In KCl and NaCl up to certain concentrations or in choline chloride even at high concentrations, in the light, the proteoliposomes with right-side-out bacteriorhodopsin did not pump protons, whereas those with inversely oriented bacteriorhodopsin did. The former began to pump only after cations were likely incorporated/permeated into the proteoliposome and reached the carboxyl terminal (cytosol) side of bacteriorhodopsin/purple membrane.

Bacteriorhodopsin; Proton pumping; Purple membrane; Proteoliposome

1. INTRODUCTION

Bacteriorhodopsin (bR) is a light-driven proton pump [1] which transports protons from the cytosolic side of the plasma membrane (the carboxyl-terminal side of the molecule [2]) to the external medium (the amino-terminal side). The mechanism of proton pumping is not fully understood, although various pieces of information have been accumulated, such as the involvement of essential carboxyl residues [3].

The bR/purple membrane binds stoichiometric amounts of divalent cations which are removable by cation exchange [4] or repeated washing [4,5]. Removal of the bound cations causes a shift in the absorption maximum from 568 nm (purple membrane) to 600 nm (blue membrane) together with disruption of the photocycle and proton pumping [6]. The blue membrane can be titrated back to purple membrane with cations [4,5], including organic cations and cationic dyes [7]. The bound cations, 5–6 per bR [5,8], are considered to be involved in electrochemical phenomena on the purple/blue membrane surface [4,8]. Some, but not all of the bound cations may also be involved in proton pumping [9], although internal carboxyl groups are little affected by cation binding [10].

bR/purple membrane can be incorporated into the liposome membrane of phospholipids [11]. These proteoliposomes can accumulate protons under actinic illumination, because of the inverted orientation (with respect to the in vivo orientation) of the bR/purple membrane in the liposomal membrane [11]. This inverted orientation is believed to be due to the excess negative charges on the cytosolic side of the bR/purple membrane [2] in ordinary preparation media at around neutral pH. Happe et al. [12] reported that at pH 2.5 (where fewer negative charges were expected to be present), proteoliposomes with bR/purple membrane

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in the right-side-out orientation could be formed by sonication for 30 s. However, formation of those right-side-out proteoliposomes showed poor reproducibility and bR was unstable in the preparative process as noted by the authors.

We were able to prepare right-side-out proteoliposomes of much better reproducibility, by sonication of a mixture of the blue membrane and phospholipids for a rather long time. Here, we describe a preparative method for such proteoliposomes.

We also report that with right-side-out (as in living cells with the C-termini exposed inside) proteoliposomes, light-driven proton pumping was detected only when cations (except protons) permeated inside the vesicles.

2. MATERIALS AND METHODS

bR/purple membrane was separated from the membrane fraction of *Halobacterium halobium (salinarium)* R_1 as in [13]. bR/purple membrane was then deionized on a column (15 × 10 cm) of Dowex C-50 similarly to the method in [4], the blue membrane formed being washed with and suspended in deionized water in a plastic vessel.

Liposomes were prepared by sonication (Heat Systems-Ultrasonics, W-225R; 1/2′ flat tip, output 5, duty cycle 50%) for 2 min, 4 times with 3-min intervals at 10°C with desiccated lipids and deionized water (10 ml) in a flat-bottomed vial (Wheaton, 20 ml). The lipid composition for a routine batch was 100 mg phosphatidylcholine (Sigma, St. Louis, type IV-S) and 2 mg dicetyl phosphate (Sigma). Proteoliposomes were then reconstituted by sonication (power 3, microtip, 1 min, 4 times with 3-min intervals; other conditions as for liposome preparation) of a mixture of liposomes and freshly prepared blue membranes (about 1 mg bR/batch of 3 ml; lipid/protein ratio 30–50) at a given pH (adjusted with HCl). The pH of the proteoliposome suspension was then brought to neutral with NaOH and 5 mM NaCl was further added for improving stability during storage.

Proton pump activity was investigated after the proteoliposome suspension had been diluted with an adequate volume of various salt solutions. The diluted suspension, in a glass vessel at 20°C, was illuminated using a 750 W projector (>500 nm, about 10^5 lux). The pH of the suspension was recorded with an assembly of a combination electrode, amplifier and recorder. The initial pH of the suspension was adjusted to around neutral with NaOH solution.

Proteoliposomes (0.3 mg protein/ml) in 10 mM Tris and 1 mM dithiothreitol at pH 7.0 were digested with papain (Boehringer, 4 μg/ml) for 9 h at 30°C as in [14]. Digested samples were then dissolved in SDS (1%) and electrophoresed in polyacrylamide gel (12.5%) in the presence of 0.1% SDS according to Laemmli [15]. The gel was stained with Coomassie brilliant blue, destained by an ordinary procedure, and analysed on a gel densitometer (Advantec-Toyo, Tokyo, DMU-33C).

Valinomycin and A23187 were purchased from Sigma, and all reagents used were of the highest grade available from Yashima Chemicals, Osaka.

3. RESULTS AND DISCUSSION

The proteoliposomes reconstituted at three different pH values showed different patterns of the light-induced pH change in 0.5 M KCl (fig.1). Upon actinic illumination, those prepared at pH 3.52 demonstrated a pH decrease (proton efflux) and those at pH 6.71 a pH increase (proton influx). The former suggested the predominance of right-side-out oriented over inversely oriented bR/purple membranes and vice versa for the latter. The inverted orientation has been observed in ordinary reconstitution experiments [11]. Proteoliposomes prepared between these pH values showed complex patterns; the pH 4.10 preparation initially showed a pH increase and then spontaneously a decrease in the light. The complex pattern suggested a mixture of bR/purple membranes oriented in two directions. The direction of the pH change suggested that the higher reconstitution pH (more negative charges on bR/purple membrane) resulted in a larger population of inverted bR/purple membranes.

It was noted that the initial rate of pH increase was greater than that of pH decrease (see below). This difference appeared to be the cause of the complex pH change patterns. The steady-state pH changes were within some hundreds of nmol H^+/mg bR in both directions, within the ranges reported for ordinary proteoliposomes with inversely oriented bR/purple membranes prepared by sonication [16].

The SDS-PAGE patterns of papain-digested proteoliposomes showed that a polypeptide band of lower M_r appeared to a greater extent in proteoliposomes reconstituted at higher pH (fig.2). This smaller peptide was formed by removal of the 17 amino acid segment from the C-terminus of bR [14]. Therefore, the densitograms suggested that reconstitution at higher pH increased the population of bR molecules with C-termini exposed to attack of papain (from outside of the vesicles). This is qualitatively consistent with the process postulated above from the direction of the light-induced pH change (fig.1).

These results are fundamentally the same as

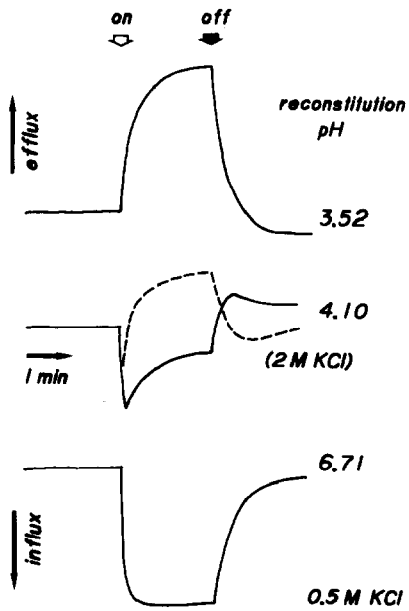

Fig.1. Dependence of light-induced pH changes of proteoliposomes on pH of reconstitution. Proteoliposomes were reconstituted with lipids and the blue membrane at the given pH values. Each preparation was suspended in 0.5 M KCl (0.05 mg protein/ml), and 3 min after the suspension was illuminated (on) for 2 min (off) and the pH change recorded. The broken line (center) shows the pH change recorded in 2 M KCl instead of 0.5 M.

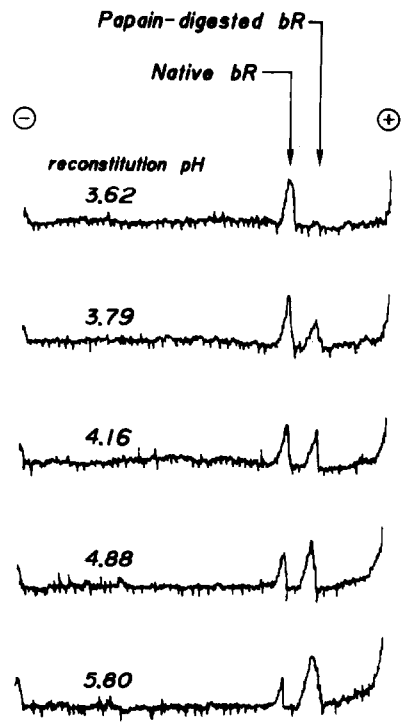

Fig.2. SDS-PAGE densitograms of bacteriorhodopsin in proteoliposomes reconstituted at different pH values and digested with papain. Proteoliposomes were reconstituted at the individual pH values, then incubated with papain under fixed conditions (section 2). Papain-treated proteoliposomes were dissolved in SDS and applied to PAGE (12.5%) in the presence of SDS (0.1%).

those of Happe et al. [12] who prepared right-side-out proteoliposomes by sonication of acidic phospholipids in 0.15 M KCl with purple membranes at pH 2.5, at which bR tends to undergo denaturation upon sonication. The difference between their preparation and ours, however, lies in the stability of the proteoliposomes and/or bR on reconstitution. Our proteoliposomes prepared with the blue membrane in deionized water at pH <3.5 were stable on reconstitution, and remained so provided salt was added for storage. Although the yield of these proteoliposomes depends on the composition of lipids used (other phospholipids could be used, but the partial involvement of acidic lipids appears to be essential) and the pH of reconstitution, the right-side-out orientation of bR/purple membranes is fairly well reproducible.

Proteoliposomes prepared at intermediate pH values (e.g. pH 4.10) showed light-induced pH changes in two directions (fig.1). As shown above, these vesicles most likely include bR/purple membranes oriented in two ways (fig.2). The complex pattern of pH changes, however, depended on KCl concentration (fig.1, broken line); the component showing pH decrease was apparently intensified in 2 M KCl. This would be caused by an increase in proton pumping activity of right-side-out bR at higher KCl concentrations, supposing that more KCl permeated into proteoliposomes. NaCl also showed similar concentration modification of pH-change patterns.

When proteoliposomes of three kinds of orientation (as in fig.1) were suspended in either KCl or choline chloride, both at 2.7 M, the pH change pattern in KCl was fundamentally the same as that in fig.1, while the pattern in choline chloride suggested that only inversely oriented bR was active (fig.3). The pH decrease component was not detected even after 12 h incubation in choline chloride, a non-permeant cation salt. If 5 mM $CaCl_2$ was added to such proteoliposomes of mix-

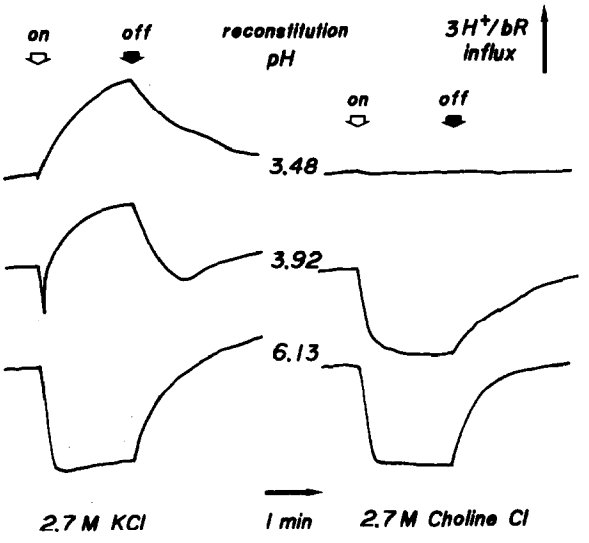

Fig.3. Dependence on salt species of light-induced pH changes of proteoliposomes reconstituted at different pH values. Proteoliposomes reconstituted at the given pH values were suspended in either 2.7 M KCl or 2.7 M choline chloride (0.03 mg protein/ml), and 3 min after the suspension was illuminated (on) for 2 min (off) and the pH change recorded.

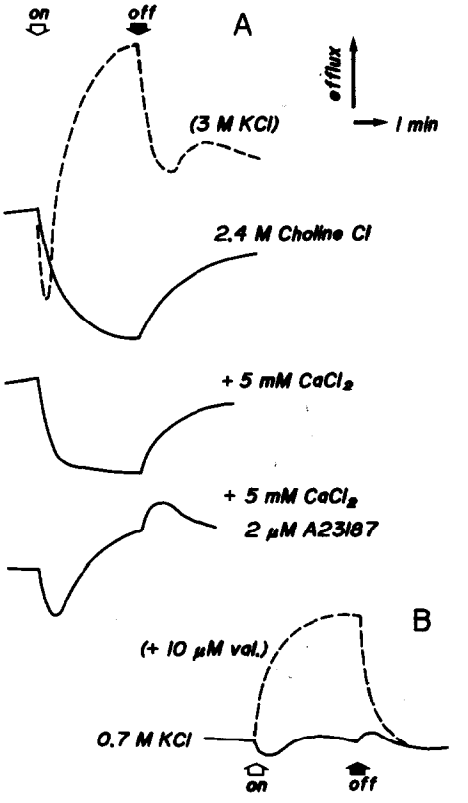

Fig.4. Ionophore enhancement of pH decrease component in light-induced pH changes of proteoliposomes containing bR oriented in both directions. (A) Proteoliposomes in 2.4 M choline chloride (or in 3 M KCl; broken line) were added with 5 mM $CaCl_2$, followed by 2 μM A23187. (B) Proteoliposomes in 0.7 M KCl were added with 10 μM valinomycin (broken line).

ed orientation in 2.4 M choline chloride and illuminated, the pH increase pattern did not change significantly (fig.4A). However, when 2 μM A23187 (Ca^{2+} ionophore) was further added, the pattern changed to show a distinct pH decrease component. Similar enhancement of the pH decrease was observed on addition of 10 μM valinomycin to proteoliposomes of similar type (right-side-out > inverted) in 0.7 M KCl (fig.4B); the light-induced pH change was initially small and bi-directional, however, on addition of valinomycin a pH decrease component became dominant. Therefore, the effectiveness of cations on pH decrease (i.e. pumping protons from the inside) depends on the permeability of individual cations through the proteoliposome membrane.

All the results on the pH changes, although qualitative, definitely show that bR in the right-side-out orientation in proteoliposome membranes pumps protons in the light only after cations become available inside the vesicles, i.e. on the carboxyl-terminal side of the bR/purple membrane. The results also suggest that Cl^- (or anions) would not be effective in pumping. The difference in pumping rates (cf. fig.1) may relate to the cation (and proton) concentration on each side of the membrane, or the electrical potential difference across the membrane. Since the effective cation concentration has not been titrated accurately vs proton pumping and the binding site(s) has not been assigned, the role of cations remains to be elucidated. Cations may be involved in the proton pumping mechanism through interaction (gating, shielding, screening, etc.) with their (specific or non-specific) binding sites on the C-terminus side of the bR/purple membrane.

The energy-transducing proton pump rhodopsins (bR and aR [17]) may be controlled by cations, while photo-sensory rhodopsins [18–20] may control cation(s) in their UV signal transduction in halobacteria.

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