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Electrogenic transport by the *Enterococcus hirae* ATPase

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A transport ATPase from *Enterococcus hirae* was reconstituted in lipid vesicles and its electrogenic action investigated with the fluorescent dye oxonol VI as membrane potential probe. Reconstitution in bacterial and in soybean phospholipid mixtures led to transport-active vesicle preparations. Inside-out oriented ATPase molecules were activated by the addition of ATP to the extravesicular medium, generating in all experiments an intravesicularly positive potential. The extravesicular pH strongly influenced the initial pumping rate and the duration of the pumping activity. At neutral pH, transient pumping activity was observed, lasting for 1–2 min, while at pH 5.6, pumping was continuous. The transport activity was not dependent on the ionic composition of the buffer on either side of the membrane. These findings can be interpreted as the action of a proton ATPase, regulated by the cytoplasmic proton concentration and electrogenically translocating protons from the cytoplasm to the extracellular space.

Introduction

We recently described the purification of a novel ATPase from the Gram-positive bacterium *Enterococcus hirae*, formerly called *Streptococcus faecalis* or *faecium* [1]. The enzyme has the following main properties: (i) it consists of a single polypeptide component of $M_r = 78\,000$, (ii) it is inhibited by micromolar concentrations of vanadate and (iii) it forms an aspartyl phosphate intermediate as part of the reaction cycle [2]. Based on these properties, this bacterial ATPase appears to belong to the class of ion-motive ATPases, chiefly represented by the eukaryotic Na^+/K^+-ATPases, Ca^{2+}-ATPases, and the fungal and plant plama membrane H^+-ATPases [3].

Until a few years ago, such ion-motive ATPases were considered to be a typically eukaryotic feature while prokaryotes regulate their ionic environment with secondary symporters or antiporters. It has now, however, become clear that ion-motive ATPases are also widespread in bacteria, and enzymes of this type have been well characterized not only in *E. hirae*, but also in *Escherichia coli* [4,5], *Staphylococcus aureus* [6], *Bacillus acidocaldarius* [7], and *Methanococcus voltae* [8].

Due to the relative ease of purification and its stability, the *E. hirae* ATPase appears to be a suitable model system for the study of fundamental aspects of ATP-driven ion translocation. We have previously reported the reconstitution of the ATPase into proteoliposomes and their functional analysis [9]. Here, we employ a sensitive optical method [10] to investigate the electrogenic properties of this enzyme.

Materials and Methods

Materials

Bacterial L-α-phosphatidylethanolamine, type IX (bacterial PE) was obtained from Sigma, St. Louis, MO. Asolectin (soybean PC) was from Associated Concentrates, Woodside, NY. Oxonol VI (bis(3-propyl-5-oxoisooxazol-4-yl)pentamethin oxonol) was from Molecular Probes, Eugene, OR. ATP (special quality) and valinomycin were supplied by Boehringer-Mannheim, F.R.G., vanadate by Ventron, Karlsruhe, F.R.G., and *n*-octyl-β-D-glucopyranoside (octyl glucoside) by Calbiochem, San Diego, CA. *n*-Decyl-β-D-maltopyranoside (decyl maltoside) was synthesized by H. Alpes [11]. All other reagents (analytical grade) were obtained from Merck, Darmstadt, F.R.G. Spectrum Medical Industries, Los Angeles, CA, was the supplier of dialysis tubing (high speed Spectra/Pore 2, 6.5 mm wide).

Cultivation of bacteria and isolation of the ATPase

E. hirae (ATCC9790, formerly called *S. faecalis* or *faecium*) was grown and ATPase was isolated and trans-

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ferred to decyl maltoside as previously described [12]. The purified enzyme had a specific ATP hydrolysis activity of 2 U/mg.

Preparation of the proteoliposomes

The bacterial PE was partially purified according to the procedure of Sone et al. [13]. 50 mg of the lipid and 160 mg of octyl glucoside were dissolved in 5 ml of a buffer containing 50 mM H_2SO_4, 20 mM Hepes, adjusted to pH 7.5 with choline base. Portions of 250 μl were stored at $-20\,^{\circ}$C and thawed immediately before use. For reconstitution, 250 μl of lipid solution and 250 μl of protein solution (0.1–0.25 U of ATPase activity, corresponding to 50–125 μg protein) were mixed on ice and transferred to dialysis tubing. Dialysis was performed at $4\,^{\circ}$C against 2 changes of 200 vol. of 50 mM Na_2SO_4, 20 mM Na-Hepes (pH 7.5), or the buffer specified in the experiment, respectively, for 6–8 and 50 h.

Fluorescence measurements

Fluorescence measurements were carried out on a Perkin Elmer 650-40 fluorescence spectrophotometer as described earlier [10], using a thermostatically controlled cuvette holder equipped with a magnetic stirrer. The excitation wavelength was set to 580 nm (slit width 20 nm) and the emission wavelength to 660 nm (slit width 20 nm). A 1 mM oxonol VI stock solution in ethanol was diluted 10-fold with buffer just before use. From this, oxonol VI was added to the sample at a final concentration of 100 nM.

Calibration of the oxonol VI fluorescence

The fluorescence change of oxonol VI in response to a membrane potential is mainly governed by an electric field dependent change in partitioning of the dye between the lipid layer and the aqueous phase. The calibration of such fluorescence signals has been described in detail for dioleoylphosphatidylcholine vesicles [10]. For the present report, these calibrations were extended to bacterial PE. A ratio of specific oxonol VI fluorescence in lipid versus water, r_f, of 7.6 ± 0.3 and an apparent partition coefficient at zero voltage, $\gamma(0)$, of 1500 ± 200 were determined for the calculation of the corresponding calibration curves (cf. ref. 10).

For each experiment, the lipid concentration was determined by comparing the oxonol VI fluorescence before addition of vesicles, F_w, with the fluorescence of the vesicle suspension, F_0 (at zero voltage, before adding ATP). The quantity F_0/F_w allows the calculation of the lipid concentration (or volume of the lipid phase) with an accuracy of 10%, which is sufficient to transform fluorescence signals into membrane potentials with the appropriate calibration curves [10]. The membrane potentials thus obtained only represent average potentials, U_{AV}, since the vesicles are heterogeneous in radii and number of active pumps [14].

The primary time-resolved fluorescence traces were digitized on a digitizing board, fed into a computer and stored for further analysis. Except for Fig. 1, all experimental traces were plotted from digitized data.

Results

Electrogenicity of the transport-ATPase

An experiment demonstrating the electrogenic effect of the pump is shown in Fig. 1. Upon addition of ATP, an increase of the fluorescence amplitude was observed. It rapidly reached a maximum after 60–70 s, followed by a slow decline. The maximum fluorescence intensity corresponds to an average membrane potential U_{AV} (max) of +70 mV. The transient nature of the electrogenic effect was caused by the transient activity of the pump, paired with passive ion leakage of the membrane. The interior positive membrane potential indicates that net positive charges were transported into the vesicles. Essentially the same results were also obtained with a cationic potential sensitive fluorescence dye, 1,3,3,1′,3′, 3′-hexamethylindodicarbocyanine (unpublished results). The orientation of the membrane potential generated by the ATPase was the same in all the experiments. Since ATP was added to the extravesicular medium and does not permeate into the vesicles, only those pumps are activated which are reconstituted inside-out. This means that in vivo, the ATPase pumps net positive charges from the cytoplasm to the extracellular medium.

To corroborate the electrogenic nature of the observed fluorescence signals, vesicles were rendered charge-permeable with valinomycin. Measurements as those in Fig. 1 were repeated in the presence of various amounts of valinomycin, a carrier for potassium and, with 1/1000 of the activity, for sodium [15]. The data shown in Fig. 2 were generated with vesicles reconstituted in 100 mM K^+ instead of Na^+ to facilitate charge movements via valinomycin; comparable results were obtained in Na^+ buffer if correspondingly higher concentrations of valinomycin were employed. Valinomycin increases the passive conductance for K^+ and thus affects the membrane potential, U, which is measured by the oxonol VI fluorescence. The relation between the change of the membrane potential, dU/dt, pump current, I_p, and leak current, I_l is given in Eqn. 1.

$$C_M \cdot \frac{dU}{dt} = I_p + I_l = n_p \cdot v_p \cdot z_p \cdot e_0 - \lambda_l \cdot U \qquad (1)$$

where C_m is the electrical capacity of the vesicle membrane, n_p the number of pumps per vesicle, v_p the turnover rate of the pump, z_p the number of net charges transferred across the membrane per pumping cycle and e_0 the elementary charge, λ_l is the leak conductance of the vesicles membrane which is ap-

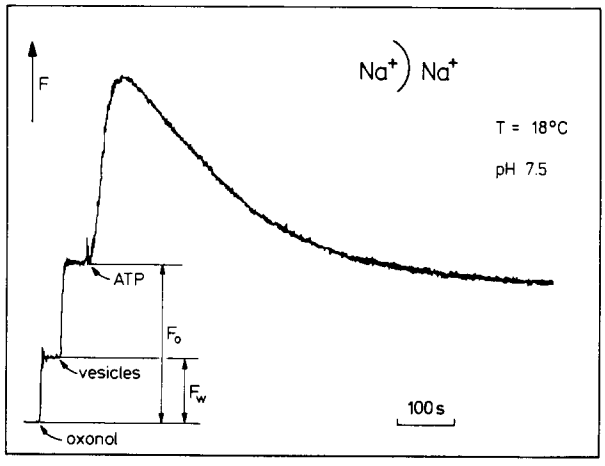

Fig. 1. Transient membrane potential generated by the ATPase. Vesicles were reconstituted in buffer containing 50 mM Na_2SO_4 and 10 mM Hepes (pH 7.5), and measured in buffer containing 50 mM Na_2SO_4, 5 mM $MgSO_4$, 10 mM Hepes (pH 7.5) and 100 nM oxonol VI. Vesicles (from bacterial PE) and 1.25 mM ATP were successively added. F is the fluorescence intensity, F_w is the fluorescence intensity of the aqueous dye solution, and F_0 that of the vesicle suspension at zero membrane voltage. The maximum rise in fluorescence intensity after addition of ATP corresponds to a membrane potential $U_{AV} \approx 70$ mV.

proximately proportional to the concentration of valinomycin [16]. A specific membrane capacity, C_M, of 1 $\mu F/cm^2$ was used in all calculations. The results presented in Fig. 2 show that the general shape of the membrane potential transient is not dependent on the overall potential. At the maximum potential, U_{AV} (max), the condition $dU/dt = 0$ is valid which means that $I_p = -I_l$. The finding that this condition is fulfilled at lower values of U_{AV} for increasing concentrations of valinomycin can be explained by an increased value of

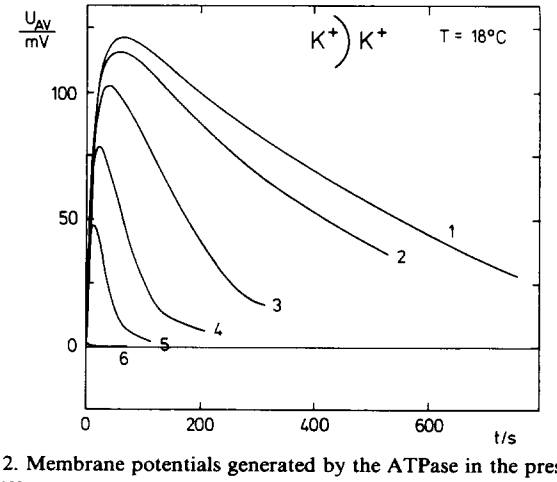

Fig. 2. Membrane potentials generated by the ATPase in the presence of different amounts of valinomycin. Vesicles were reconstituted in buffer containing 50 mM K_2SO_4 and 10 mM Hepes (pH 7.5). Valinomycin was added from ethanolic solutions before ATP. The numbers of the curves correspond to following nominal aqueous concentrations: 1, no valinomycin; 2, 1 pM; 3, 10 pM; 4, 50 pM; 5, 100 pM; 6, 1 nM.

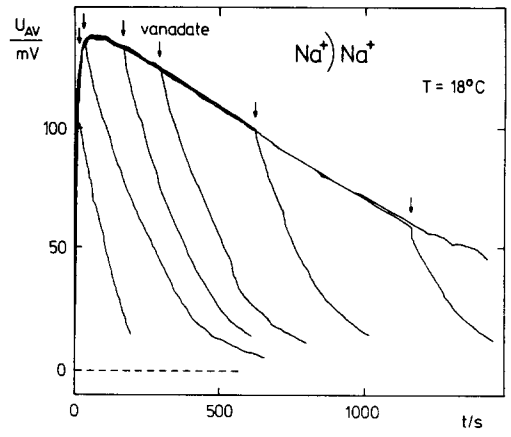

Fig. 3. Influence of vanadate on the membrane potential generated by the ATPase. Experiments as shown in Fig. 1 were performed at pH 6.0. At different times, 5 μl of 400 mM sodium vanadate were added to 1 ml reactions. Dashed line: vanadate added before ATP.

λ_l due to the action of valinomycin and a dependence of the pumping current on the membrane potential, presumably by a non-constant current voltage curve. Even at high concentrations of valinomycin when only low membrane potentials are effective, the pump reduces its activity after a short period of time and approaches a complete stop after approx. 100 s. These findings suggest that the occurrence of a transient pumping activity is not caused by membrane voltage effects. In an earlier report [9] we showed that the reconstituted ATPase could be reactivated by detergent or by valinomycin. However, the concentration of valinomycin was more than 20 000-fold higher than those employed in this work. We found that such high concentrations of the membrane soluble ionophore disturb the membrane structure in a detergent-like manner.

To demonstrate that the decay of the fluorescence signal is due to a decreased rate of pumping and not to any other secondary effects (e.g., increased membrane permeability), the pumping process was stopped with vanadate [1]. When added 10 s before ATP, vanadate inhibited the pumping activity completely (Fig. 3, dashed line); when added after the addition of ATP, the observed decay of the potentials corresponded to the passive decharging of the membrane capacity, C_M, which is an exponential process with a time constant $\tau = C_M/\lambda_l$, where λ_l is the leak conductance. The experiments in Fig. 3 show that τ remains virtually constant, yielding a nearly time-invariant conductance, λ_p, of 5–7 nS/cm^2. This value agrees well with previous estimates of λ_p of 5 nS/cm^2 for dioleoylphosphatidylcholine vesicles [14]. The observed decrease of the fluorescence in absence of vanadate therefore reflects a reduction of the number of transporting pumps and/or the turnover rate.

Although we could not pinpoint the reason for the gradual shutdown of the pumping activity of the

ATPase, we excluded a lack of ATP as a reason for the transient signals: the ATP concentration after an experiment was found to be virtually unchanged and adding more ATP at the end of the fluorescence transient was without an effect. Similarly, the accumulation of ADP, which is known to inhibit the ATPase (unpublished observations), was determined to be insignificant and could therefore not be responsible for the observed effects. An alternative explanation for the transient nature of the electrogenic effect would be the depletion of the transported ion, particularly if it were a rare ionic species that was only present as a contaminant. However, no evidence for this was found (see below). We like to speculate that the transient activity of the ATPase at neutral to alkaline pH reflects a regulatory property.

Ion specificity

The ion specificity of the ATPase was investigated by preparing vesicles in different buffers, containing alternatively 100 mM Na^+, K^+, $Tris^+$ or $choline^+$ as cations, and SO_4^{2-} or Cl^- as anions. The replacement of SO_4^{2-} by Cl^- only led to an increased leak conductance, in agreement with the higher membrane permeability of Cl^- compared to SO_4^{2-}. When vesicles were formed solely in 100 mM Tris-Hepes, essentially the same electrogenic transport properties as depicted in Fig. 1 were observed. Testing many different vesicle preparations under a variety of experimental conditions, we always observed closely similar electrogenic activities. From these findings, we conclude that the pump activity is not obligatorily coupled to the presence of either Na^+, K^+, Tris, choline, or Mg^{2+}, although any of these ions may be transported when present at high concentrations. Mg^{2+} is a necessary cofactor for enzyme activity [1] but an unchanged pumping activity was seen when Mg^{2+} was only present as Mg^{2+}-ATP.

We also added the following ions to the vesicle preparation: Ca^{2+}, Fe^{3+}, Bi^{2+}, Co^{2+}, Cu^{2+}, Zn^{2+}, Pb^{2+}, Sr^{2+}, Cd^{2+}, Ag^+, Ce^{4+}, Mn^{2+}, Al^{3+}. None of these ions showed a stimulation or prolongation of the pumping activity when added in the concentration range of 1–50 μM. When EDTA was employed to complex trace amounts of di- and trivalent cations, no effect on pumping was observed either. To resolve any differential effects of cations inside and outside the vesicle, we tested all pairwise combinations of Na^+, K^+ or $choline^+$ as inside and outside buffers. Again, no significant effects on pumping rate or maximal potential reached were apparent as shown in Fig. 4.

pH dependence

By elimination, protons appeared most likely to be involved in the electrogenic activity of the ATPase. So we investigated the pH dependence of the pumping process (Fig. 5). Varying the pH had obviously two effects. First, we found that the maximal potential,

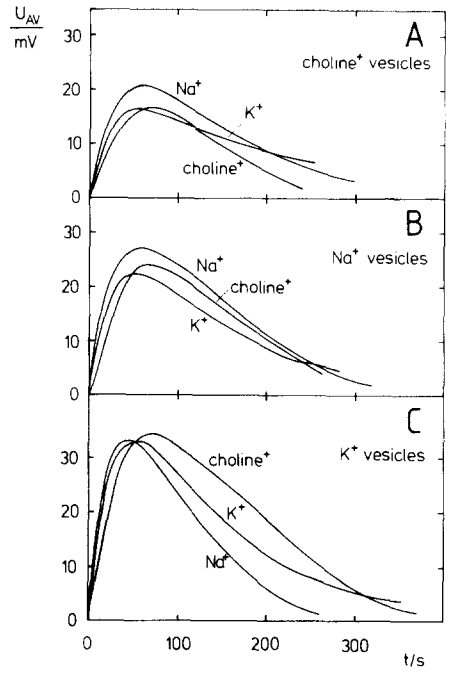

Fig. 4. Influence of buffer composition on the membrane potential generated by the transport ATPase from *E. hirae*. Vesicles were prepared in buffers containing 20 mM Hepes and 50 mM of either Na_2SO_4 or K_2SO_4 or $choline_2SO_4$, pH 7.5. The buffer in which the experiments were performed contained in addition 5 mM $MgSO_4$. $T = 19\,^{\circ}C$. Corresponding buffers are marked Na^+, K^+ and $choline^+$. A: vesicles reconstituted in $choline^+$ buffer, B: Vesicles reconstituted in Na^+ buffer, C: vesicles reconstituted in K^+ buffer.

while constant between pH 5.5 to 6.15, decreased steeply above pH 6.15 (Fig. 5A and B). These changes of $U_{AV}(max)$ are discussed further in the context of the lipid dependence. Secondly, lowering the pH markedly reduced the initial rate of rise in membrane potential, dU_0/dt (cf. Eqn. 1), and thus increased the time to reach $U_{AV}(max)$. Concomitantly, the plateau potential persisted for a longer time. At pH 5.5, the membrane potential was maintained near $U_{AV}(max)$ for more than 50 min. The potential, however, collapsed rapidly when the pH was raised to 7.5 by the addition of NaOH to the medium, leading to a fast inactivation of the pump. Since the passive leak rate of the vesicles remained practically constant in the pH range studied (not shown), these observations must be interpreted as prolonged pumping activity of the ATPase at pH 5.5. Essentially the same results were obtained if the vesicles were prepared in K^+, $choline^+$, or $Tris^+$ buffers instead of Na^+ buffer.

In the experiment of Fig. 6, the pumping activity of the ATPase was started at pH 7.5, where the electrogenic transient ends after approx. 10 min. Then, an appropriate amount of H_2SO_4 was added to lower the pH to 5.8. Reactivation of the pumping activity was observed, again raising the membrane potential for the next 25 min. Conversely, when pumping was started at

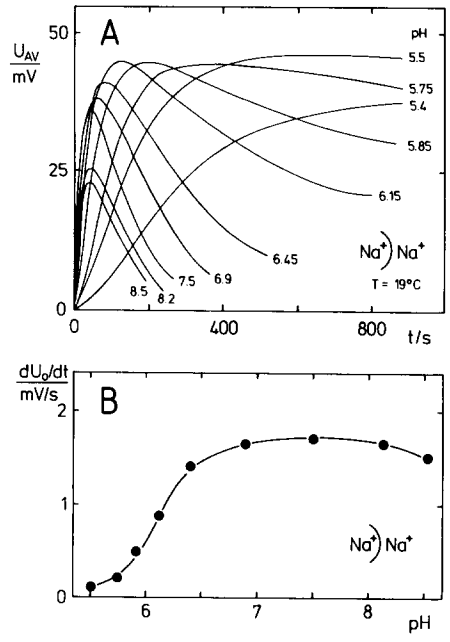

Fig. 5. Influence of the pH on the membrane potential generated by the ATPase. With the exception of pH the conditions of the experiment were as in Fig. 1. The pH was adjusted to the indicated value with H_2SO_4 or NaOH and confirmed to be stable during the time of the experiment. A: time-course of the average membrane potential, U_{AV}, at different pH values. B: initial slope of the potential signal, dU_0/dt, as a function of the pH. According to Eqn. 1, this parameter is proportional to $n_p \cdot v_p \cdot z_p$, the product of the number of active pumps, n_p, the turnover rate, v_p, and the pump stoichiometry factor, z_p.

pH 5.85, the membrane potential transient persisted and finally merged after 36 min with that of vesicles reactivated by a pH drop. Clearly, the same states of pumping activity were eventually reached, independent of the different starting conditions. Thus, the arrest of pumping at high pH is not due to irreversible inhibition or protein denaturation; rather, it may reflect a physiological regulatory mechanism. Control experiments performed in the absence of ATP exhibited only a very slow increase in fluorescence in response to the induced pH change. This effect was due to the generation of a proton diffusion potential caused by leakage (Fig. 6).

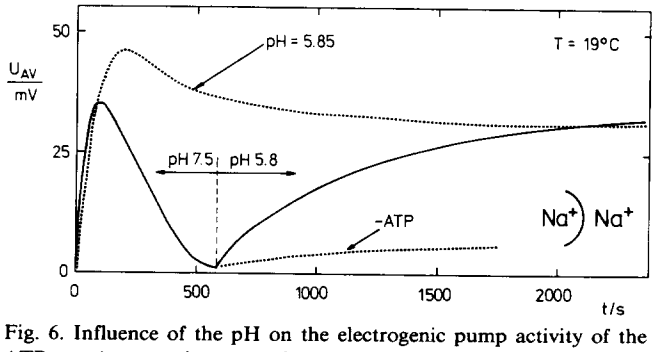

Fig. 6. Influence of the pH on the electrogenic pump activity of the ATPase. An experiment performed in buffer of pH 5.85 (upper dashed line) is compared with an experiment at pH 7.5 (solid line). When the pumping transient had almost ended after approx. 10 min, H_2SO_4 was added to lower the external pH to 5.8. The lower dashed line indicates the proton diffusion potential in the absence of ATP, generated by proton leakage due to the pH gradient. All other conditions were as in Fig. 5.

Lipid dependence

We also found that the enzymatic activity was dependent on the lipid composition used for the reconstitution of the ATPase. Two different lipid preparations that produced transport active vesicles were investigated in more detail: asolectin, a soy bean phospholipid mixture, with phosphatidylcholine as the main component, and *Escherichia coli* lipids, with phosphatidylethanolamine as the main component. While both of these lipid preparations led to electrogenic pumping activity, a striking difference was found in the dependence of the activity on the external pH. In Fig. 7, the pH dependence of the maximum fluorescence intensities of the transients are depicted as relative values of $\Delta F_{max}/F_0$. With bacterial PE, $\Delta F_{max}/F_0$ increased when the pH was lowered, with a plateau between pH 5.5 and 6.2, and a sharp decrease below pH 5.4 (Fig. 7A). When the ATPase was reconstituted in asolectin, the maximum of $\Delta F_{max}/F_0$ was shifted to pH 7.3 (Fig. 7B) and the

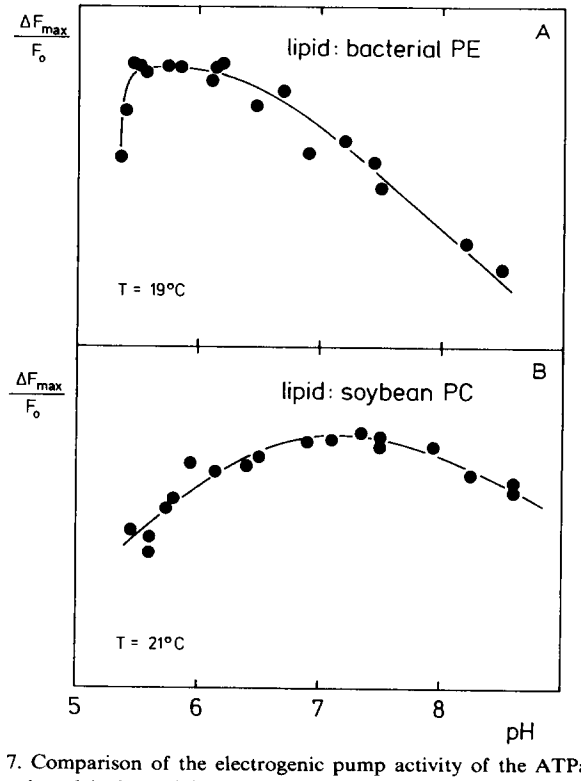

Fig. 7. Comparison of the electrogenic pump activity of the ATPase reconstituted in bacterial PE (A) and soybean PC (B). Experiments performed in 50 mM Na_2SO_4 and 10 mM Hepes, pH adjusted with H_2SO_4 or NaOH. Since the experiments were performed with different vesicle preparations with variable enzymatic activities the fluorescence intensities, $\Delta F_{max}/F_0$, were normalized to the same maximum value for each data set. The lines connecting the data points are drawn to guide the eye.

overall pH dependence of the fluorescence amplitudes was smoother than in the bacterial lipids. Longer lasting transients at low pH could also be observed in soybean vesicles.

Discussion

The purified, reconstituted ATPase from *E. hirae* clearly is capable of electrogenic ion translocation. Based on the indirect evidence discussed below, the ATPase most likely translocates protons electrogenically from the cytoplasm to the extracellular space, or, indistinguishably, hydroxyl ions in the opposite direction.

The maximal steady state potential, $U_{AV}(\max)$, generated by the ATPase depended on the number of transport active pumps, n_p, reconstituted per vesicle (cf. eqn. 1). Typically, potentials in the order of 100 mV could be obtained. With a leak conductance of 5 nS/cm^2, the leak current Φ_l is 500 pA/cm^2 at $U = 100$ mV. The surface area, A, of a vesicle with radius $r = 50$ nm is $3.1 \cdot 10^{-10}$ cm^2. This results in a leak current $I_l = \Phi_l \cdot A = 1.6 \cdot 10^{-19} \cdot A$, or 1 elementary charge per s. At the maximal pumping rate, an initial slope, dU_0/dt, of 1.7 mV/s was observed. This corresponds, on the average, to 3 elementary charges per s moved across the membrane of a vesicle. Due to the inherent heterogeneity of the vesicles, a reliable estimate of the pump stoichiometry as number of charges translocated per ATP hydrolyzed cannot be derived. The comparison of the number of charges transported per second by the pump at zero and at 100 mV yields a ratio of 3, thus indicating a voltage dependence of the pump.

The finding of net transport of positive charges into the vesicles could in principle be due to various actions of the pump: (1) cations are transported into the vesicles, (2) cations are moved both into and out of the vesicles but at different stoichiometries (as in the case of Na,K-ATPase [17]), (3) anions are transported out of the vesicles, or (4) modes 1 and 3 occur simultaneously.

Although the high sensitivity of valinomycin for potassium is sometimes used to establish the ion specificity of transport processes, this was not possible in our experiments: the ATPase of *E. hirae* pumps at relatively low rates and valinomycin exhibits sufficient conductance for protons or sodium so as to shortcircuit such currents too. The monovalent cations Na^+, K^+ and choline$^+$ could be used interchangeably in reconstitutions and measurements without a significant effect on the electrogenic pumping. These observations strongly argue against the obligatory participation of either sodium or potassium (and obviously also choline and Tris) in the transport process.

We had earlier reported the *E. hirae* ATPase to be a potassium pump, based on the direct measurements of potassium extrusion from vesicles [9]. To reconcile the previous data with the presents results, two explanations appear plausible: (1) the potassium efflux we had observed was due to passive potassium (leak) currents, driven by the pump-generated membrane potential, or (2) potassium is actively moved by the pump in counterflux to protons (mode 2), but only at high potassium concentrations and without changing pumping rates. Neither mode of potassium movement at high potassium concentrations can be excluded at present.

To evaluate the influence of anions, vesicles were prepared in the presence of sulfate or chloride, and Tris instead of Hepes. The replacement of sulfate by chloride only led to an increased leak conductance; the pumping activity was not influenced. Also the absence of Hepes was without effect. Gradients of anions, e.g. sulfate inside and chloride outside, did not change the electrogenic activity of the ATPase. Similarly, electrogenic pumping was observed in buffers that contained only Tris-Hepes and Mg^{2+}-ATP. We take these findings to indicate that the ATPase does not transport anions. Therefore modes 3 and 4 should be excluded.

Besides Mg^{2+} all other di- or trivalent cations can be present only in the μmolar range or lower when EDTA is added. But neither the addition of those trace element ions nor their chelation by EDTA influenced the observed electrogenic effects. Especially the longlasting pumping at low pH cannot be explained by transport of these ions. Since Mg^{2+} has always to be present as a necessary cofactor of ATP, only direct tracerflux experiments will rigorously rule out direct participation of this ion in transport. However, the direction of charge movements by the ATPase strongly argues against Mg^{2+} as the transported species: Mg^{2+} would be pumped out of the cell.

The only ion with a strong effect on the transport activity in our experiments was the proton (see Fig. 5). The observed pH effects were independent of the type of other cations present. Since the pH was varied by titrating the extravesicular pH, the role of the intravesicular (extracellular) pH remains unclear. The buffer capacity of the vesicle interior is high, since it is not only governed by aequeous buffer substances, but also by the lipids, which may contribute up to 100 mM of buffering capacity, depending on the membrane surface/volume ratio of the vesicles [18]. Control experiments with intravesicularly applied pH indicators like BCDCF or pyranine showed no detectable changes in pH (unpublished results). The slowly increasing proton diffusion potential at an external pH of 5.8 shown in Fig. 6 indicates the existence of a pH gradient across the membrane. This passive effect is even smaller when an intravesicular positive potential is generated by pumping activity. Thus, the intravesicular pH in these experiments seems to remain unchanged. All these findings can be accommodated by assuming that the ATPase pumps protons into the vesicles and that the pumping activity is regulated by the extravesicular (cytoplasmic)

pH. In vivo, the pump would thus translocate protons from the cytoplasm to the outside and be regulated by the cytoplasmic pH.

At physiological pH, the reconstituted ATPase showed only transient activity before assuming an 'idle' state. We attempted to estimate the average number of turnovers performed by a single pump at pH 7.5 before it comes to a stop. The number of charges transported across the membranes in a complete pumping transient was determined from the trace in Fig. 1 by integrating the charges to load the membrane capacitor to the corresponding voltage, and the current necessary to overcome the leakage. Assuming an average vesicle radius of 50 nm, 360 elementary charges, n_t, were obtained. The pump density χ in the bilayer may be estimated by the relation [19]

$$\chi = L \cdot f \cdot \beta \cdot \rho_L \cdot d / M \qquad (2)$$

where L is Avogadro's number, $f \approx 0.5$ the fraction of inside-out oriented pumps, $\beta \approx 0.04$ the protein/lipid ratio (wt/wt), $\rho_L \approx 1$ g/cm^3 the density of the lipid, $d \approx 5$ nm the membrane thickness and $M \approx 78.000$ g/mol the molar mass of the protein. χ was calculated to be $750 \cdot \mu m^{-2}$. The number of pumps, n_p, per vesicle with $r = 50$ nm will then be 18. An estimated number of 20 turnovers ($n_t/n_p = 20$) would thus be necessary to generate the fluorescence transient depicted in Fig. 1. Mechanistic implications of this behaviour will be discussed below.

The ATPase activity was crucially dependent on the lipid composition of the vesicles. While pure dioleoylphosphatidylcholine did not yield transport-active proteoliposomes, the use of bacterial PE or soy bean PC resulted in transport-competent preparations. This points to the need of the ATPase for a special lipid environment. For the Na,K-ATPase it is known that the membrane thickness and the content of double bonds in the fatty acid chains can strongly influence the pumping activity [20]. Similarly, the lipid headgroups and the electrostatic properties of membranes can influence protein function [21]. The use of bacterial PE or soybean PC resulted in different responses of the *E. hirae* ATPase to changes in pH (see Fig. 6), indicating that lipids could play an important role in the regulation of this pump. But electrostatic effects also cannot be discounted. Asolectin contains lipids with negatively charged headgroups, such as phosphatidyl serine [22], which are evidenced by the significantly smaller apparent partition coefficient, $\gamma(0)$, for the negatively charged oxonol VI with soy bean PC vesicles compared to bacterial PE vesicles (unpublished results). Negative charges at the membrane interface generate, due to a Gouy-Chapman effect, a locally enhanced proton concentration [21] and, as a consequence, can shift any pH effects to higher bulk pH values, as seen in Fig. 7.

We have previously shown that the *E. hirae* ATPase forms an acyl phosphate intermediate as part of the reaction cycle [2] and thus belongs to the class of P-ATPases [23]; the P-type ATPases have previously been called E_1E_2-ATPases, based on two intermediate states, E_1 and E_2, these enzymes can form in the reaction cycle [24]. Fig. 8 outlines a minimal model for the reaction cycle of the *E. hirae* ATPase: the high-affinity form of the enzyme, E_1, binds a cytoplasmic proton, H^+_{cyt}, to form HE_1; ATP is then used to generate the high-energy intermediate $(H)E_1 \sim P$, where the bracket indicates that the proton may be 'occluded' (i.e., not readily exchangeable with bulk protons (e.g., Ref. 25)). Following transition to $P\text{-}E_2H$ with relaxation of the high energy intermediate $\sim$ P to -P and conversion of the enzyme to the low-affinity form E_2, the proton can be expelled to the exterior. Finally, inorganic phosphate, P_i, is released and E_2 returns to the initial form E_1.

A simple mechanism for the down-regulation of the ATPase at high cytoplasmic pH would be deprotonation of a functional group. Since 20 turnovers can take place before the activity is inhibited, this deprotonation would have to occur either in a shortlived intermediate or from a slowly exchanging site such as the "occluded" proton binding site in $(H)E_1 \sim P$. This would result in the resting state $(\ )E_1 \sim P$, which could be reactivated by acidic pH. Prolonged storage of the ATPase in the absence of ATP (as during purification) could, even at high pH, conceivably lead to a slow depopulation of the state $(\ )E_1 \sim P$ by direct pH-independent conversion of inactive $(\ )E_1 \sim P$ to the active E_1-form (Fig. 8, dashed arrow). Accumulated E_1 would then be responsible for the observed transient actitvity at neutral pH values. Since the resting form of the enzyme, $(\ )E_1 \sim P$, is not an obligatory intermediate of the reaction cycle, an active enzyme molecule could go through several reaction cycles until it is bypassed into the $(\ )E_1 \sim P$-trap. Clearly, this model rests to some extent on conjectures,

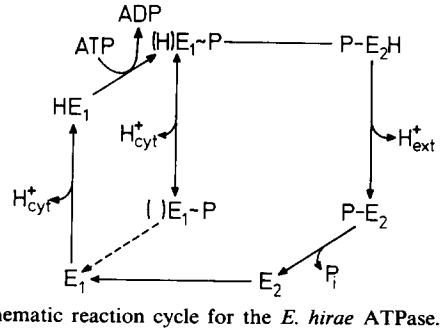

Fig. 8. Schematic reaction cycle for the *E. hirae* ATPase. E_1 and E_2 are conformations of the enzyme with the proton binding site(s) exposed to the cytoplasm and the extracellular medium, respectively. In the 'occluded' states $(H)E_1 \sim P$ and $(\ )E_1 \sim P$ the proton cannot directly exchange between binding site and aqueous phase.

yet, it encompasses testable predictions that will form the basis for future experimentation.

E. hirae belongs to the lactic acid bacteria and can generate ATP only by arginine metabolism or glycolysis. The latter process generates lactic acid and leads to the acidification of the bacterial environment. Obviously, survival and growth of *E. hirae* critically depend on an efficient system to regulate the cytoplasmic pH. The F_1F_0-ATPase as well as a putative K,H-ATPase have been implicated in pH homeostasis [26–30]. The ATPase we describe here could well serve in pH regulation. In growing cells with a cytoplasmic pH near neutrality, the pump would remain mostly in an idle state, while at acidic cytoplasmic pH, the pump would be activated and would increase the cytoplasmic pH and polarize the membrane. Future experiments aimed at the disruption of the gene of the ATPase by molecular biology tools should yield further clues on the in vivo function of this enzyme.

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