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TRANSMEMBRANE pH GRADIENTS AND FUNCTIONAL HETEROGENEITY IN RECONSTITUTED VESICLE SYSTEMS

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The pH-sensitive, membrane impermeant fluorescence probes 8-hydroxy-1,3,6-pyrenetrisulfonate (pyranine; $pK_a = 7.2$) and 1-naphthol-3,6-disulfonate (Naps $pK_a = 8.2$) can be simultaneously entrapped within the intravesicular aqueous compartment of unilamellar vesicles and reconstituted proteoliposomes, where they function as reliable reporters of the intravesicular pH. Because the two probes are sensitive to pH over different but overlapping ranges, the useful monitoring range for the co-trapped probe pair extends from pH 6.5 to 9. In vesicles pre-equilibrated at a given pH and then subjected to a sudden change in external pH, the rate and extent of the subsequent change in internal pH are identical at all times during the re-equilibration, regardless of which probe is used to monitor the change. However, in reconstituted bacteriorhodopsin proteoliposomes, the size of the transmembrane pH gradient generated in the light always appears greater when pyranine is used to monitor internal pH. This discrepancy can most readily be understood in terms of heterogeneity in the vesicle suspension, with at least two populations of vesicles, one active in proton and one inactive. A simple algorithm was developed which generates, from the observed internal pH changes for two probes of different pK_a, the percentage of vesicles which are inactive, as well as the actual internal pH of the active fraction. The applicability of this algorithm was subsequently confirmed using a suspension of vesicles in which the level of heterogeneity was deliberately altered by the addition of various amounts of gramicidin. The apparent transmembrane pH gradient for the vesicle population as a whole decreased with increasing gramicidin, as did the calculated percentage of vesicles able to maintain a pH gradient, while the transmembrane gradient calculated for the active vesicle fraction only was essentially unaffected by gramicidin.

Introduction

The maintenance and regulation of ion gradients across biological membranes are crucial factors in normal cellular function [1]. Transmembrane proton gradients are especially important as driving forces for endergonic reactions such as active transport and ATP formation in cells and organelles [2,3]. Furthermore, the activities of most enzymes are sensitive to pH; and changes in pH within cells or organelles can have important con-

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† The mention of firm names or trade products does not imply that they are endorsed or recommended by the U.S. Department of Agriculture over other firms or similar products not mentioned.

Abbreviations: Pyranine, 8-hydroxy-1,3,6-pyrenetrisulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Tricine, *N*-tris(hydroxymethyl)methylglycine; Naps, 1-naphthol-3,6-disulfonic acid.

sequences for the metabolic function of such systems.

The movement of charged ions across cell and organelle boundaries is limited mainly by the phospholipid membrane barrier. Much of what is presently known about the movement of ions across membranes has come from studies employing model membrane systems in the form of small unilamellar vesicles prepared by sonication [4–7] or larger vesicles prepared by evaporation or dilution of some solvent [8–10]. Fluxes of hydrogen ions in such systems can be detected by various methods, including (i) direct measurement of external pH changes with an electrode [8–10], and (ii) changes in the optical properties (absorption, fluorescence) of pH-sensitive probe molecules [4–7,9,11]. In the latter case, the probe molecule can exist in either the external [9] or the internal [4–6,11] aqueous compartment, or in the membrane itself [7].

Quantitative data obtained by each of the techniques mentioned above must be interpreted within the constraints of the unverified, implicit assumption that the vesicle population being studied is both physically and kinetically homogeneous. Electron microscopy studies have provided convincing evidence of a uniform vesicle size for certain preparations [12], but a similar uniformity in the kinetic behavior of the various dynamic processes in a population of vesicles has heretofore only been assumed. There is, however, a considerable potential for heterogeneity in reconstituted systems, where the distribution and orientation of proteins throughout the vesicle population must also be considered. For example, Hwang and Stoeckenius [12] have estimated that, in their preparations of bacteriorhodopsin proteoliposomes formed by sonication, less than 80% of the vesicles actually contained the protein. The presence of an indeterminate population of inactive vesicles contaminating a population of active vesicles will necessarily lead to errors in the determination of the true magnitude of ion gradients and consequently to errors in determining the quantitative relationship between these gradients and the reactions to which ion fluxes are coupled.

In previous studies [4–6,11,13] it has been shown that the highly charged membrane-impermeant fluorescence probe 8-hydroxy-1,3,6-pyrenetrisulfonate (pyranine) could be used as a reliable real-time indicator of intravesicular pH in small unilamellar vesicles over the pH range 6–8.5 ($pK_a = 7.2$). We now report the use of a second membrane-impermeant, pH-sensitive fluorescence probe, 1-naphthol-3,6-disulfonate (Naps) which has properties similar to pyranine, but is useful in more alkaline regimes ($pK_a = 8.2$). Furthermore, we have found that this probe can be trapped inside vesicles along with pyranine, and the two probes monitored simultaneously. The use of these 'co-trapped' probes not only extends the useful pH range of the entrapped-probe technique, but also provides data which may be interpreted in terms of heterogeneity within the vesicle population and makes possible a determination of the actual internal pH in the active fraction of the vesicle population.

Materials and Methods

Vesicles were prepared from purified soybean lipids (asolectin) by a sonication procedure described previously [4]. Briefly, 10 mg of asolectin dissolved in chloroform was evaporated onto the inside of a test-tube under a stream of nitrogen. A solution (0.5 ml) containing 0.1 M KCl, 5 mM Mes, 5 mM Tricine (pH 8.2) and the indicated concentration of fluorescent probe(s) was added to the tube. The mixture was sonicated (under N_2) in a sonic bath (Laboratory Supplies, Co., Hicksville, N.Y.) until the solution was uniformly clear (approx. 30 min). For vesicles reconstituted with bacteriorhodopsin, purified bacteriorhodopsin was added to the sonicated vesicle suspension, and the mixture was re-sonicated for an additional 30 min. The vesicles were then passed through a Sephadex G-25 column to remove the untrapped fluorescent probe [4]. Vesicle preparations were normally used within 4 h of their preparation.

Naps fluorescence was excited with a 1000 W xenon lamp equipped with a Baird Atomic 344 nm interference filter. Pyranine fluorescence was excited with a 6 V tungsten lamp and the beam was filtered with a 467 nm interference filter. The emitted fluorescence of the two probes was detected by a photomultiplier tube through a 510 nm interference filter. Data were recorded with a stripchart recorder (Heath/Schlumberger Model

204). Alternations between the two excitation wavelengths were performed manually using a shutter.

In experiments employing vesicles reconstituted with bacteriorhodopsin, actinic light was provided by a 1000 W tungsten–halogen lamp and an orange cut-off filter (transmission over > 550 nm). The light intensity at the upper surface of the solution was 45 $mW \cdot cm^{-2}$.

Excitation and emission spectra were determined with a SLM 8000 spectrofluorimeter.

Cells of *Halobacterium halobium* R1 were grown according to Oesterhelt and Stoeckenius [14]. Bacteriorhodopsin was isolated and purified as described by Becher and Cassim [15]. Stock solutions of bacteriorhodopsin were made up in 0.15 M KCl and stored in a refrigerator.

Pyranine and Naps were obtained from Eastman Chemicals.

Results

Previous studies with pyranine [4–6,11] have shown the fluorescence of that probe to be extremely sensitive to pH, with the level of fluorescence (excitation = 460 nm; emission-520 nm) decreasing sharply as the solution pH is lowered. The fluorescence level decreases from its maximum value at pH > 8 to nearly zero at pH < 6.5, with an apparent midpoint at pH 7.2. Like pyranine, the absorption and fluorescence properties of the water-soluble naphthol derivative 1-naphthol-3,6-disulfonate (Naps) are also extremely sensitive to pH (Fig. 1). Alkaline solutions containing Naps exhibit an absorption maximum at 350 nm, the intensity of which falls off dramatically at lower pH. This change in absorbance is reflected in a 90% decrease in the level of 490 nm fluorescence, which is observed when the solution pH is lowered from 10 to 4 (Fig. 1B). Titration of Naps fluorescence as a function of pH (Fig. 2) yields the expected sigmoidal relationship with an apparent midpoint at pH = 8.2, suggesting that these fluorescence changes are associated with the protonation state of the 1-hydroxyl group. When small unilamellar vesicles are prepared in a solution of Naps and then passed through a Sephadex G-25 column, a portion of the probe molecules elute with the vesicle fraction. Vesicles prepared in the absence of Naps, and then incubated in a solution of the probe, are not fluorescent after passsage through a Sephadex G-25 column (see Methods). This indicates that Naps is being trapped within the intravesicular aqueous com-

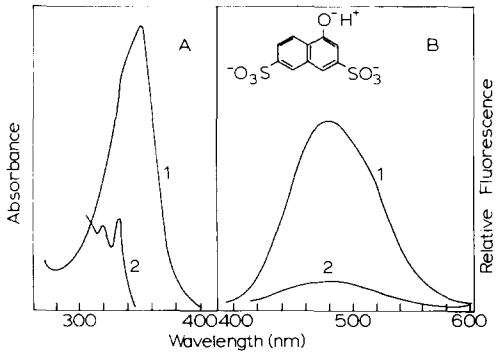

Fig. 1. Absorption and fluorescence emission spectra of Naps. (A) Absorption spectrum of an aqueous solution at pH 10.0 (trace 1) and pH 7.0 (trace 2). (B) Emission spectra at pH 10.0 (trace 1) and pH 4.0 (trace 2). Excitation wavelength was 350 nm. The structure of Naps is shown in panel B.

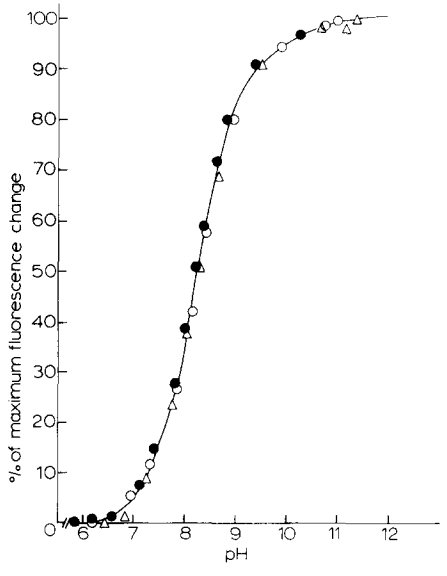

Fig. 2. pH-dependent changes in the fluorescence intensity of Naps. Excitation wavelength was 334 nm, emission wavelength was 510 nm. (●———●), 20 μM Naps in buffer alone (see Methods); (○———○), Naps entrapped in asolectin vesicles; (△———△), Naps entrapped in asolectin vesicles reconstituted with bacteriorhodopsin. Gramicidin (10 μM) was added to facilitate transmembrane H^+ equilibration.

partment. Once entrapped, the highly charged Naps does not readily leak out of the vesicles (leak rate under 5%/day), and the probe's fluorescence response to pH remains indistinguishable from the response in bulk solution. Incorporation of the *Halobacterium halobium* proton pump, bacteriorhodopsin into the vesicles also has no detectable effect on the fluorescence response of the vesicle-entrapped probe. Thus, like pyranine, which exhibits similar behavior [4,6], Naps can be used as a reliable reporter of internal aqueous hydrogen ion concentration in small unilamellar vesicles and reconstituted systems.

Vesicles were also prepared containing both pyranine and Naps entrapped within the same internal compartment. Under these conditions, the individual pH sensitivity of each of the 'co-trapped' probes was identical to that observed for the same probe in bulk solution (Fig. 3), even when one probe was in large excess over the other. This allowed the use of Naps in most of the subsequent experiments at a ratio of 30:1 in relation to pyranine. This was necessary in order to compensate for differences in quantum efficiencies of the two probes and because in the presence of pyranine, it was necessary to excite Naps considerably off-peak in order to avoid interfering changes from pyranine [11].

The response of vesicle entrapped pyranine and Naps to a sudden change in external pH is shown in Fig. 4. in which a suspension of vesicles containing the co-trapped probes was equilibrated at pH 7, and then suddenly adjusted to pH 9 by the rapid addition of KOH. The resulting proton efflux (or OH^- influx) was followed simultaneously for each probe, and the pH (determined from a subsequent titration curve, e.g., Fig. 3) plotted as a function of time. It is clear that both probes indicated the same pH as the internal hydrogen ion concentration was equilibrated with the external medium, even though the two probes differ in pK_a by about 1 pH unit. Similar results were also obtained after HCl additions (data not shown). These results would be expected only if the H^+/OH^- permeability of essentially all of the vesicles in the suspension were about the same (see below). Thus, as far as passive H^+/OH^- move-

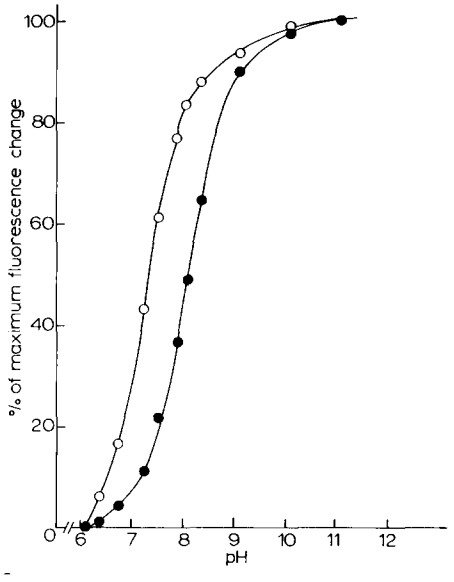

Fig. 3. pH-dependent changes in the fluorescence associated with vesicles containing entrapped Naps (60 mM) and pyranine (2 mM). Gramicidin (10 μM) was added to facilitate transmembrane H^+ equilibration. (○———○), excitation at 467 nm (pyranine); (●———●), excitation at 334 nm (Naps). Fluorescence emission was detected at 510 nm for both probes.

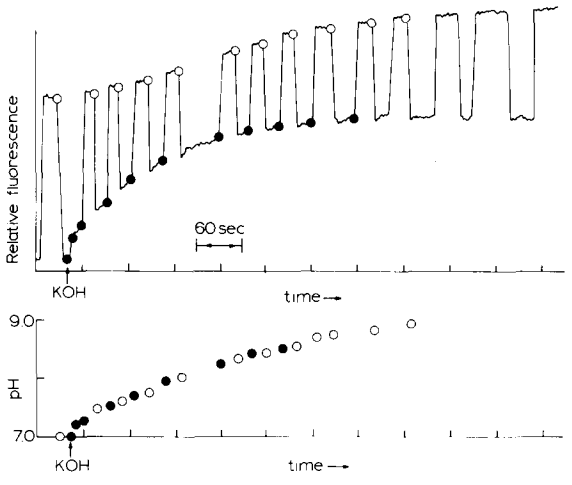

Fig. 4. Changes in the fluorescence intensity of pyranine and Naps (co-trapped within unilamellar asolectin vesicles) following a rapid increase in the external pH. (Top panel) The combined output of the fluorescence from the Naps probe (excitation 334 nm, upper trace) and pyranine (excitation 467nm, lower trace). Vesicles were pre-equilibrated at pH 7 before the KOH pulse. The final pH after the pulse was 9.0. (Bottom panel) Time-dependent changes in the internal pH of the vesicle suspension. Values were determined by comparison to a titration performed immediately following the experiment shown in the top panel.

ments are concerned, these vesicles appear to be quite homogeneous.

However, the likelihood for heterogeneity in a population of vesicles almost certainly increases dramatically for reconstituted systems, in which the activity, distribution and orientation of the reconstituted protein must also be considered. The data presented in Fig. 5 demonstrate that the kinetics and magnitude of light-induced changes in the internal pH of bacteriorhodopsin proteoliposomes containing pyranine can be easily monitored by following changes in the fluorescence intensity of the entrapped probe. Thus, actinic illumination of the proteoliposome suspension causes a decrease in pyranine fluorescence (internal acidification), which is reversed in the dark as the accumulated protons leak back out into the medium. The kinetics of the observed changes are very similar to those reported by Hwang and Stoeckenius [12] based on measurements of external (medium) pH changes. The light-dependent changes in pyranine fluorescence were completely abolished by gramicidin and by nigericin, indicating that these changes are, in fact, caused by the formation of a light-induced pH gradient.

In order to minimize the chances of heterogeneity in the proteoliposome preparations, reconstitution conditions were optimized with respect to protein : lipid ratio and sonication time (Fig. 6). Using optimal conditions (0.7 mg bacteriorhodopsin/mg lipid; 30 min sonication), proteoliposomes were then prepared containing simultaneously entrapped pyranine and Naps. Upon illumination of the bacteriorhodopsin, both probes exhibited fluorescence changes indicative of an intravesicular pH drop. However, the measured

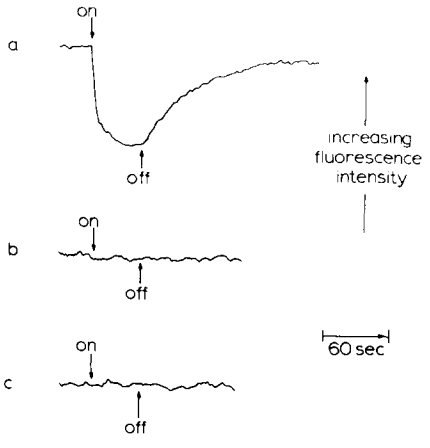

Fig. 5. Light-induced changes in the fluorescence intensity of pyranine entrapped within bacteriorhodopsin proteoliposomes. The 2.0 ml reaction mixture contained 0.1 M KCl, 5 mM Mes/KOH, 5 mM Tricine/KOH (pH 7.8), and proteoliposomes equivalent to 4.8 mg phospholipid. Proteoliposomes were prepared by sonicating 0.7 mg bacteriorhodopsin/mg asolectin for 30 min in the above buffer containing 2 mM pyranine as described in Materials and Methods. The K^+ ionophore valinomycin was added (1.0 μM) to minimize membrane potential effects. The intensity of the actinic illumination (arrows) was approx. $2 \cdot 10^6$ erg·cm^2·s^{-1}. Fluorescence emission was measured at 520 nm. (a) pyranine fluorescence was excited at 460 nm; (b) as in (a), except that gramicidin (10 μg/ml) was added to the proteoliposome suspension prior to actinic illumination; (c) as in (a) except that nigericin (1.25 μg/ml) was added prior to actinic illumination. The steady-state ΔpH established in (a) was approx. 0.4 pH units.

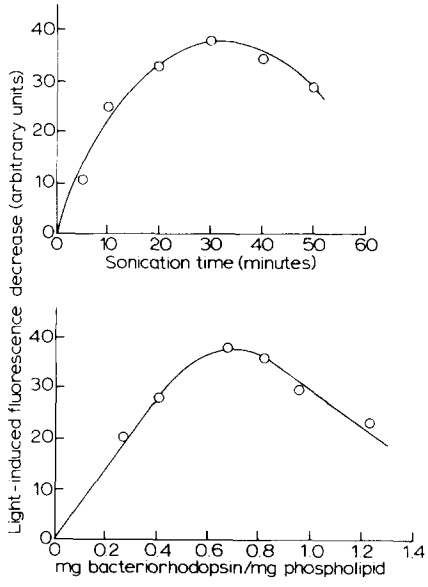

Fig. 6. Effect of reconstitution conditions on the apparent magnitude of the light-induced proton gradient in bacteriorhodopsin proteoliposomes. The maximum ΔpH established was 0.4 pH units. (Top panel) Bacteriorhodopsin and asolectin vesicles (0.7 mg protein/mg lipid) were sonicated under N_2 in a bath type sonicator at 20°C for the indicated times in a solution containing 0.1 M KCl, 5 mM Mes/KOH, 5 mM Tricine/KOH (pH 8.2) and 2 mM pyranine. (Bottom panel) Conditions were the same as above except the sonication time was 30 min and the protein : lipid ratio was varied as indicated. Changes in intravesicular pH were monitored as changes in the fluorescence of entrapped pyranine as described in Fig. 5a.

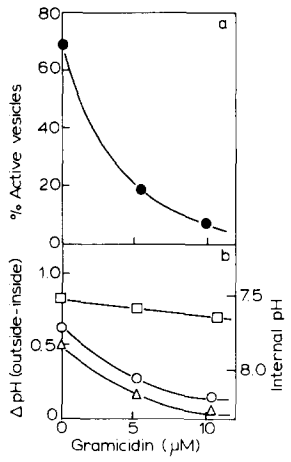

Fig. 7. Effect of gramicidin apparent on the magnitude of the transmembrane pH gradient and the degree of vesicle heterogeneity in illuminated bacteriorhodopsin proteoliposomes. Experimental conditions were similar to those described in the legend for Fig. 5, except that the proteoliposomes contained both 60 mM Naps and 2 mM pyranine. Internal pH values were determined from the maximum extent of the light-induced fluorescence change by comparison with a titration curve done in the dark. The initial pH (dark) was 8.35 for all experiments. (a) Gramicidin-dependent decrease in the proportion of the total proteoliposome population able to maintain a transmembrane gradient calculated as described in the text. (b) Apparent changes in the magnitude of the transmembrane H^+ gradient and internal pH as determined directly from changes in pyranine (○) or Naps (△) fluorescence. The squares (□) represent the calculated transmembrane gradient for only the active vesicle fraction after correction for the inactive vesicle fraction as described in the text.

internal pH after actinic irradiation was different, depending upon which probe was being measured (Fig. 7b). Since the two probes do not appear to interact when trapped within the same vesicle this difference can be best explained as resulting from the presence of a heterogeneous population of vesicles.

To analyze such a system, we assume the presence of two subpopulations of vesicles: an active fraction made up of vesicles in which protons are being accumulated as a result of light-driven proton pumping activity, F_{act}, and a second inactive fraction made up of vesicles containing probes but whose internal pH remains unchanged from the bulk pH, $(1-F_{act})$.

The data in Fig. 3 provide values of probe fluorescence intensity as a function of pH for a population of vesicles equilibrated with the external phase. These intensities may be defined as I^0_{pH} for Naps and $I^{0\prime}_{pH}$ for pyranine. At the beginning of the experiment, prior to illumination of the proteoliposomes, the internal and external proton concentrations are equivalent for all vesicles (pH = 8.35, Fig. 7). Hence, the measured starting intensities of the two probes may be written as:

$$I_{mea} = I^0_{8.35} \text{ and } I'_{mea} = I^{0\prime}_{8.35} \quad (1)$$

As the bactoriorhodopsin pumps protons into the vesicles, the internal pH of the active fraction – but not that of the inactive remainder – will change and the measured probe intensities of the system will become:

$$I_{mea} = I^0_{pH} F_{act} + I^0_{8.35}(1 - F_{act}) \quad (2)$$

$$I'_{mea} = I^{0\prime}_{pH} F_{act} + I^{0\prime}_{8.35}(1 - F_{act}) \quad (3)$$

It may be readily seen that these measured intensities are functions of two variables: internal pH and active fraction. It may further be seen that a given set of these two variables will not necessarily yield values of I_{mea} and I'_{mea} from which the same apparent pH values (on the original assumption of a homogeneous system) might be calculated. Conversely, however, it is possible to fit both I_{mea} and I'_{mea}, determined experimentally, to unique values of internal pH and active fraction.

Although the titration curves presented in Fig. 3 could, in theory, be expressed in equation form under ideal conditions *, we have chosen to use the real data and obtain the best fit by numerical methods. The algorithm which searches for this best fit operates as follows. The value of I_{mea} corresponding to a particular experiment is used in conjuction with an initial trial pH (internal) to calculate F_{act} from a file containing 500 data points defining the appropriate curve from Fig. 3. The resulting set of pH and F_{act} values is used to generate a trial value of I'_{mea} from the appropriate curve in Fig. 3. Finally, an error or difference

* Detailed listings of Fortran and Basic programs to analyze double probe experiments are available from the first author. These programs use equation-derived titrations of fluorescence intensity.

between the trial and experimental values of I'_{mea}, ΔI, is plotted as a function of the assumed pH. The assumed value of pH is incremented and the calculated repeated until ΔI equals 0.

Using data obtained from an illuminated suspension of bacteriorhodopsin proteoliposomes, the actual internal pH of the active fraction was found to be significantly lower than the apparent internal pH measured by either probe (Fig. 7). Furthermore, only 68% of the vesicles appeared to be actively generating a pH gradient when irradiated.

The accuracy and reliability of this method was further tested by deliberately altering the proportion of inactive vesicles in the proteoliposome population by introducing small quantities of gramicidin. Previous studies [13] have shown that, once incorporated into a vesicle, gramicidin forms a transmembrane H^+ channel which will not exchange into another vesicle, even after several hours. Thus, gramicidin will short-circuit any transmembrane pH gradient that may be generated inthe vesicle into which it has been incorporated, and that vesicle will become effectively inactive. In other words, increasing additions of gramicidin to bacteriorhodopsin proteoliposomes should decrease the size of the active fraction without altering the magnitude of the transmembrane pH gradient calculated for the active vesicles. The data in Fig. 7 clearly indicate that this is in fact the case. Using the algorithm outline above, it was calculated that 10.7 μM gramicidin decreased the active fraction to only 7.5% of the vesicle population, while the calculated internal pH for the active vesicles remained practically unchanged. This is in contrast to the apparent internal pH determined directly from the fluorescence changes of the entrapped probes, which showed a large decrease from the value obtained in the absence of gramicidin.

Discussion

The advantages of the entrapped-probe technique include simplicity, sensitivity and the ability to detect internal pH changes in real time [4–6]. Like pyranine [4–6,11], Naps has been found to be an effective and reliable probe of the internal pH of small unilamellar vesicles and reconstituted proteoliposomes. In addition, we have shown that pyranine and Naps can be trapped within the same vesicle at the same time. Because the probes have significantly different pK_a values (7.2 and 8.2, respectively), the useful pH range for this probe combination spans the region of most biological interest from pH 6.5 to 9.0. Although other matched probe pairs can be developed for this or other pH regimes, the pyranine/Naps pair offers the convenience of operating with a single monitoring emission wavelength. Thus, only one photomultiplier is required, and the monitoring probe can be selected by simply switching the excitation wavelength (e.g., Fig. 4).

Perhaps the most important consequence of using co-trapped probes with different pK_a values is the resulting facility to detect and quantify the degree of vesicle heterogeneity in a suspension. This becomes critically important in assessing the quantitative relationships between pH gradients and the reactions to which proton fluxes are coupled, especially in reconstituted systems where heterogeneity is likely to be significant. Furthermore, the dual-probe technique also provides a rapid method for analyzing the factors which might contribute to vesicle heterogeneity in reconstituted vesicle populations, including sonication time, protein : lipid ratio and phospholipid composition.

In this paper we have illustrated the procedure for determining vesicle heterogeneity in a reconstituted system of bacteriorhodopsin proteoliposomes. It should be emphasized that, in our analysis, we have assumed that only two types of vesicle exist in our preparations – fully active and fully inactive. Although the data determined using this assumption agree reasonably well with the theoretically expected results, there is no proof that this represents a complete description of the system. Indeed, it seems reasonable to expect that some active vesicles may generate larger pH gradients than others. Nevertheless, it is clear the the internal pH values determined by assuming two-population heterogeneity provide insights into the light-induced behavior of proteoliposomes that are not available from the pH values obtained assuming homogeneity. This becomes especially true when the active fraction of the vesicle population is a small percentage of the total (Fig. 7). We have made no attempt to determine the reasons for the apparent heterogeneity of the reconstituted

bacteriorhodopsin vesicles used in this study. Even though reconstitution conditions were optimized, it is possible that some of the vesicles did not incorporate the protein, or that some of the protein was inactive or incorporated in a reversed or scrambled orientation, or that some of the vesicles were rendered leaky to H^+ by natural ionophores contaminating the bacteriorhodopsin preparation. Indeed, under certain conditions bacteriorhodopsin can reconstitute in an orientation reversed from normal, so that in the light it pumps protons out of the vesicles (Gree, R.V., personal communication). We attempted to assess the extent of any reversed orientation in our system by measuring the fluorescence response of illuminated bacteriorhodopsin proteoliposomes containing pyranine at a pH below the probe pK_a. At pH 6.5, for example, any vesicles containing bacteriorhodopsin pumping protons outward would experience a net internal alkalinization, detectable as an increase in pyranine fluorescence. However, no such increase was observed. Only fluorescence decreases (or no changes) were detected at all pH values (data not shown). Thus we conclude that the proportion of reversed orientation vesicles in our preparation is very small, and cannot account for the observed level of heterogeneity.

Regardless of its cause, it is obvious that the very existence of multiple populations of vesicles can introduce serious errors into quantitative measurements of transmembrane pH gradients in vesicular systems. Indeed, it seems unlikely that any suspension of organelles or reconstituted vesicles is homogeneous to the point where the introduced heterogeneity error is negligible, although at the moment there is very little direct information on the homogeneity of any particular preparation. Given the presence of significant heterogeneity, the measured pH gradients in such mixed populations must be considered as underestimations of the actual gradient in fully active vesicles. The exact extent of the error, which can be quite large (Fig. 7), can be evaluated only when the relative proportion of active and inactive vesicles has been determined. In the absence of such information, it is clear that measured values for transmembrane pH gradients should be interpreted with great caution.

Acknowledgments

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