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MEASUREMENT OF PROTON/M₄₁₂ RATIOS IN SUSPENSIONS OF PURPLE AND WHITE MEMBRANE FROM HALOBACTERIUM HALOBIIUM

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SUMMARY

We have studied isolated purple membrane from Halobacterium halobium strain S₉ and white membrane from the over-producing mutant strain JW-5, making careful measurements of the proton to M₄₁₂ stoichiometry of our preparations, and also examining them by Quasi Elastic Light Scattering (QELS) and by negative staining electron microscopy (EM), to assess extent of aggregation and hydrodynamic radius.

In the past decade there have been varying reports of the stoichiometry of proton release from purple membrane in suspension and protons pumped after its incorporation into phospholipid vesicles. Interpretation of the results, especially when by a group other than that which carried out the experiments, has sometimes been clouded by a misunderstanding of the differences between the various measurements involved and also by a lack of recognition that the aggregation state of membranes will affect the measured ratios.

In an attempt to resolve these misunderstandings, we have investigated the H⁺/M₄₁₂ ratio, measured in suspensions of purple membranes by the laser flash induced response of pH indicator dyes. We have found this ratio to reflect the aggregation state of the membrane, assessed on the same preparations by QELS (and EM, see accompanying chapter: Lefort-Tran et al.). We have also found our measurements of proton release stoichiometry on retinal reconstituted white membrane suspensions to give much higher H⁺/M₄₁₂ ratios than the same measurements on purple membrane suspensions.

INTRODUCTION

Bacteriorhodopsin is a small (approx. 26kD) protein found in the plasma membrane of the bacterium Halobacterium halobium (1). Its function is to pump protons out of the cytoplasm into the surrounding medium, using the energy provided by sunlight. This electrochemical

gradient is then utilised by a number of other membrane proteins, e.g. for ATP production and solute transport. Bacteriorhodopsin is produced in large quantities by the organism when the oxygen content of the medium falls so low as to make conventional respiration growth limiting, a situation which occurs, for example, when the salt concentration in evaporating sea-water pools becomes very high. H. halobium is thus able to grow well even in saturated salt solution.

The reported stoichiometry of protons released per M_{412} intermediate formed in the photocycle is variable. It has been reported (2) that, in the presence of high salt concentrations and at low light intensity, the H^+/M_{412} ratio approaches three, but that in distilled water, the ratio is 0.3-0.5 at pH 7.5. Lozier et al. (3) found the H^+/M_{412} ratio for membrane sheets in water to be 1.0 at the same pH. Using flash photolysis with pH indicator dyes, Govindjee et al. (4) reported H^+/M_{412} for purple membranes in suspension to vary with salt concentration, unlike membranes incorporated into liposomes, which have a consistent H^+/M_{412} for pumping of about 2.0, and that proteolytic cleavage of the C-terminal tail of bacteriorhodopsin by trypsin specifically lowers the H^+/M_{412} stoichiometry (5). Several laboratories (6) (7) (8) (9), however, report that the removal of the carboxyl tail region does not affect proton pumping of the purple membranes after incorporation into liposomes.

CLARIFICATION OF THE PARAMETERS

In the treatment of the figures on proton stoichiometry, it must first be understood that each of the techniques used looks at a somewhat different event.

In aqueous suspension, the laser/indicator dye technique measures the average transient release of protons from the two faces of the membrane. In this case the proton pumping cannot be detected, only net proton release from the membrane. This observed proton release is, most likely, due to a difference of pK of the two faces of the membrane (10) and as such, is probably an indicator of varying chemical nature of the two sides.

The dyes commonly used will partition into the membrane interface region due to their high hydrophobicity, and thus the figure obtained will be weighted toward the transient changes in this region and perhaps more importantly, modulated by differing surface charges of preparations due to the influence of the Gouy-Chapman double layer. These are problems in calibration of the dye response (which is carried out by adding protons to the bulk aqueous phase and measuring an equilibrium response) and means that figures cannot easily be compared between preparations having different surface charge densities, nor absolute values for H^+/M_{412} easily obtained. If a probe with a large permanent charge of the same polarity as the surface is used, then this latter problem is considerably reduced, if not eliminated.

Measurements by the pH electrode method, because of the longer time scale of the electrode response, are a response to the equilibrating proton release into the bulk aqueous phase, leading to a steady-state. This will be an even more complicated function of the titratable groups at the surface, since in the intervening time the released protons will have diffused to other areas of the surface, as well as into the bulk phase. The calibration in this case, however, is under the comparable conditions. Once again, a pumped proton gives no response.

In liposomes, proton pumping is the primary object of the measurement techniques. In the laser technique, dye is normally added to the external aqueous phase so that the measurement detects alkalinization due to protons pumped into the liposomes. As before, it is a transient response which is detected and subject to the same influence of surface charge of both bacteriorhodopsin and lipid on the dye measurement (If the dye is also present inside the liposomes, then the data obtained would reflect average proton release from the internal and external faces of the membrane). Because of the differing interior and exterior volumes and consequent difficulties of calibration, however, this is probably not a useful measurement.

In liposomal suspensions, the pH electrode measures a similar event to the laser technique, but once again responding to the pH change on a rather slower time scale to that technique.

METHODS

Membranes

Purple membranes from H. halobium Sg were isolate as previously described (11). Membranes were bleached by hydroxylamine plus light (12). White membranes from the JW-5 strain were reconstituted with all-trans retinal to form functional bacteriorhodopsin (13). White and purple membranes were suspended in 10mM azide at pH7 and kept in the cold; before use the suspensions were washed into test media.

Trypsin treatment

1mg/ml of purple or white membrane, suspended in 50mM Tris-Cl, 30mM NaCl, 10mM CaCl₂ pH7.5 buffer solution were treated with trypsin, suspended in the same buffer. The final concentration of trypsin was 0.017mg/ml for purple membrane and 0.0334mg/ml for white membrane. Samples were incubated for four hours at 37°C, and reaction stopped by dilution with double-distilled water and centrifugation (35000 x g, 30min, 5°C). Two additional washes completed removal of the lighter cleavage product.

Laser Apparatus

Measurements were made at a controlled temperature of 20 C in a 1cm path length cell. Actinic light was provided by flashes from a Phase-R DL 1100 dye laser, using Rhodamine 575, the flash duration was 350ns and the flash artifact less than 40µs. The photo-current from a photomultiplier was recorded using a Gould Biomation Model 4500 digital oscilloscope. Essentially noise-free traces were obtained on averaging of 16 flash responses.

Proton Release

Proton release from purple or white membrane sheet preparations was measured using the pH indicator dye, 7-hydroxycoumarin. The working concentrations were 0.312mg/ml membranes in distilled water, and 0.1mM hydroxycoumarin. The dye, dissolved in ethanol, was added in 30µl to 3ml of sample. The pH was adjusted to 7.5 between each measurement. Laser flash photolysis measurements were made at 412nm without and at 365nm with and without the dye. Transient absorption differences at 365nm were assessed at the time of maximum difference. The response of the dye (r) at 365nm to added protons was determined for each sample by careful titration with small aliquots of standard HCl, following absorption changes at 365nm using a Perkin Elmer Lambda 5 spectrophotometer. The final calculations of H^+/M_{412} were made using the following equation.

$$\text{Ratio} = \frac{\Delta I_{365}^{\text{dye}} \times \epsilon_{365}^{\text{M}}}{\Delta I_{365}^{\text{prot}} \times r_{365}^{\text{dye}}}$$

the extinction due to M_{412} at 365nm was calculated using:

$$\epsilon_{365}^{\text{M}} = \frac{\epsilon_{412}^{\text{M}} \times \Delta I_{365}^{\text{protein}}}{\Delta I_{412}^{\text{protein}}}$$

The $\epsilon_{412}(\text{protein})$ used was $23,000 \text{ M}^{-1}\text{cm}^{-1}$.

Since the measurements on the sample with and without the dye are made under the same conditions and the response due to M_{412} calibrated at 365nm, all other variables involved in the calculation of absorbances from the photomultiplier current measurements cancel out. There is also the advantage that reproducibility of flashes and electronic response is only required over the short period of time necessary to make the three measurements. In fact reproducibility is excellent so long as the instrument is closely watched and calibration of the dye response is very carefully carried out for each sample. Samples of white membranes reconstituted with retinal and measured on different days, for example, gave results differing by less than $0.1 \text{ H}^+/\text{M}_{412}$.

QELS Studies

QELS (14) allows determination of the translational diffusion coefficient (D_t), from which the hydrodynamic radius (R) for spherical particles can be calculated, using the following equation:

$$D_t = \frac{k T}{6 \pi \eta R}$$

where k is the Boltzman constant; T and η the absolute temperature and viscosity of the medium, respectively. The apparatus for QELS studies has recently been described for characterization of sarcoplasmic reticulum (15) and purple membranes (16).

RESULTS AND DISCUSSION

Proton release figures for native and trypsin treated membrane preparations are given in Fig. 1. It can be seen that, upon trypsin treatment, the proton release activity of purple membranes declines from an initial value over $1.5 \text{ H}^+/\text{M}_{412}$ to a value below 0.5. Retinal reconstituted white membrane, on the other hand, starts with a much higher proton release stoichiometry, close to $4.0 \text{ H}^+/\text{M}_{412}$, and even after trypsin treatment it retains most of this activity. In summary, whilst purple membrane loses two thirds of its activity, retinal reconstituted white membrane loses less than one tenth of its activity.

Whilst the absolute $\text{H}^+/\text{M}_{412}$ figures presented here cannot be directly compared, due to the surface charge considerations discussed earlier, the relative figures are most informative. One must remember that, on cleavage of the C-terminal tail, the surface loses considerable negative charge, which will in turn increase the amount of deprotonated hydroxycoumarin in the interface region and so cause an increase in sensitivity of the dye to protons released into this region, decreasing any lowering

of H^+/M_{412} stoichiometry upon trypsin treatment. We are confident, however, that our trypsin treatment procedure causes a similar extent of cleavage in both membrane preparations, so this increase in sensitivity should affect each of the samples equally. The fact is that the white membranes show a much smaller decline in activity than the purple membranes. What, then, is the reason for the decrease?

In Fig. 2 and Table 1, some representative results of a series of QELS experiments on the same samples are presented. It can be seen, from the close agreement with the superimposed theoretical curves, that the samples have good size homogeneity, except for some deviations in the low frequency region where some much larger particles make a contribution.

Native purple membrane consists of a suspension of particles with homogeneous size distribution in a rather narrow range (around 260nm). Upon trypsin treatment the membranes become extensively aggregated, with a QELS pattern more characteristic of a gel than of a suspension of particles. The appearance of such large aggregates gives us a clue as to why the H^+/M_{412} figures decline so much on trypsin treatment. With the membranes stacked and confined, as they must then be, it will be impossible for protons to be released into a region where they can be detected by the dye, or indeed by any other measurement technique. The light flash will still, however, generate M_{412} so that the observed ratio of H^+/M_{412} must decline.

In the case of white membranes there is a quantitatively different effect. In the first place, white membrane patches are rather smaller (38nm radius), even before trypsin treatment. Following treatment, they too aggregate, but to a rather lesser extent than do purple membranes

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Figure 2. QELS data on membrane preparations.

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TABLE 1
Translocational diffusion coefficients and hydrodynamic radii of
purple and white membrane preparations

Membrane Preparation	Diffusion Coefficient $\times 10^{-9} \text{ (cm}^2\text{s}^{-1}\text{)}$	Hydrodynamic Radius ^a (nm)
native purple	8.5	260 $\pm$ 20
trypsin treated purple	0.63	see text
white	24.4	88 $\pm$ 50
white after retinal reconstitution	24.4	88 $\pm$ 50
white, + retinal trypsin treated	4.3	515 $\pm$ 50

^a mean $\pm$ standard deviation, conditions as in figure 1.

(final radius 515nm, and homogeneous) and perhaps in a rather more open structure. The H^+/M_{412} ratio remains quite high, as a consequence.

Such aggregation after proteolysis has been previously observed by ourselves and others (17) and attributed to the reduction in electrostatic repulsion allowing the hydrophobic forces to induce aggregation. We have found that untreated preparations of purple membrane, over a range of concentration between 0.02 and 9mg/ml, stored for many months at 2°C showed no sign of aggregation by QELS. We and others (18) have also observed, however, that in some preparations of purple membrane, long term storage allows the development of protease activity, most likely arising from bacterial contamination. This activity is inhibited by benzamidine, indicating that it is trypsin-like (19). After proper washing of cell homogenates and purification of purple membrane by sucrose density gradient, little or no benzamidine inhibited protease activity is present. Thus, it is apparent that in order to get good H^+/M_{412} figures one must take great pains to exclude all bacterial contamination.

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