Use of the fluorescent pH probe pyranine to detect heterogeneous directions of proton movement in bacteriorhodopsin reconstituted large liposomes

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Light-induced proton pumping was measured in bacteriorhodopsin reconstituted large proteoliposomes (prepared by reverse-phase evaporation) using the fluorescence of internally trapped pyranine. From the effect of the one-sided inhibitor La^{3+}, the presence of both inside-out and right-side-out oriented bacteriorhodopsin was demonstrated. The use of pyranine at acidic pH allowed us to detect two distinct populations of proteoliposomes which exhibited oppositely directed proton pumping. This suggests that the two orientations of bacteriorhodopsin are at least partially distributed in different proteoliposomes.

Bacteriorhodopsin *Reconstitution* *Protein orientation* *Proton pumping* *Pyranine*

1. INTRODUCTION

Reconstitution of purified ion pump proteins into liposomes is a powerful approach to study the mechanisms of active transport (reviews [1,2]). An important parameter when studying ion movements is the transmembrane orientation of protein in reconstituted proteoliposomes. In the case of bacteriorhodopsin (BR), the light-driven proton pump of *Halobacterium halobium* [3,4], most reconstitution procedures have yielded small vesicles in which the protein is mainly oriented inside-out relative to intact bacteria (i.e. carboxylterminus facing outside) with associated light-induced inward proton pumping [5,6]. Recently we have described a new reconstitution procedure for BR using reverse-phase evaporation which yields large unilamellar (0.2 μm average diameter) proteoliposomes [7,8]. From proteolysis and proton pumping experiments, a largely inside-out orientation was inferred, although the presence of a small fraction of right-side-out protein could not be excluded [7,8].

A limitation of the usual structural and functional tests for protein orientation is that they give no information concerning the relative distribution of the distinct protein orientations among proteoliposomes (except for possibly freeze-fracture electron microscopy). The presence of distinct proteoliposome populations having predominantly different protein orientations may complicate transport studies.

In this study, we make use of the pH-sensitive probe pyranine [9–11] entrapped inside the intraliposomal aqueous space to monitor light-induced proton pumping in our large reconstituted BR proteoliposomes. We show that pyranine can be used to detect bulk internal pH variations associated with pumping, show the existence of 2 distinct orientations of BR inside proteoliposomes and demonstrate a non-homogeneous distribution of these 2 BR orientations among proteoliposomes, namely 2 populations of proteoliposomes which exhibit oppositely directed proton pumping.

Abbreviations: pH, transmembrane pH gradient; Pipes, 1,4-piperazineethanesulfonic acid

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2. MATERIALS AND METHODS

The preparation of BR and phospholipids as well as the reconstitution procedure using the reverse phase evaporation method have been described [7,8]. Here proteoliposomes were formed using either pure egg phosphatidylcholine (EPC) or a mixture of EPC and phosphatidic acid (PA) (mole ratio 9:1) with a phospholipid to BR weight ratio of 80:1 in 30 mM K_2SO_4, 100 mM Pipes buffer, pH 7.1, containing 400 μM pyranine. After extrusion through 0.4 and 0.2 μM polycarbonate membranes [7,8], the preparation was dialysed at 5°C against the same buffer, once for 5 h and once overnight to remove external pyranine.

Fluorescence intensity measurements were performed on a Perkin Elmer MPF 44A spectrofluorimeter. Excitation was performed at 460 nm and emission was recorded at 510 nm using 10 nm slit widths. A 510 nm interference optical filter was positioned along the emission path to remove further stray and diffuse light arising from BR illumination.

For fluorescence measurements, dialysed proteoliposomes were diluted in buffer to a concentration of 4 μg BR/ml in a fluorescence quartz cuvette maintained at 20°C. Illumination of BR was performed with an Intralux 6000 xenon lamp equipped with a heat filter and a high pass filter (550 nm $< \lambda <$ 800 nm) through a flexible light guide whose aperture was positioned above the quartz cuvette. The optimal light intensity and valinomycin concentrations were chosen as those yielding the maximum apparent initial rate of proton pumping as determined from pyranine fluorescence.

3. RESULTS

When the non-permeant fluorescent probe pyranine is present during reconstitution, it partially becomes trapped inside the proteoliposomes. External pyranine can be removed by dialysis. Calibration of the internal pyranine fluorescence intensity vs pH inside proteoliposomes can be effected by permeabilizing the membrane to protons with nigericin and adjusting the external pH to the desired value. As shown in fig.1, the pH dependence of the fluorescence intensity is basically similar for the probe inside proteoliposomes and free in solution. A slight alkaline shift in pK_a occurs in the case of proteoliposomes formed from EPC alone. This effect has already been observed with other membranes and is attributed to binding of a fraction of the probe to the membrane surface [9–11]. In contrast, with BR-EPC-PA proteoliposomes, no pK_a shift is observed, suggesting that the net negative charge of PA prevents the membrane binding of the probe. Proteoliposomes prepared in the absence of pyranine, incubated in a solution of the probe, are not fluorescent after dialysis. Once entrapped, the highly charged pyranine cannot readily diffuse through the phospholipid bilayer (typical rates of leakage of 2.5% per day). All these data indicate that pyranine can be used as a reliable reporter of internal pH in bacteriorhodopsin large unilamellar liposomes.

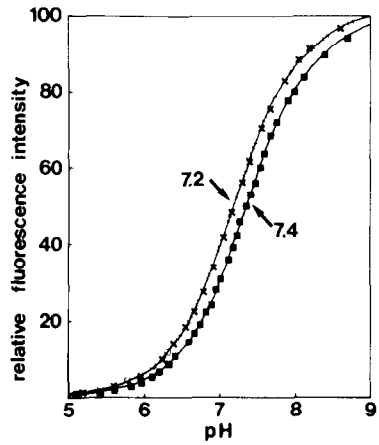

Fig.1. pH-dependent changes in the fluorescence intensity of pyranine. (○) 1 μM pyranine in buffer alone, (×) pyranine (400 μM) entrapped in BR-EPC-EPA vesicles, (■), pyranine (400 μM) entrapped in BR-EPC vesicles. The proteoliposome samples (4 μg BR/ml) contained nigericin (1 μg/ml) to facilitate transmembrane H^+ equilibration. Numbers indicate pK_a values, Excitation wavelength was 460 nm and the emission wavelength was 510 nm.

In this study, we made use of the trivalent cation La^{3+} which seems to interact strongly with negative lipids leading to aggregation of the BR-EPC-PA proteoliposomes. For this reason, we mainly developed results obtained with proteoliposomes formed from pure EPC.

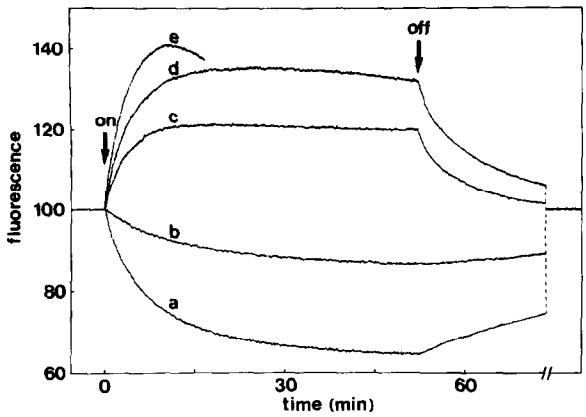

Fig.2. Light-induced changes in the fluorescence intensity of pyranine trapped inside BR-EPC proteoliposomes at pH 7.1. Liposomes pretreated with valinomycin (0.2 μM) were incubated in the presence of La $(NO_3)_3$ at different concentrations (mM): (a) 0, (b) 1, (c) 2.5, (d) 5, (e) 10. First arrow, light on; second arrow, light off.

Actinic illumination of BR-EPC proteoliposomes at pH 7.1 in the presence of valinomycin induced a time-dependent decrease of fluorescence intensity of trapped pyranine to a steady level corresponding to a 38% decrease (fig.2, curve a). The effect slowly reversed in the dark. The response was strongly diminished in the absence of valinomycin (maximum decrease 5%) and was abolished in the presence of nigericin (1 μg/ml). Since in the absence of valinomycin or in the presence of nigericin the main contribution of the light-induced transmembrane electrochemical proton gradient is the electrical potential $\Delta\psi$ (not shown) [7,8,12], our results indicate that the changes in fluorescence intensity observed in fig.2 are only due to the formation of a light-induced pH gradient. Thus the overall effect of proton pumping by BR is internal acidification, confirming the predominant inside-out orientation of the protein in the proteoliposomes.

To detect whether a significant fraction of right-side-out oriented BR exists in our proteoliposomes, we used the inhibitor La^{3+} to block inside-out BR. Skulachev and collaborators [13–15] have reported that La^{3+} inhibits proton pumping only when facing the C-terminal side of BR. As shown in fig.2 for BR-EPC proteoliposomes, increasing concentrations of La^{3+} first reduced the amplitude of the light-induced negative fluorescence response of trapped pyranine (curve b) and finally made the response positive (curves c–e). La^{3+} concentrations higher than 10 mM were not tested since aggregation and precipitation of the proteoliposomes became too significant (the effect of such aggregation can already be observed as a slow intensity decrease which follows the rapid positive response at the higher La^{3+} concentrations shown in fig.2). With negatively charged BR-EPC-PA proteoliposomes, aggregation and precipitation were more rapid. Nevertheless, qualitatively similar effects of La^{3+} could be observed, namely inversion of the pyranine fluorescence response. In all cases where aggregation was not significant, the effect of La^{3+} could be reversed by EGTA (not shown).

Thus, inhibition of inside-out BR by La^{3+} allows one to detect a light-induced internal pH increase using pyranine fluorescence. This clearly indicates that proteoliposomes contain a significant fraction of BR which pumps protons outwardly and is thus oriented right-side-out.

This result raises the problem of the relative distribution of these 2 oppositely oriented BR fractions among proteoliposomes. Namely, are right-side-out and inside-out BR molecules homogeneously mixed in the same proteoliposomes or are they heterogeneously distributed in distinct proteoliposome populations (i.e. partially or totally segregated)? An answer was obtained from the following experiments in which BR-EPC proteoliposomes prepared at pH 7.1 as above were brought to a more acidic pH (6 or 5.5) and allowed to equilibrate in the presence of valinomycin before testing proton transport. As can be seen (fig.3, curves a and c), at such acidic pH, the fluorescence response to BR illumination was biphasic with an initial rapid decrease followed by a slower rise to a stationary level. The biphasic character was more pronounced at pH 5.5 than at pH 6. In both cases, the dark response was also biphasic, the intensity first decreasing and then increasing to the initial level. At such pH values, the effect of La^{3+} was to reduce the contribution of the negative early phase of the light-induced biphasic fluorescence response considerably so that the latter rapidly became positive (fig.3, curves b and d), as observed at pH 7.1. Qualitatively similar results were obtained with BR-EPC-PA proteoliposomes.

These data can be explained by considering that the 2 opposite orientations of BR demonstrated

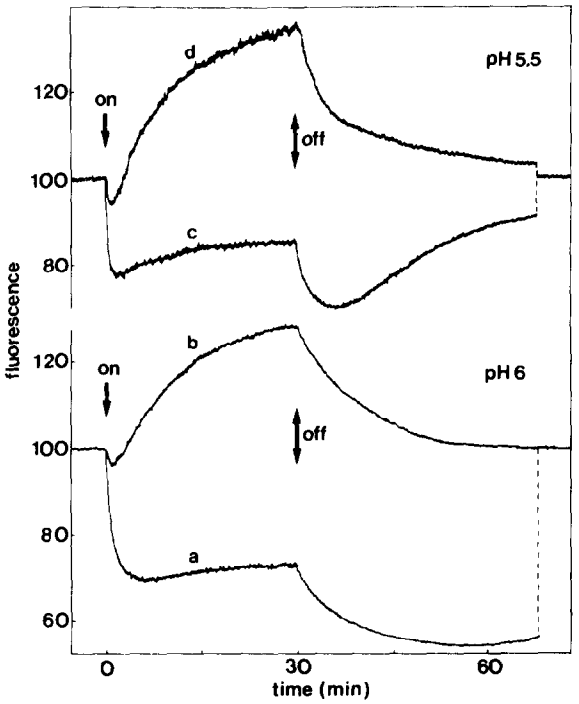

Fig.3. Light-induced changes in the fluorescence intensity of pyranine trapped inside BR-EPC proteoliposomes at pH 6 and pH 5.5. Liposomes prepared at pH 7.1 were adjusted to pH 6 (a,b) or 5.5 (c,d) with concentrated H_2SO_4 and allowed to equilibrate in the presence of 0.2 μM valinomycin. Equilibrated liposomes were then incubated in the presence (b,d) or absence (a,c) of 10 mM La $(NO_3)_3$. First arrow, light on; second arrow, light off.

previously are non-homogeneously distributed among proteoliposomes. Namely, in our preparation 2 populations of proteoliposomes exist; one having BR mainly oriented inside-out and the other having BR mainly oriented right-side-out. Upon illumination, one population undergoes internal acidification due to net inward proton pumping, while the other undergoes internal alkalinization due to net outward proton pumping. Thus, upon pumping, the pyranine fluorescence intensity associated with the former population decreases while that associated with the latter increases. The total fluorescence response is the sum of the 2 contributions and is greatly influenced by the non-linear dependence of the pyranine fluorescence intensity upon pH (see fig.1). At pH 7.1, the fluorescence contribution of the inwardly pumping proteoliposome population, which is predominant, determines the overall kinetics so that one observed a monphasic fluorescence decrease. On the other hand, at pH 6 or 5.5, it can be seen from fig. 1 that the fluorescence intensity associated with these proteoliposomes is expected to vary more slowly upon acidification and to reach very low values. Thus, at acidic pH, the fluorescence intensity contribution of inwardly pumping proteoliposomes initially dominates the kinetics but becomes rapidly so low that it is finally outweighed by the contribution of outwardly pumping proteoliposomes which rises in parallel. Thus one observes a biphasic fluorescence kinetics. Similar arguments can be used to explain the dark response observed at acidic pH. It may be noted that the fact that the fluorescence initially decreases in the dark is by itself proof that part of the proteoliposomes have undergone internal alkalinization upon pumping. The fact that the biphasic kinetics corresponds to pumping by 2 opposite orientations of BR is confirmed by the effect of LA^{3+} at acidic pH. Indeed, La^{3+}, which inhibits inside-out BR, appears to reduce the initial negative phase of the biphasic light-induced kinetics. The inhibitory effect of La^{3+} appears to be less pronounced at acid pH, since a concentration as high as 10 mM cannot completely erase the contribution of inside-out BR. A similar pH dependence has been reported by Drachev et al. [15].

4. DISCUSSION

This work indicates that pyranine can be used to monitor internal pH variations associated with light-induced proton pumping in bacteriorhodopsin large proteoliposomes. In the case of proteoliposomes formed from EPC as the only lipid, the presence of probe binding may possibly hinder an exact evaluation of internal pH values. However, here, a qualitative description of the proton pumping kinetics is sufficient for our purpose. Furthermore, it must be stressed that results similar to those described in detail for BR-EPC proteoliposomes could be observed with negatively charged BR-EPC-PA proteoliposomes in which pyranine membrane binding does not occur.

In our previous papers [7,8], papain and α-chymotrypsin proteolysis were used to demonstrate that BR was largely oriented inside-out in the proteoliposomes. No definite conclusion

could be drawn concerning the possible occurrence of a small fraction (about 15–20%) of right-side-out protein. Here we have used the non-penetrating one-sided inhibitor La^{3+} to demonstrate directly the presence of right-side-out BR molecules, pumping protons outwardly. Furthermore, experiments performed at acidic pH indicate the presence of 2 functionally distinct populations of proteoliposomes which undergo opposite internal pH variations upon pumping. This shows that the 2 orientations of BR are heterogeneously distributed among proteoliposomes in such a way that both inwardly and outwardly pumping proteoliposomes occur (with more than 50% BR inside-out and right-side-out, respectively).

The occurrence of these 2 populations of proteoliposomes cannot be accounted for by simple binomial statistics of distribution of BR orientations among proteoliposomes. Indeed, with average values of 80–85% BR inside-out (from proteolysis data) and 100 copies of BR per liposome (corresponding to a lipid/BR weight ratio of 80), this would lead to a negligible amount of proteoliposomes having more than 50% BR right-side-out. Thus, it must be assumed that, due to some particular mechanism, inside-out and right-side-out BR molecules are at least partially segregated in different proteolipisomes during reconstitution. Work is presently in progress to determine the origin of this segregation as well as its exact nature (i.e. partial or total).

This study indicates that knowledge of only the average transmembrane orientation of a transport protein is not always sufficient. The relative distribution of the 2 different orientations among proteoliposomes is also important for characterization of the reconstituted system. Indeed, in the case of BR, the occurrence of inwardly and outwardly pumping proteoliposomes can introduce serious errors in measurements of the components of the electrochemical proton gradient.

The method introduced here can be used to detect such heterogeneous proton movement directions in membranes containing proton transport systems (Recently Bell et al. [16], using 2 co-trapped probes, pyranine and 1-naphthol-3,6-disulfonate, have detected another type of heterogeneity in a suspension of sonicated BR liposomes. They analyzed their results assuming ping and fully inactive vesicles. However, the presence of outwardly pumping vesicles in their reconstituted system cannot be excluded. In our case, the presence of inactive proteoliposomes cannot explain our results and is unlikely since sucrose gradient and electron microscopy experiments indicate that no pure lipidic vesicles are present [7,8]. Nevertheless, our results and those of Bell et al. [16] indicate that the heterogeneity of the preparation must be considered for a precise analysis of pyranine data.) The rationale is that, due to the non-linear dependence of pyranine fluorescence upon pH, the response of either inwardly or outwardly pumping membrane vesicles can be favored by appropriately choosing the initial pH. The method may be applicable to many membrane preparations (either natural or reconstituted), provided that the energizing substrate has access to both sides of the membrane.

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REFERENCES

[1] Eytan, G.D. (1982) Biochim. Biophys. Acta 694, 185–202.
[2] Darszon, A. (1983) J. Bioenerg. Biomembranes 15, 321–334.
[3] Stoeckenius, W. and Bogomolni, R.A. (1982) Annu. Rev. Biochem. 52, 587–616.
[4] Dencher, N.A. (1983) Photochem. Photobiol. 38, 753–767.
[5] Van Dijck, P.W.M. and Van Dam, K. (1982) Methods Enzymol. 88, 17–25.
[6] Heyn, M.P. and Dencher, N.A. (1982) Methods Enzymol. 88, 31–35.
[7] Rigaud, J.L., Bluzat, A. and Büschlen, S. (1983) Biochem. Biophys. Res. Commun. 111, 373–382.
[8] Rigaud, J.L., Bluzat, A. and Büschlen, S. (1983) in: Physical Chemistry of Transmembrane Ion Motions (Spach, G. ed.) pp. 319–327, Elsevier Amsterdam, New York.
[9] Kano, K. and Fendler, J.H. (1978) Biochim. Biophys. Acta 509, 389–399.
[10] Clement, N.R. and Gould, J.M. (1981)

[11] Damiano, E., Basilana, M., Rigaud, J.L. and Leblanc, G. (1984) FEBS Lett. 166, 120-124.
[12] Hellingwerf, J.K., Arents, J.C., Scholke, B.J. and Westerhoff, H.V. (1979) Biochim. Biophys. Acta 547, 561-582.
[13] Drachev, L.A., Kaulen, A.D. and Skulachev, V.P. (1978) FEBS Lett. 87, 161-167.
[14] Drachev, L.A., Kaulen, A.D., Khitrina, L.V. and Skulachev, V.P. (1981) Eur. J. Biochem. 117, 461-470.
[15] Drachev, A.L., Drachev, L.A., Kaulen, A.D. and Khitrina, L.V. (1984) Eur. J. Biochem. 138, 349-356.
[16] Bell, D.H., Patterson, L.K. and Gould, J.M. (1983) Biochim. Biophys. Acta 725, 368-375.