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Partial separation of inwardly pumping and outwardly pumping bacteriorhodopsin reconstituted liposomes by gel filtration

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Reconstitution of bacteriorhodopsin using the reverse-phase evaporation technique leads to the formation of both inwardly proton pumping and outwardly proton pumping liposomes [(1985) FEBS Lett. 188, 101–106]. The reconstituted preparation was size fractionated using gel filtration chromatography on Sephacryl S1000. The direction of proton pumping was assessed in various fractions using the pH sensitive fluorescence probe pyranine. The proportion of outwardly pumping liposomes was shown to increase with decreasing liposome size. The larger liposomes (diameter larger than 200 nm) were shown to be pure inwardly pumping liposomes with almost homogeneous bacteriorhodopsin orientation.

Bacteriorhodopsin; Reconstitution; Protein orientation; Liposome size; Gel filtration; Pyranine

1. INTRODUCTION

Much insight into the mechanism of active ion transport has been gained from the use of reconstituted liposomes containing ion pump proteins (review [1,2]). Several functionally important properties must be fulfilled by such reconstituted liposomes for quantitative transport studies to be performed. These include a large and relatively uniform size as well as an homogeneous repartition and a well characterized transmembrane orientation of the protein. The latter parameter is possibly the most difficult to control and to quantitate. Only in a very limited number of cases has reconstitution of a protein with a single orientation been achieved [3–7]. When two orientations are present, the only knowledge of their average relative proportions is not always sufficient for functional characterisation of the reconstituted system. Indeed, the two orientations may be unevenly distributed among liposomes, leading to heterogeneity in transport directions and efficiency.

In the case of BR, the light-driven proton pump of *Halobacterium halobium* [8,9], we have recently described a reconstitution procedure using reverse-phase evaporation [10]. This method yields large (average diameter 200 nm) liposomes of homogeneous composition. Both proteolysis and proton pumping experiments demonstrated that two orientations of the protein were present, although the inside-out orientation (i.e. carboxyl-terminus facing outside) was predominant (approx. 80% of total) [10,11]. Furthermore, by using a pH-sensitive fluorescence probe approach, we were able to demonstrate that these two orientations of the protein were at least partially segregated in different subclasses of liposomes. This leads to the presence of both inwardly and outwardly proton pumping liposomes in the reconstituted preparation [11]. Despite this heterogeneity, we have shown that these liposomes can be used to study the kinetics of proton passive permeability [12] and of light-driven proton transport [13] under conditions which closely match the physiological situation, due to their large internal volume, homogeneous lipid to protein ratio and low proton leakiness. However, a quantitative evaluation of

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Abbreviations: BR, bacteriorhodopsin; Pipes, 1,4-piperazine ethane sulfonate; pyranine, 8-hydroxy-1,3,6-pyrenetrisulfonic acid

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proton pumping initial rates and ΔpH values was partially hampered by the occurrence of the two liposome populations pumping in opposite directions.

In the present study, we show that these two functionally distinct subclasses of liposomes can be partially separated using gel filtration chromatography, suggesting that these possess different size distribution. This allows us to obtain liposomes of large and homogeneous size with quasi-uniform orientation of BR.

2. MATERIALS AND METHODS

2.1. *Reconstitution of BR*

The preparation of BR and phospholipids as well as the reconstitution procedure using reverse-phase evaporation have been reported [10,11]. Here reconstituted liposomes were formed using a mixture of egg phosphatidylcholine and phosphatidic acid (mole ratio 9:1) with a phospholipid to BR weight ratio of 80:1 in 125 mM K_2SO_4, 5 mM Pipes buffer, pH 6.8, containing 400 μM pyranine. The preparation was then passed through a small Sephadex G25 column (PD 10, Pharmacia) to remove external pyranine.

2.2. *Gel filtration*

A Sephacryl S1000 (Pharmacia) column (100 × 1.6 cm) was allowed to pack ascendingly at a flow rate of 0.6 ml · min^{-1} in the same buffer. The column was saturated with lipids by overnight closed circuit circulation of 50 mg of egg phosphatidylcholine sonicated vesicles. A 1.5 ml volume of BR reconstituted liposomes (lipid concentration 8 mg/ml) was applied to the column and eluted at a flow rate of 0.3 ml · min^{-1}. Fractions of 1 ml were collected.

2.3. *Fluorescence measurements*

The fluorimetric assay of light-induced proton pumping using pyranine fluorescence has been described [11,14]. Before assay, fractions eluted from gel chromatography were diluted twice with iso-osmotic 30 mM K_2SO_4, 100 mM Pipes buffer. Valinomycin was added from a concentrated ethanol solution at a final ratio of 0.25 mmol per mol lipid which provided optimal proton pumping activity.

2.4. *Electron microscopy*

Freeze-fracture electron microscopy studies were performed as in our previous publication [10].

3. RESULTS

Reconstitution of BR using the reverse-phase evaporation method leads to a preparation in which both inwardly and outwardly pumping liposomes simultaneously occur. This conclusion is based on the use of the pH-sensitive fluorescence probe pyranine entrapped inside the liposomes to monitor proton pumping [11]. We shall recall briefly the rationale of these experiments since the same approach is used below to assay the fractions eluted from gel chromatography.

At neutral pH, light induced proton pumping by BR leads to a decrease of the fluorescence of liposome-entrapped pyranine indicating that the major effect is an internal pH decrease (fig.1, curve a). This indicates a predominant inside-out orientation of BR (i.e. carboxyl-terminus facing outside) with inward proton pumping. A similar experiment performed in the presence of millimolar concentrations of lanthanide trivalent cation leads to a fluorescence increase indicating an internal pH increase (Fig.1, curve b). Drachev et al. [15] have shown by experiments using planar bilayers containing BR that the lanthanide

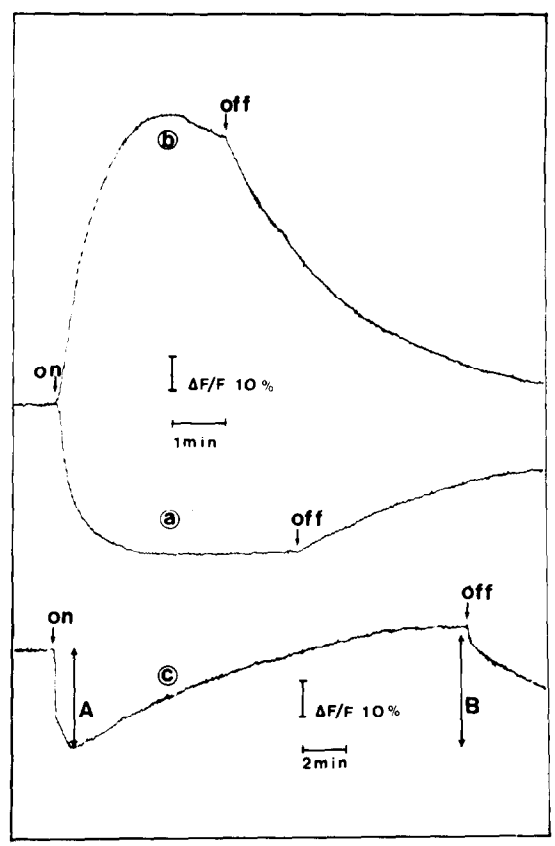

Fig.1. Light-induced fluorescence responses of pyranine trapped inside BR reconstituted liposomes (lipid concentration 0.08 mg/ml) at pH 6.8 in the absence (curve a) and presence (curve b) of 5 mM Gd $(NO_3)_3$ and at pH 5.5 (curve c). First arrow, light on; second arrow, light off.

trivalent cation La^{3+} inhibits proton pumping only when facing the carboxyl-terminus of the protein with no effect on the amino-terminal side. Thus La^{3+} appears to act as a one-sided non-permeant inhibitor of BR. Thus our experiments (see below) indicate that a significant amount of right-side out orientation of BR is also present in the liposomes, with associated outward proton pumping.

In our former study, La^{3+} was used as inhibitor. However, experiments were hampered by the precipitating effect of the cation upon liposomes, especially when the latter contained phosphatidic acid [11]. In the present work such charged liposomes were necessary since these proved, in preliminary experiments, to be more stable during chromatography. We found that Gd^{3+} was a more potent inhibitor than La^{3+} since lower concentrations were necessary. By using a low buffering power inside the liposomes to obtain a more rapid pumping kinetics, we were able to monitor the fluorescence response in the presence of 5 mM Gd^{3+} without much influence from precipitating effects. As can be seen from fig.1 (curve b), the rapid fluorescence rise associated with proton pumping is only minimally affected by the slower decrease due to precipitation of the liposome. Although a variability of approx. 20% was found in the maximum amplitude of the fluorescence rise, this approach gives a reasonably quantitative measurement of pumping by inside-out BR. Gd^{3+} concentrations higher than 5 mM yielded a similar positive response (although precipitation was more rapid) suggesting that this concentration completely inhibits inside-out BR.

When proton pumping is assayed in BR liposomes pre-equilibrated at pH 5.5, i.e. in conditions where pyranine is much more sensitive to an increase of internal pH than to its decrease [11], a biphasic fluorescence response is observed (fig.1, curve c). This is taken to indicate the presence of both inwardly pumping and outwardly pumping liposomes to which the early negative phase and late positive phase of the response are, respectively, attributed (the biphasic character is due to the fact that the fluorescence of the inwardly pumping liposomes rapidly reaches a near-zero level while that of the outwardly pumping liposomes ultimately reaches high values, see [11] for details). This indicates that the two orientations of BR are, at least partially, segregated in distinct subclasses of liposomes. The biphasic character of the response can be conveniently characterised by the ratio *B/A* of the amplitudes of the negative and positive phase (see fig.1).

The reconstituted BR preparation containing trapped pyranine was submitted to gel filtration chromatography over Sephacryl S1000. A representative elution profile is shown in fig.2A. Two peaks are present, corresponding to excluded and fractionated material. The eluted fractions were assessed for the direction of proton pumping as described above. Fluorescence responses at pH 6.8 in the absence and presence of 5 mM Gd^{3+} and at pH 5.5 are shown in fig.3A–C, respectively, for representative fractions. Results obtained for the whole elution profile are summarized in fig.2B and C. The relative amplitude of the fluorescence responses at pH 6.8 in the presence and absence of Gd^{3+} and the parameter *B/A* which characterizes

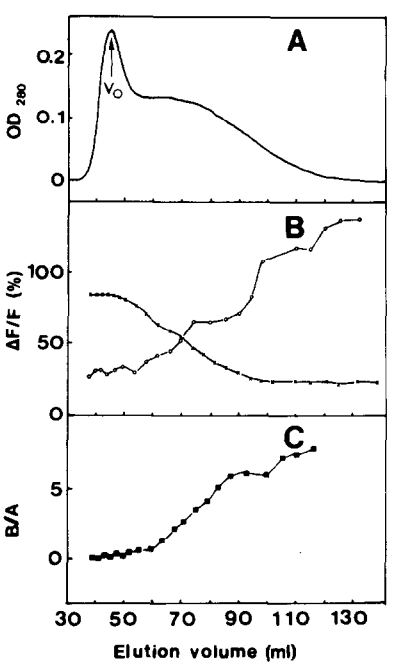

Fig.2. (A) Elution profile of BR reconstituted liposomes on Sephacryl S1000. V_0, void volume. (B) Relative amplitude of the light-induced internal pyranine fluorescence response at pH 6.8 for liposome fractions in the absence (×) and presence (○) of 5 mM Gd $(NO_3)_3$ (relative amplitudes are plotted in absolute values and are negative in the absence and positive in the presence of Gd^{3+}). (C) *B/A* ratio of the amplitudes of the early negative and late positive phases (see fig.1) of the light-induced internal pyranine fluorescence response at pH 5.5 for liposome fractions.

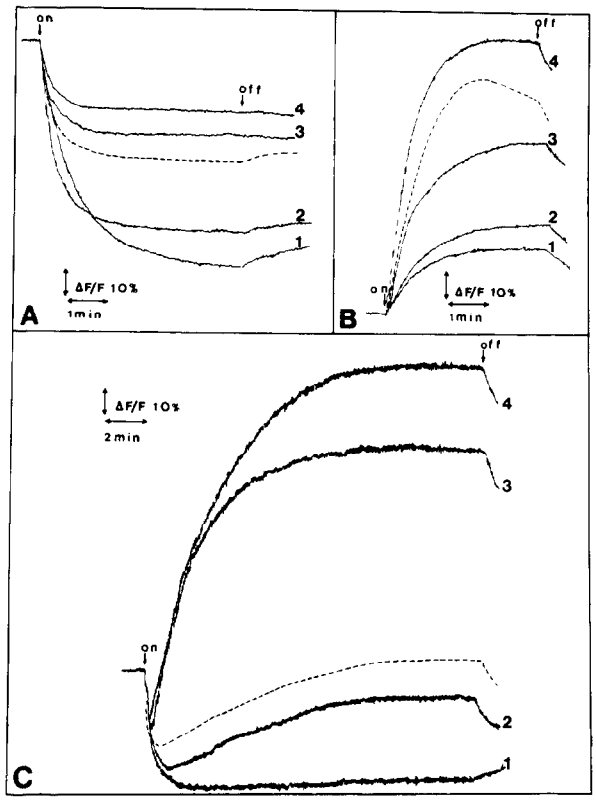

Fig.3. Light-induced fluorescence responses of internal pyranine for various liposome fractions eluted from gel chromatography at pH 6.8 in the absence (A) and presence (B) of 5 mM Gd $(NO_3)_3$ and at pH 5.5 (C). The elution volume of the fractions are void volume, i.e. 44 ml (1), 64 ml (2), 92 ml (3), 116 ml (4). The corresponding responses for the original reconstituted liposome preparation are shown as dotted lines for comparison. First arrow, light on; second arrow, light off.

the biphasicity of the pH 5.5 response are plotted as a function of the elution volume of the fractions. As can be seen, at pH 6.8, the first eluted fractions (i.e. void volume fractions) yield a very important negative fluorescence response in the absence of Gd^{3+} and a very low positive fluorescence response in the presence of Gd^{3+} as compared to the original preparation. For the fractions eluted subsequently, the responses observed in the absence and presence of Gd^{3+} become respectively lower and higher with increasing elution volume. This suggests that the relative amount of right-side out BR in liposomes increases with elution volume. An estimation of the percentage of right-side out BR can be obtained from the initial rates of the fluorescence responses with and without Gd^{3+}. This percentage increases from 12% for the first fraction to 42% for the last one (note that such an estimation cannot be made on the original preparation due to the heterogeneity of liposome sizes). At pH 5.5, the response decreases monophasically for the void volume fractions, but progressively becomes more and more biphasic with increasing elution volume, the late positive phase growing in amplitude. Thus, the amount of outwardly pumping liposomes appears to increase with elution volume in parallel with the amount of right-side out BR.

The reconstituted BR preparation was also assayed for liposome size using freeze-fracture electron microscopy. The histogram of fracture sizes for the initial sample and various fractions eluted from gel chromatography is shown in fig.4.

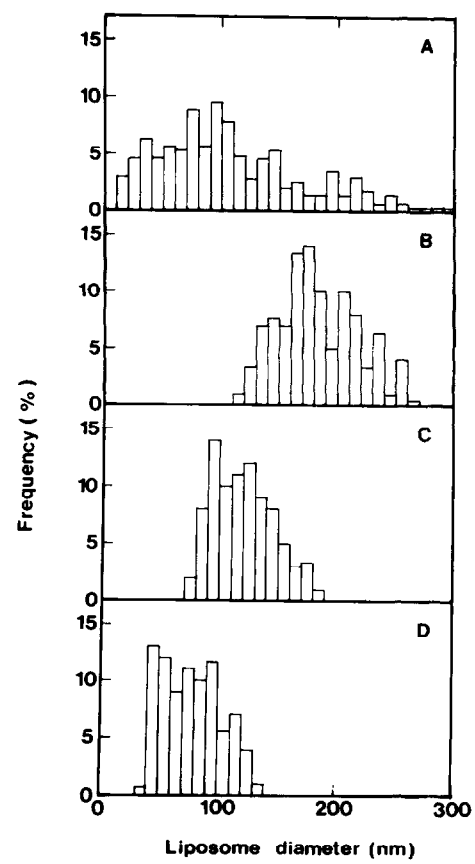

Fig.4. Size histogram of liposome sizes obtained from freeze-fracture electron microscopy for (A) original BR liposomes; (B) void volume, i.e. 44 ml; (C) 64 ml; and (D) 92 ml elution volume fractionated BR liposomes.

As can be seen, the original preparation is relatively heterogeneous in size with fracture diameters ranging from 20 to 300 nm (fig.4A). On the other hand, the fraction corresponding to the top of the void volume peak (fig.4B) yields a much narrower distribution of fracture diameters ranging from 130 to 230 nm (larger fracture diameters were found in the leading edge of the peak, however it was found to be slightly contaminated with multilamellar material). As expected, for fractions corresponding to larger elution volumes, the fracture diameter distribution shifts to lower values (fig.4C and D, note however that fractions corresponding to the trailing end of the elution profile could not be assayed for fracture diameter distribution, being too diluted for a sufficient amount of vesicles to be counted).

4. DISCUSSION

Gel filtration over Sephacryl S1000 has recently been shown to be an efficient technique for size fractionation of liposomes [16,17]. It was reported that liposomes with diameters smaller than about 200 nm are fractionated. Our freeze-fracture electron microscopy studies of the various eluted fractions are in agreement with this view. Of particular interest to us are the liposomes eluted in the void volume. These appear to yield a fairly narrow distribution of fracture diameter, i.e. 130–250 nm. Furthermore, the size homogeneity of these liposomes is likely to be underestimated by the freeze-fracture technique due to the multiplicity of fracture planes. Considering the exclusion limit of about 180 nm for the Sephacryl S1000 gel [14], it appears that the void volume liposomes are more likely to have diameters ranging from 200 to 250 nm and are thus highly homogeneous in size.

Our previous results indicated that the reconstituted BR preparation contains both inwardly pumping liposomes (with predominantly inside-out BR) and outwardly pumping liposomes (with predominantly right-side-out BR). Here, we confirm this and show that these two subclasses of liposomes can be partially separated using gel filtration. Indeed, the larger liposomes which are excluded from the gel appear to be pure or nearly pure inwardly pumping liposomes with very few right-side-out BR. On the other hand, the smaller liposomes are mixtures more and more enriched in outwardly pumping liposomes with increasing right-side-out BR as their size decreases.

The question arises whether the segregation of the two BR orientations in distinct liposomes subclasses is partial or total. In particular, do inwardly pumping liposomes contain only inside-out BR or are small amounts of right-side-out BR also present? In this regard, it may be remarked that the larger liposomes display no biphasic fluorescence response at pH 5.5 while they yield a low but significant positive response in the presence of Gd^{3+} at pH 6.8. Thus, it seems that these large liposomes are pure inwardly pumping liposomes but nevertheless contain low amounts of right-side-out BR (of the order of 10% as estimated from the initial rates of fluorescence response in the absence and presence of Gd^{3+}). More generally, for the original preparation as well as for all chromatographic fractions, the absolute increase of fluorescence observed upon proton pumping in the presence of Gd^{3+} is invariably larger than the residual level of fluorescence observed at the steady state without Gd^{3+} at pH 6.8. This suggests that some of the liposomes which pump protons inwardly in the absence of Gd^{3+}, pump protons outwardly in its presence and thus contain some right-side-out BR. This conclusion could not be attained in our previous study since the use of La^{3+} did not allow us to completely inhibit inside-out BR [11]. Thus, there appears to be a partial scrambling of BR orientations between liposomes.

One conclusion of this work is that the two functionally distinct liposome subclasses present in the reconstituted BR preparation have different size distributions. Namely, inwardly pumping liposomes have, on average, larger diameters than outwardly pumping liposomes. It is at this present stage difficult to provide an explanation of how two such types of liposomes are formed in parallel, since the details of the mechanisms of vesicle formation by reverse-phase evaporation are practically unknown [18].

The size distributions of the two liposome subclasses are not sufficiently different to allow a total separation by gel filtration. However, liposomes excluded from the gel are characterized by a large and relatively homogeneous size distribution (200–350 nm diameter), a quasi-uniform protein orientation and a single direction

of proton pumping. These may prove particularly useful in quantitative studies on the kinetics of proton pumping by BR, since, from the extent of the pyranine fluorescence response of these liposomes as well as from our previous studies [13], it appears that ΔpH of the order of 2 units can be generated, i.e. values which are close to physiological conditions.

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