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Ratiometric fluorescence measurements of membrane potential generated by yeast plasma membrane H^+-ATPase reconstituted into vesicles

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Abstract

Potential-sensitive fluorescent probes oxonol V and oxonol VI were employed for monitoring membrane potential ($\Delta\psi$) generated by the *Schizosaccharomyces pombe* plasma membrane H^+-ATPase reconstituted into vesicles. Oxonol VI was used for quantitative measurements of the $\Delta\psi$ because its response to membrane potential changes can be easily calibrated, which is not possible with oxonol V. However, oxonol V has a superior sensitivity to $\Delta\psi$ at very low concentration of reconstituted vesicles, and thus it is useful for testing quality of the reconstitution. Oxonol VI was found to be a good emission-ratiometric probe. We have shown that the reconstituted H^+-ATPase generates $\Delta\psi$ of about 160 mV on the vesicle membrane. The generated $\Delta\psi$ was stable at least over tens of minutes. An influence of the H^+ membrane permeability on the $\Delta\psi$ buildup was demonstrated by manipulating the H^+ permeability with the protonophore CCCP. Ratiometric measurements with oxonol VI thus offer a promising tool for studying processes accompanying the yeast plasma membrane H^+-ATPase-mediated $\Delta\psi$ buildup.
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Keywords: Proteoliposome; Oxonol V; Oxonol VI; K^+/valinomycin calibration; Electrogenic pump; Proton permeability

1. Introduction

Electrochemical gradient of protons, $\Delta\mu_H^+$, generated on cell membranes is indispensable for energizing many life-essential functions. It consists of two components—membrane potential $\Delta\psi$ and concentration difference of protons ΔpH. In yeast, plasma membrane Mg^{2+}-dependent H^+-ATPase plays a crucial role in generation and maintenance of $\Delta\mu_H^+$ [1–3]. This electrogenic pump from the family of P-ATPases translocates one proton across the membrane concomitantly with splitting one molecule of ATP [4,5]. Its transport action can be conveniently studied when the enzyme is reconstituted into vesicles [6,7]. The plasma membrane H^+-ATPase from *Schizosaccharomyces pombe* was previously successfully reconstituted directly from crude plasma membranes after being solubilized by *n*-octyl glucoside [8]. The membrane potential generated by reconstituted H^+-ATPase can be monitored with the aid of potential-sensitive fluorescent probes [9]. Redistribution dyes from the group of oxonols, oxonol V and oxonol VI have been frequently used in studies with various types of charge-translocating systems [10–13]. Because these dyes carry a negative electric charge, both are suitable for monitoring inside-positive membrane potentials in the vesicles. To evaluate $\Delta\psi$ generated by H^+-ATPase, the fluorescence response of the dye to $\Delta\psi$ can be calibrated by membrane potentials created after imposition of a potassium gradient by valinomycin-facilitated potassium diffusion [10,14]. So far, the response of oxonols to $\Delta\psi$ has been monitored by measuring the fluorescence intensity at a single wavelength [8,10,15]. In this method, however, part of the information obtained from fluorescence measurements is being lost. When the whole fluorescence spectrum is tracked, fluorescence quenching can be distinguished from spectral shifts caused by the binding of the dye to the membrane. For this purpose, we introduced a method of monitoring the ratio of fluorescence intensities measured at two suitable wavelengths. In this study, we demonstrate that this method is applicable to quantitative estimation of the $\Delta\psi$ generated by reconsti-

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tuted H^+-ATPase and provides a useful tool for studies of factors affecting the $\Delta\psi$ buildup.

2. Materials and methods

2.1. Chemicals

Egg yolk lecithin (L-α-phosphatidylcholine from dried egg yolk, type X-E), ATP (adenosine 5′-triphosphate disodium salt), hexokinase (from baker's yeast, type III), *n*-octyl glucoside (*N*-octyl β-D-glucopyranoside), CCCP (carbonyl cyanide *m*-chlorophenylhydrazone) and MES (2-(*N*-morpholino) ethanesulfonic acid) were purchased from Sigma, valinomycin from Fluka, oxonol V (bis-(3-phenyl-5-oxoisoxazol-4-yl)pentamethine oxonol) and oxonol VI (bis-(3-propyl-5-oxoisoxazol-4-yl)pentamethine oxonol) from Molecular Probes.

2.2. Strains, media and growth conditions

The yeast *S. pombe* 972h$^-$ was grown in a minimal medium [16] containing 2.5% glucose at 38 °C. Cells in the early stationary phase were harvested and homogenized as described in Ref. [17].

2.3. Isolation of plasma membranes

Membranes were isolated essentially as described in Refs. [8,18] without additional solubilization and purification. The protein content of the plasma membrane fraction was determined according to Ref. [19] with bovine serum albumin as a standard.

2.4. Preparation of proteoliposomes with reconstituted H^+-ATPase

Octylglucoside-mediated reconstitution with detergent removal by dialysis was carried out according to Ref. [8] with slight modifications: egg yolk lecithin (20 mg/ml) was dissolved together with 40 mg octylglucoside in 1 ml of chloroform, dried by nitrogen gas on the walls of a tube and desiccated in vacuum for 45 min. The resulting film was hydrated by an appropriate volume of 10 mM MES (pH 6.0 by NaOH) and plasma membranes were added to the detergent–phospholipid mixture to a total volume of 1 ml so that the protein content in the mixture was 200 μg/ml. The proteoliposomes were thus prepared in lipid/protein ratio (w/w) 100:1. The mixture was dialysed in the "Mini-Lipoprep" apparatus (Diachema AG, Zürich) at 4 °C against 0.5 l of 10 mM MES (pH 6.0). The buffer was replaced after 1.5 h dialysis and the reconstitution was complete after 3 h.

The resulting proteoliposomes were collected, rapidly frozen in liquid nitrogen, thawed at room temperature, sonicated by a probe type sonicator (Cole-Palmer Instruments Co.) two times for 2–3 s and kept on ice. When $MgCl_2$, KCl or choline chloride were required to supplement the composition of the reconstitution buffer, the ions were added to the suspension of thawed proteoliposomes and the freeze–thaw sonication procedure was repeated two times.

2.5. Preparation of liposomes

Liposomes from egg yolk lecithin (20 mg/ml) were prepared similarly as proteoliposomes except that plasma membranes were not added.

2.6. Fluorescence measurements

Fluorescence measurements were performed on a Fluoromax 2 spectrofluorometer (photomultiplier tube photocatode sensitive to 700 nm) at an excitation wavelength of 560 nm selected from the xenon lamp spectrum. The scattered excitation light was eliminated using a red cut-off filter with full transparency from about 600 nm.

2.7. Fluorescence monitoring of H^+-ATPase-generated $\Delta\psi$

The potential-sensitive fluorescent probes oxonol V and oxonol VI were used for monitoring H^+-ATPase-generated $\Delta\psi$. Potential-dependent accumulation of dye molecules inside the vesicles is accompanied by changes of fluorescence characteristics due to the binding of the dye to the liposomal membrane. The emission spectrum of both dyes shifts upon binding about 20 nm toward longer wavelengths (a red shift). Moreover, oxonol V exhibits pronounced quenching of its fluorescence probably due to aggregation of dye molecules. Hence, $\Delta\psi$ generation can be monitored by tracking the position of emission spectrum or by measuring fluorescence intensity at a defined wavelength. Intensity ratio measurements at two wavelengths (640 nm/620 nm for oxonol V and 640 nm/615 nm for oxonol VI) were found to be sufficient for tracking the spectrum position.

The redistribution probes respond to $\Delta\psi$ by shifting their emission spectrum, only when both bound and free dye forms contribute sufficiently to the overall fluorescence spectrum. When one fluorescence component predominates, the response of the spectrum to changes in $\Delta\psi$ is suppressed. Concentrations of proteoliposomes for the measurement therefore have to be chosen properly to ensure the appropriate contribution of the bound dye fluorescence to the overall fluorescence. Because oxonol V binds much more extensively to the membrane than oxonol VI, much (about 35 times) lower concentration of the proteoliposomes is needed for proper $\Delta\psi$ sensing. The use of oxonol V thus improves the efficiency of testing for proteoliposome tightness and quality of reconstitution. Moreover, it allows also to reduce the residual detergent level in a sample by a massive dilution of the proteoliposomes before the fluorescence measurement.

2.8. Calibration of the fluorescence response to membrane potential on proteoliposomes

The calibration of the dye response to $\Delta\psi$, for example, by K^+ diffusion membrane potentials, is a prerequisite for evaluating the $\Delta\psi$ to which the electrogenic proton-motive pump charges the proteoliposomal membrane. When defined K^+ gradients are imposed on the membrane, the K^+ permeability of which is selectively increased by the ionophore valinomycin, the created $\Delta\psi$ can be calculated from the Nernst equation, $\Delta\psi=(RT/F)\ln([K^+]_{out}/[K^+]_{in})$. The potassium/choline system was used for osmotic equilibration of the lumen of the proteoliposomes with the medium when different K^+ gradients were applied [20]. Appropriate K^+ gradients across the proteoliposomal membrane were adjusted so that proteoliposomes prepared in 0.5 mM KCl, 149.5 mM choline chloride and 10 mM MES (pH 6.0) were diluted in the same MES containing graded KCl concentrations and corresponding choline chloride concentrations to reach a total of 150 mM. Valinomycin (1 nM) was then added to proteoliposomes stained by one of the dyes and the appropriate fluorescence intensity ratio was measured.

3. Results

3.1. Monitoring of H^+-ATPase-generated $\Delta\psi$ by potential-sensitive fluorescent dyes

Fluorescence responses of oxonol V and oxonol VI to the H^+-ATPase-generated $\Delta\psi$ differ. Oxonol V responds to the energization of the proteoliposomes by ATP addition mainly by fluorescence quenching (Figs. 1 and 3). The position of its emission spectrum does not change markedly, whereas oxonol VI exhibits a pronounced red shift in its spectrum (Fig. 2) manifested by an increase in the fluorescence intensity ratio ($R=I_{640}/I_{620}$ in Fig. 3). Both dyes are sensitive to the dissipation of the generated $\Delta\psi$ brought about either by the ATPase inhibitor vanadate or the protonophore CCCP (Fig. 3). CCCP transfers protons across the membrane, and thus increases selectively the membrane proton permeability. As seen in Figs. 1–3, the ATPase can no longer generate $\Delta\psi$ in the presence of 3.3 μM CCCP, which thus short-circuits the membrane. Vanadate affects the generated $\Delta\psi$ more slowly than the protonophore and its effect is dependent on its concentration relative to the actual content of the ATPase in samples. Both vanadate- or CCCP-induced $\Delta\psi$ dissipations observed with fluorescent probes confirm that the probes indeed report on $\Delta\psi$ generated by the H^+-ATPase. A $\Delta\psi$ dissipation following depletion of ATP by hexokinase in the presence of glucose observed with oxonol VI is another confirmation (see inset in Fig. 7A).

Two factors have to be considered when measuring $\Delta\psi$ by fluorescent probes. (1) The binding of the dyes to liposomal membrane is affected by the presence of ions in the medium.

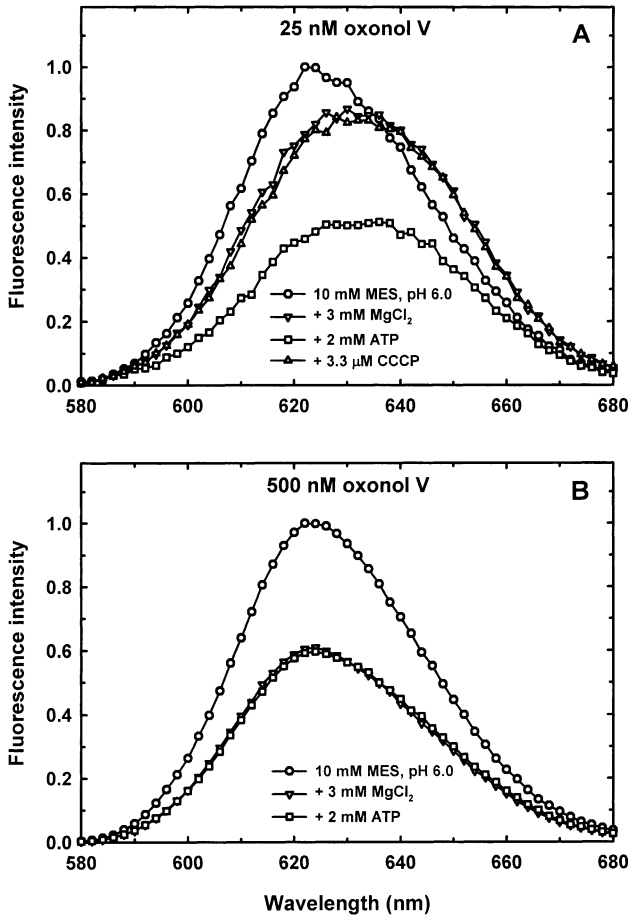

Fig. 1. Fluorescence response of 25 nM (A) and 500 nM (B) oxonol V to energization of proteoliposomes containing incorporated plasma membrane H^+-ATPase. The dye fluorescence spectra measured in a pure suspension of proteoliposomes in 10 mM MES at pH 6.0 (○) are compared with those measured after addition of 3 mM $MgCl_2$ (▽), 2 mM ATP (□) and 3.3 μM CCCP (△). Lipid concentration in (A) was 2 μg/ml, in (B) 6.6 μg/ml; content of plasma membrane proteins in (A) was 20 ng/ml, in (B) 66 ng/ml.

Ions such as $MgCl_2$, KCl or choline chloride cause that more dye binds to the membrane, and the contribution of the bound dye to overall fluorescence is thus enhanced. The emission spectrum of both probes is then red shifted (Figs. 1 and 2). As tested in experiments with liposomes (data not shown), the same concentration of KCl and choline chloride (150 mM) causes nearly the same change in the dye emission spectrum. The K^+ diffusion-based calibration can then be used for an evaluation of the generated $\Delta\psi$ only when a uniform overall ionic strength is ensured during the calibration and monitoring of the $\Delta\psi$ generated by H^+-ATPase. For this reason, the H^+-ATPase-generated $\Delta\psi$ was monitored in the reconstitution buffer supplemented by choline chloride (149.5 mM choline chloride, 0.5 mM KCl). (2) Since the potential-sensitive fluorescent probes carry an electric charge, they participate in the electrochemical equilibrium across the membrane. The $\Delta\psi$ dissipating effect of the charged dye molecules is demonstrated in Figs. 1 and 2. Whereas 25 nM oxonol V or 20 nM oxonol VI responds

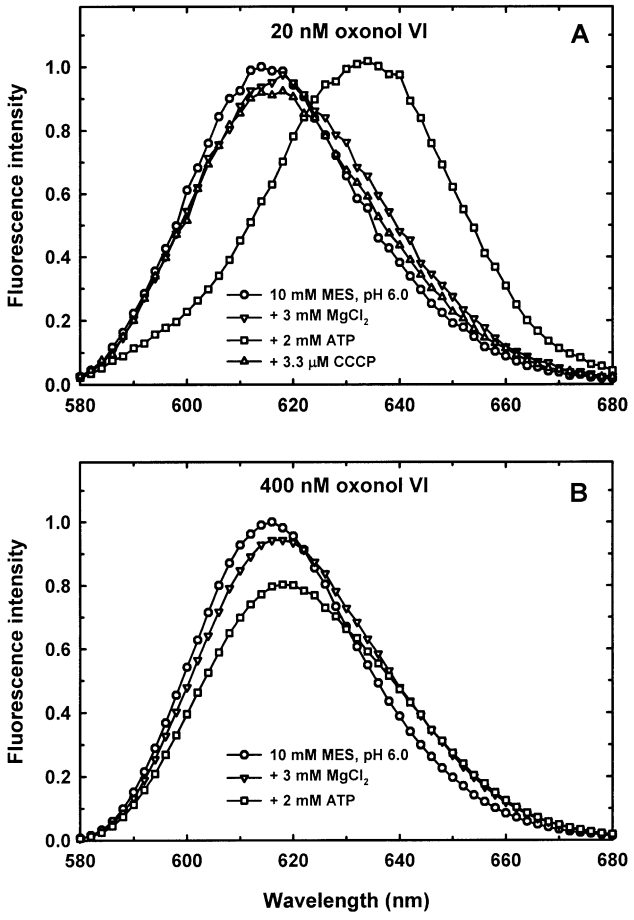

Fig. 2. Fluorescence response of 20 nM (A) and 400 nM (B) oxonol VI to energization of proteoliposomes containing incorporated plasma membrane H^+-ATPase. The dye fluorescence spectra measured in a pure suspension of proteoliposomes in 10 mM MES at pH 6.0 (○) are compared with those measured after addition of 3 mM $MgCl_2$ (▽), 2 mM ATP (□) and 3.3 μM CCCP (△). Lipid concentration in the sample was 70 μg/ml, content of plasma membrane proteins 0.7 μg/ml.

markedly to the $\Delta\psi$ buildup upon ATP addition, there is no significant change in emission spectra for 20-fold higher concentrations of the dyes. This cannot be simply explained by saturation of dye-binding sites on the membrane surface, because the dye (oxonol VI) at the same concentration is still sensitive to membrane potentials created by K^+ diffusion (data not shown). A similar dissipating effect was reported for radioactive potential-sensitive probes [21]. It was also shown in Ref. [13] that the generated $\Delta\psi$ is dissipated by permeant anions such as NO_3^-, SCN^- or Cl^-. When $\Delta\psi$ is created as a consequence of K^+ gradient, ~ 1 μM dye concentration is low compared to, for example, 150 mM ambient K^+ concentration, and charged dye molecules thus do not play a significant role in ion fluxes across the membrane. On the other hand, such a dye concentration matches proton concentration at the ambient pH of 6.0 and a role of the dye molecules cannot be omitted when $\Delta\psi$ is generated by proton transport. Potential-sensitive dyes have to be therefore used at sufficiently low concentrations.

Ratiometric measurements of H^+-ATPase generated $\Delta\psi$ and calibrations of proteoliposomes by K^+ diffusion-created membrane potentials performed with oxonol V and oxonol VI are shown in Figs. 4 and 5. The proteoliposomes were energized and the $\Delta\psi$ generation was started by the addition of 2 mM ATP. The generated $\Delta\psi$ was abolished by the addition of 3.3 μM CCCP. Using appropriate K^+ gradients in the presence of 1 nM valinomycin, a set of membrane potentials was adjusted on proteoliposomes identical to those on which $\Delta\psi$ generated by the H^+-ATPase was monitored. As tested in experiments with liposomes (data not shown), the presence of ATP, and also of CCCP and valinomycin, does not significantly affect the emission spectrum of the dyes. Thus, there is a good reason to

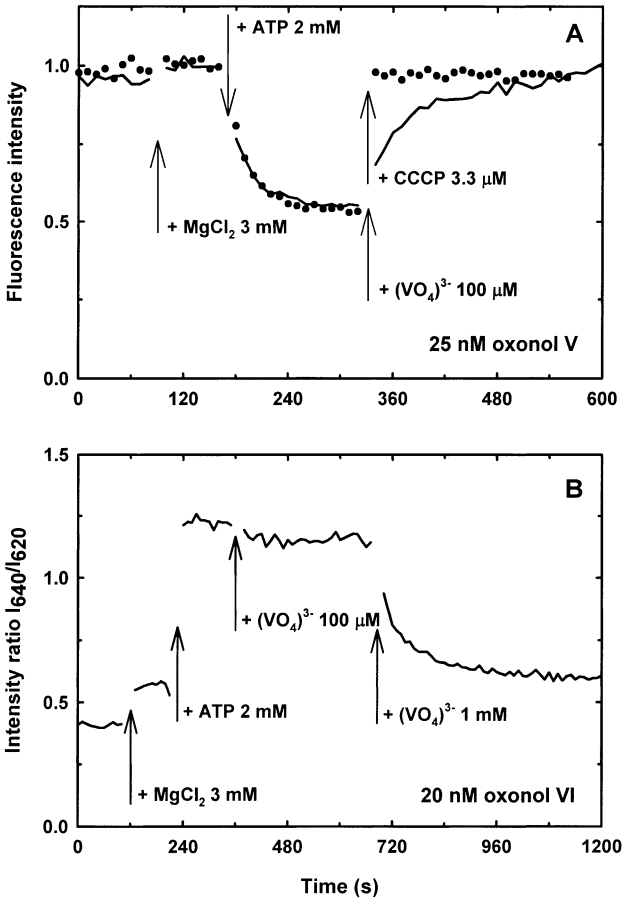

Fig. 3. Comparison of different fluorescence responses of oxonol V and oxonol VI to H^+-ATPase-generated $\Delta\psi$ measured in a suspension of proteoliposomes in 10 mM MES at pH 6.0. (A) Fluorescence intensity of oxonol V at 635 nm I_{635} is monitored after an addition of 3 mM $MgCl_2$ and 2 mM ATP. Then the dye response is changed either by adding 100 μM vanadate (—) to block ATPase function or 3.3 μM CCCP (●) to collapse the generated $\Delta\psi$. Oxonol V concentration in the samples was 25 nM, content of plasma membrane proteins 20 ng/ml and lipid concentration 2 μg/ml. (B) Fluorescence ratio I_{640}/I_{620} of oxonol VI is monitored after an addition of 3 mM $MgCl_2$ and 2 mM ATP. Due to a higher concentration of proteoliposomes 1 mM vanadate was used to block the ATPase function. Oxonol VI concentration in the sample was 20 nM, content of plasma membrane proteins 0.7 μg/ml and lipid concentration 70 μg/ml.

presume that changes in the dye emission that follow the addition of these agents report solely on $\Delta\psi$. The binding of positively charged dyes to the K^+–valinomycin complex accompanied by changes in dye fluorescence was reported previously [22]. This binding was supposed to be a cause of unreliability of calibrations based on K^+ diffusion membrane potentials. In our experiments either with pure liposomes or proteoliposomes, we did not observe any effect of the dye binding to the complex on the fluorescence spectrum position. Only a minor effect on the fluorescence intensity was noticeable (data not shown), which could be explained by quenching accompanying the formation of the putative complex. The minute extent of this effect may be due to the low concentration of valinomycin used in our experiments (1 nM). Moreover, it should be noted that the ratiometric measurements report on spectral position, not on fluorescence intensity, and thus the incidental decrease in fluorescence intensity does not interfere with monitoring fluorescence response to the $\Delta\psi$ buildup.

Because we performed the fluorescence response calibration measurements on the same system as the measurements of the $\Delta\psi$ generated by H^+-ATPase, we assume that the calibration values can be easily used for evaluating the H^+-ATPase-generated $\Delta\psi$. This assumption takes for granted that no sealed vesicles without H^+-ATPase incorporated in the membrane are present in the proteoliposome suspension. The presence of such vesicles would result in underestimation of the $\Delta\psi$ because these vesicles carry the K^+ diffusion potential during calibration, and would thus contribute to the fluorescence response of the system to an imposed calibration $\Delta\psi$ but not to the fluorescence response to H^+-ATPase-generated $\Delta\psi$. Theoretically, the population of vesicles is homogeneous and the vesicles have the same number of H^+-ATPase molecules incorporated in the membrane in the same orientation. This is not fulfilled for real proteoliposomes and the $\Delta\psi$ value measured in the real system is thus a mean value for the heterogeneous proteoliposome population.

3.2. Oxonol V response to H^+-ATPase-generated $\Delta\psi$

It was previously reported that oxonol V fluorescence responds differently to $\Delta\psi$ passively created by the K^+ diffusion and to $\Delta\psi$ actively generated by a proton-translocating ATPase [12,23]. In the former case, the emission spectrum of oxonol V exhibits a red shift similar to that of oxonol VI. In the latter case, the $\Delta\psi$ buildup is accompanied by a pronounced decrease of oxonol V fluorescence intensity, but not by a marked shift of emission spectrum (Figs. 3 and 4). The decrease of intensity is relatively slow (tens of seconds), whereas the intensity ratio increase follows immediately upon ATP addition and then it holds. On comparing calibrations performed with the two oxonols (Figs. 4A and 5A), ratiometric measurements lead for either dye to a different magnitude of H^+-ATPase-generated $\Delta\psi$, oxonol VI indicating a much higher $\Delta\psi$ than oxonol V. Fig. 4B shows that the intensity ratio response to the H^+-ATPase-generated $\Delta\psi$ becomes more marked on decreasing the oxonol V concentration from 25 to 3 nM. As the dye response to the K^+ diffusion-created $\Delta\psi$ of 55 mV remains nearly the same, the measurement at lower concentrations gives a higher $\Delta\psi$. The anomalous response of oxonol V to actively generated membrane potentials was previously explained by fluorescence quenching due to an aggregation of the dye molecules around the electrically charged part of the proton pump [23]. The higher intensity ratio response to the H^+-ATPase-generated $\Delta\psi$ at lower dye concentrations can be explained by smaller dissipation of $\Delta\psi$ due to the flux of dye molecules across the membrane or by suppression of the dye aggregation. Fig. 4B shows that the fluorescence intensity reports on the presence of $\Delta\psi$ also for the highest dye concentration used here (25 nM) where the intensity ratio response is negligible. This observation supports the latter explanation. Generally, it can be said that

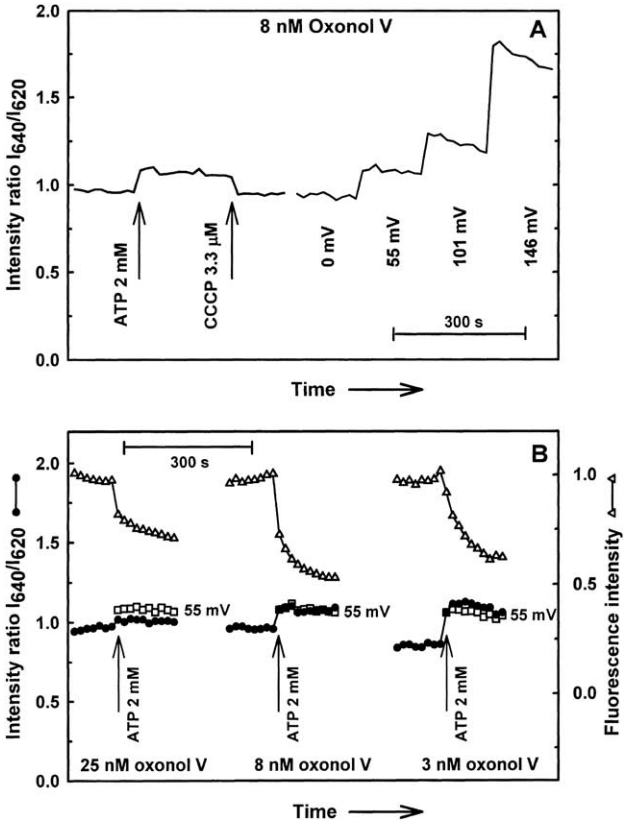

Fig. 4. Fluorescence response of oxonol V to H^+-ATPase-generated $\Delta\psi$ and its K^+ gradient-based calibration. (A) Fluorescence intensity ratio I_{640}/I_{620} of 8 nM oxonol V was monitored. $\Delta\psi$ generated upon addition of 2 mM ATP was collapsed by 3.3 μM CCCP. (B) Fluorescence intensity ratio response of 25 nM (left), 8 nM (middle) and 3 nM (right) oxonol V to the energization of the proteoliposomes by 2 mM ATP (●) as compared with the response to $\Delta\psi$ adjusted to 55 mV by K^+ diffusion (□). For the same dye concentrations, a quenching of fluorescence intensity I_{635} which follows an ATP addition is shown (△) in the upper part of the figure. Lipid concentration in the samples was 2 μg/ml, content of plasma membrane proteins 20 ng/ml.

although the discrepancy between the two dyes becomes less marked with their decreasing concentration, the oxonol V measurement of H^+-ATPase-generated $\Delta\psi$ gives unreliable results when membrane potentials created by K^+ diffusion are used for calibration.

3.3. Oxonol VI response to H^+-ATPase-generated $\Delta\psi$ and its calibration

Because oxonol VI does not exhibit such fluorescence quenching in energized proteoliposomes as oxonol V, it was employed for evaluation of $\Delta\psi$ generated by the reconstituted H^+-ATPase. When fluorescence response to $\Delta\psi$ is calibrated by K^+ diffusion-based membrane potentials, one has to keep in mind that only a limited value of $\Delta\psi$ can be created owing to the diffusion. A practical upper limit of the voltage range in which fluorescence signals can be calibrated by the Nernst-potential method was set at 140 mV by Ref. [10] or at 130 mV by Ref. [14]. This limitation stems from the fact that K^+ cations creating the membrane potential penetrate into the proteoliposomal lumen and change the actual inner K^+ concentration. Using the equation $Q = AC_mU$ (A is the total membrane area and $C_m \equiv 1$ $\mu F/cm^2$ is the specific membrane capacitance) [10], one can calculate the electric charge Q which has to move across the membrane to charge up the membrane capacitance AC_m to reach the voltage $U = 150$ mV. Considering a proteoliposome diameter of 100 nm, nearly 300 K^+ cations have to move across the membrane. For comparison, 160 K^+ cations are present in the proteoliposomal lumen when internal K^+ concentration is 0.5 mM. As the inner K^+ concentration increases, the ratio $[K^+]_{out}/[K^+]_{in}$, present in the Nernst equation drops, and so does the membrane potential. In contrast to Ref. [10], we do not assume that every K^+ cation penetrating through the membrane immediately enters the lumen. It should stay on an inner surface as part of an electrical layer that charges the membrane capacitance. In such case, the K^+ concentration in the lumen should increase stepwise from the original concentration and the $\Delta\psi$ decay should start from the level given by the Nernst equation for an imposed K^+ gradient. As shown later, the stability of the K^+ diffusion potentials depends on the proton permeability of the membrane. This indicates that the rate at which K^+ ions enter the lumen and, hence, at which the inner K^+ concentration is changed, is determined by counter fluxes against the K^+ flux charging the membrane.

Fig. 5 compares calibrations done on proteoliposomes from two different preparations. It can be seen that the intensity ratio at higher calibration membrane potentials decays within minutes for proteoliposomes from preparation 2, whereas calibration membrane potentials on proteoliposomes from preparation 1 are relatively stable. The decay most probably reflects changes in inner concentration of K^+ cations. A similar decay appears when the protonophore CCCP is added (Fig. 6), which indicates that the instability of K^+ diffusion membrane potentials is related to proton

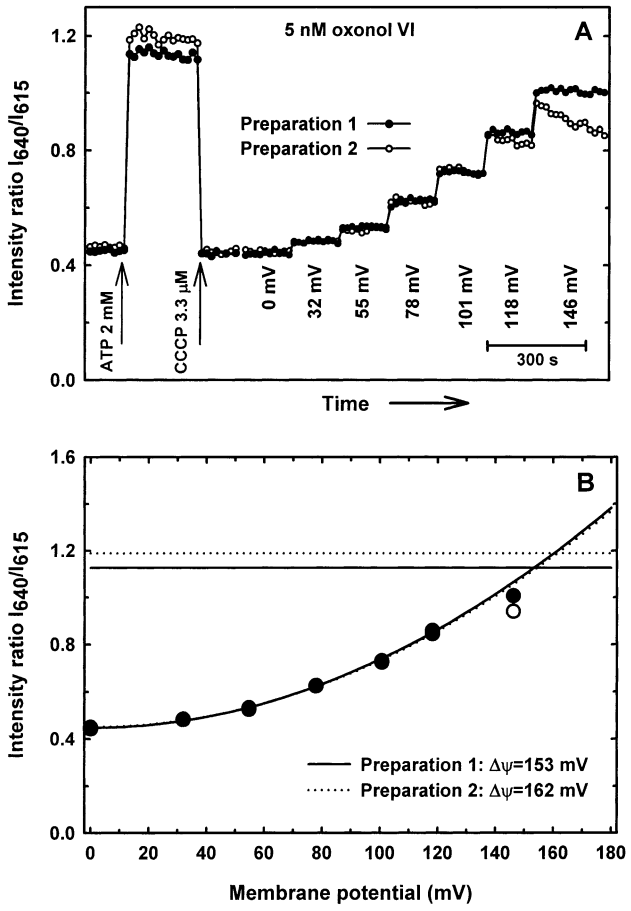

Fig. 5. Estimation of the H^+-ATPase-generated $\Delta\psi$ by ratiometric oxonol VI fluorescence measurement using K^+ gradient-based calibration. Two sets of experimental data obtained from two different preparations of proteoliposomes are presented. (A) Intensity ratio I_{640}/I_{615} was monitored for 5 nM oxonol VI. $\Delta\psi$ generated upon addition of 2 mM ATP was collapsed by 3.3 μM CCCP. Lipid concentration in the samples was 70 μg/ml, content of plasma membrane proteins 0.7 μg/ml. (B) Calibration points obtained from measurement presented in A and corresponding to $\Delta\psi$ lower than 125 mV were fitted by a polynomial of the second order. The intensity ratios corresponding to H^+-ATPase-generated $\Delta\psi$ are represented by horizontal lines. Values of the generated $\Delta\psi$ were determined as the $\Delta\psi$-coordinate of intersection of the calibration curve with the appropriate horizontal line.

permeability of the membrane. It was also observed that the decay was enhanced with increasing the dye concentration (10 nM and higher, data not shown). The different ability of proteoliposomes from different preparations to maintain K^+ diffusion-created $\Delta\psi$ can be ascribed, for example, to their different proton permeability due to an inhomogeneity of egg yolk lecithin used for the reconstitution. Calibration curves drawn through the calibration points are presented in Fig. 5B. The calibration points save those at 146 mV lie on a parabolic curve. The points at 146 mV deviate from the curve and the deviation is larger for proteoliposomes from preparation 2, which is consistent with the assumption that their proton permeability is higher. The deviation indicates that the actual $\Delta\psi$ created by the highest imposed K^+

gradient is lower than the $\Delta\psi$ calculated from the Nernst equation (146 mV). This is in accordance with limits stated for K^+ diffusion-based calibrations in Refs. [10,14]. The calibration points measured for $\Delta\psi$ of 146 mV were therefore not used in the calculation of the calibration curve. The calibration points corresponding to $\Delta\psi$ lower than 125 mV were fitted by a polynomial of the second order.

3.4. Evaluation of H^+-ATPase-generated $\Delta\psi$

Fig. 5 clearly demonstrates that the H^+-ATPase-generated $\Delta\psi$ is stable and is higher than the highest K^+ diffusion-created $\Delta\psi$. Because the generated $\Delta\psi$ exceeds the range of the calibration, its value can be merely estimated by extrapolation, that is, as the $\Delta\psi$-coordinate of intersection of the calibration curve with a horizontal line that stands for an intensity ratio measured for the generated $\Delta\psi$. The estimated values were around 160 mV. The $\Delta\psi$ should depend on the activity of the reconstituted ATPase, purity of the lipid, quality of reconstitution, etc. The estimated value corresponds to the particular lipid/protein ratio used in preparation. Provided that H^+-ATPase forms 50% of total of plasma membrane proteins [24], a rough estimate can be made that one proteoliposome 100 nm in diameter contains six ATPase molecules in its membrane.

Because we tested an influence of dye concentration on the measured value of $\Delta\psi$, we tried to find an optimal dye concentration in which the dye does not interfere with the $\Delta\psi$ generation. The limits of the spectrofluorometer sensitivity were reached on going to the dye concentration of 5 nM. This concentration was thus used for the calibration.

As seen in inset of Fig. 6A, 1 nM CCCP caused a decay of the K^+-diffusion $\Delta\psi$ (146 mV, calculated from the Nernst equation). This documents the fact that the instability of K^+ diffusion-based membrane potentials is associated with the proton permeability of the membrane. The same amount of CCCP caused a drop of about 16 mV in the H^+-ATPase-generated $\Delta\psi$ (see Fig. 6A and next chapter). These two observations indicate that the same factors that induce the K^+-diffusion $\Delta\psi$ decay have a significant influence on the H^+-ATPase-generated $\Delta\psi$. On the other hand, there is a good reason to assume that the H^+-ATPase-generated $\Delta\psi$ unaffected by additional factors is monitored when the calibration K^+-diffusion membrane potentials are stable. We can thus conclude that the best results are obtained when care is taken to exclude factors potentially affecting the $\Delta\psi$ value such as impurities in the lipid or unsuitable concentration of the dye.

3.5. The role of membrane proton permeability in $\Delta\psi$ generation

The role of membrane permeability for protons in $\Delta\psi$ generation by the H^+-ATPase was studied by using the protonophore CCCP. Defined amounts of CCCP successively added to proteoliposomes gradually increased their

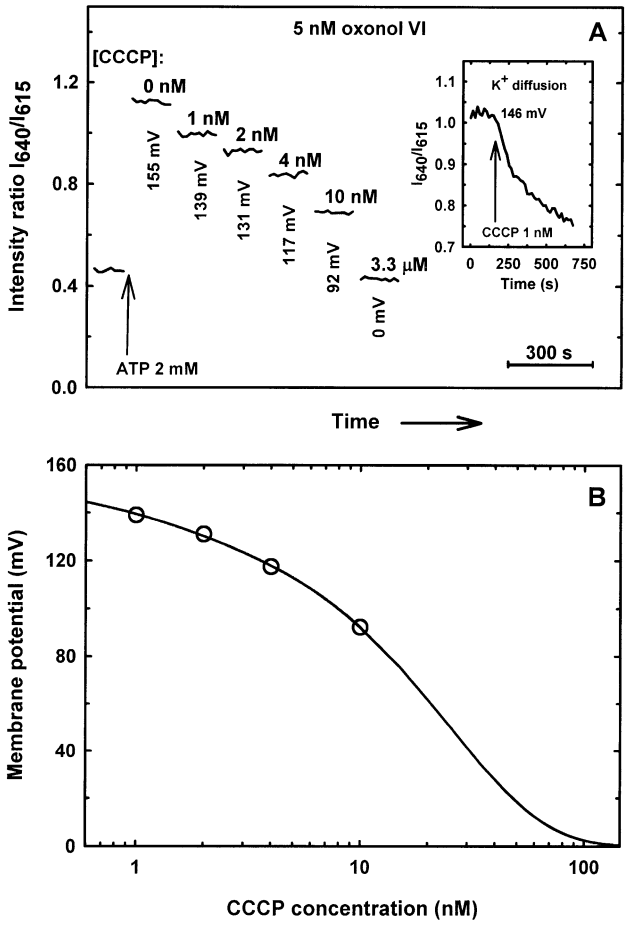

Fig. 6. Changes of the H^+-ATPase-generated $\Delta\psi$ induced by protonophore CCCP. (A) Time dependence of intensity ratio I_{640}/I_{615} of 5 nM oxonol VI was monitored in a suspension of proteoliposomes (preparation 2). Proteoliposomes were energized by an addition of 2 mM ATP and then defined amounts of CCCP were successively added. Inset shows a decay of the fluorescence intensity ratio caused by an addition of 1 nM CCCP to proteoliposomes from preparation 1 with K^+ diffusion-created $\Delta\psi$ of 146 mV. (B) The dependence of $\Delta\psi$ values determined by the calibration curve drawn in Fig. 5B for preparation 2 on CCCP concentration was fitted by the sum of two exponential functions. Lipid concentration in the samples was 70 μg/ml, content of plasma membrane proteins 0.7 μg/ml.

proton permeability (Fig. 6) and progressively decreased the H^+-ATPase-generated $\Delta\psi$ by inducing proton counterflux across the membrane. The $\Delta\psi$ levels were stable and no intensity ratio decay was observed. CCCP can thus serve as a means for adjusting defined membrane potentials on proteoliposomes, stable for several minutes. The $\Delta\psi$ generated at different CCCP concentrations was determined by using the calibration curve drawn in Fig. 5. The curve of $\Delta\psi$ versus CCCP concentration, fitted by two exponentials (Fig. 6B), documents that CCCP concentrations higher than about 100 nM abolish the membrane potential.

Fig. 7A demonstrates that the H^+-ATPase-generated $\Delta\psi$ holds for at least 50 min. This is in accordance with the ATP consumption during proton pumping calculated for the actual concentration of H^+-ATPase; ATP should persist

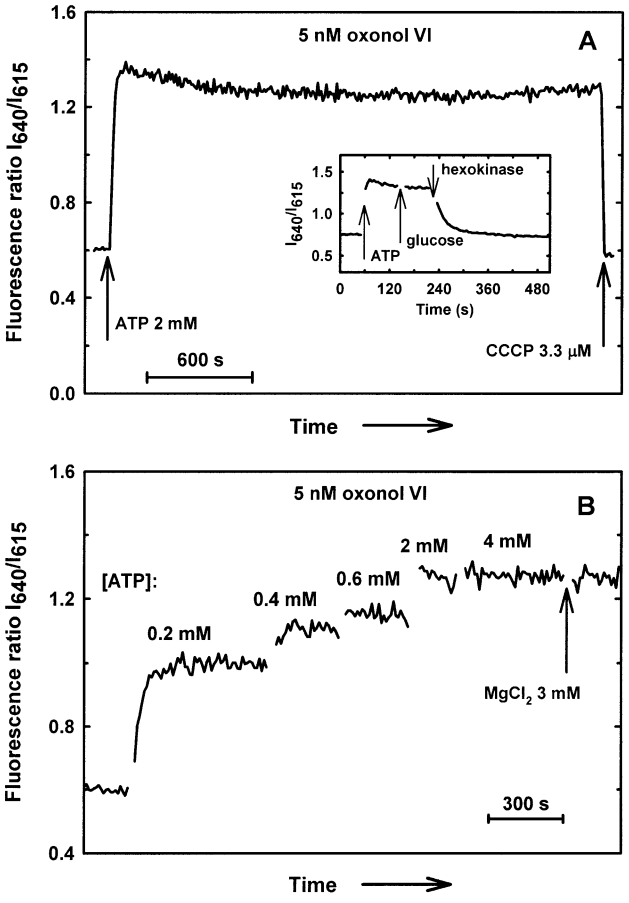

Fig. 7. Stability of the H^+-ATPase-generated $\Delta\psi$ and its dependence on ATP concentration. (A) Time dependence of intensity ratio I_{640}/I_{615} of 5 nM oxonol VI was monitored in a suspension of proteoliposomes prepared and diluted in 10 mM MES (pH 6.0), 10 mM KCl and 140 mM choline chloride. Proteoliposomes were energized by an addition of 2 mM ATP and then the fluorescence measurement was carried out for about 50 min. At last, the $\Delta\psi$ was collapsed by 3.3 μM CCCP. Inset shows dissipation of the generated $\Delta\psi$ following depletion of ATP by the action of hexokinase. The proteoliposome suspension was supplemented by 25 mM glucose and 22 units of hexokinase were added to 3 ml of the sample. (B) In the same proteoliposomes as in A, $\Delta\psi$ generated by H^+-ATPase was monitored by 5 nM oxonol VI for different concentrations of ATP that was successively added to the sample. At last, $MgCl_2$ was added to increase its actual concentration in the suspension from 3 to 6 mM to exceed the ATP concentration, which was 4 mM at that moment. Lipid concentration in the samples was 70 μg/ml, content of plasma membrane proteins 0.7 μg/ml.

for hours. Depletion of ATP by hexokinase in the presence of glucose results in the dissipation of the generated membrane potential signified by a decrease in the fluorescence intensity ratio (inset of Fig. 7A). Fig. 7B shows how the generated $\Delta\psi$ depends on ATP concentration. ATP concentration determines the rate of ATP splitting by the H^+-ATPase and thus the rate of proton transport across the membrane. At 2 mM ATP, that is, concentration of ATP used in our experiments, the generated $\Delta\psi$ did not increase upon further ATP addition. This means that the value of H^+-ATPase-generated $\Delta\psi$ measured in our experiments corresponds to the maximum $\Delta\psi$ that can be generated on the proteoliposomes reconstituted under our conditions.

The extraordinary stability observed for H^+-ATPase-generated $\Delta\psi$ shows that the $\Delta\psi$ buildup is either unaffected by changes in inner pH which are supposed to take place during proton pumping, or these changes are minor. This observation can be interpreted as indicating that the protons transported by the pump stay at the membrane surface or return back through the membrane. Thus, the inner pH need not be markedly affected during the pumping. To confirm this hypothesis, measurements of the inner pH by pH-sensitive fluorescent indicators are now being performed on reconstituted proteoliposomes.

4. Conclusion

Oxonol V detects the $\Delta\psi$ generated by H^+-ATPase even at low concentrations of proteoliposomes. Its response to the generated $\Delta\psi$ consists mainly in the pronounced quenching of its fluorescence. To K^+ diffusion-based membrane potentials, oxonol V responds moreover by the marked red shift of the emission spectrum. The different dye behavior in the presence of $\Delta\psi$ generated actively by H^+-ATPase or created passively owing to K^+ gradient does not allow the use of the K^+ diffusion-based membrane potentials for calibration of the probe response to $\Delta\psi$. Because it is not therefore clear how to calibrate the fluorescence response, oxonol V cannot be used for quantitative measurements of the $\Delta\psi$ generated by H^+-ATPase. On the other hand, oxonol VI does not exhibit such pronounced quenching of fluorescence as oxonol V upon binding to the membrane. This dye responds to both types of $\Delta\psi$ similarly. The marked red shift reports on the built $\Delta\psi$. Oxonol VI therefore allows evaluating the H^+-ATPase-generated $\Delta\psi$ by using the K^+/valinomycin calibration method. The ratiometric measurements can be advantageously employed for this purpose.

Ratiometric measurements of oxonol VI fluorescence offer a useful tool for studying membrane potential generated by the vesicle-reconstituted yeast plasma membrane H^+-ATPase. We have shown that the H^+-ATPase builds up $\Delta\psi$ about 160 mV across the proteoliposomal membrane, which is stable over tens of minutes. The crucial role of the membrane proton permeability in the $\Delta\psi$ generation was demonstrated by manipulating the membrane permeability with the protonophore CCCP. Defined membrane potentials, stable over minutes, were adjusted by different concentrations of the protonophore.

This study documents that even in the case of vesicles, care has to be taken to find an optimal combination of a particular $\Delta\psi$-sensitive fluorescent probe with a particular technique of fluorescence monitoring. Obviously, much more complicated situation occurs in intact cells in which the probe behavior cannot be easily described. At present, there is no generally applicable method for deciding which dye/technique combination suits a specific system under

study but, in principle, (i) the redistribution fluorescent probes should be used in very low concentrations so as not to interfere with the studied system (a particular concentration is often determined by limits of spectrofluorometer sensitivity), (ii) full spectral approach should be chosen for elucidating how the selected fluorescence probe senses the membrane potential.

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