

Reconstitution of a Plasma-Membrane H^+ -ATPase Into Bilayer Lipid Membrane

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Abstract. The plasma membrane H^+ -ATPase of *Neurospora* has been reconstituted into planar lipid bilayer membranes by means of the vesicle-fusion technique described by Finkelstein and his collaborators (Zimmerberg et al., 1980; Cohen et al., 1980, 1984; Akabas et al., 1984). Enzyme was first transferred from isolated plasma membrane fragments into asolectin vesicles by a detergent-dialysis procedure (Perlin et al., 1984). After H^+ -pumping activity had been checked by quenching of acridine orange fluorescence, the vesicles were fused into preformed bilayers. Critical features of the fusion process include (i) attachment of the vesicles to the bilayer in the presence of divalent cations (Mg^{++}), and (ii) rapid osmotic swelling, which was enhanced by prior sonication or freeze-thawing of the vesicles, and/or by inclusions of physiologic channels. Enough proton pumps could be thus incorporated into bilayers to achieve ATP-driven, vanadate-sensitive currents of 0.04–0.4 pA. Aqueous solutions of low ionic strength were used to suppress conductance fluctuations due to the channels, and when that precaution was taken, we could demonstrate the proton pump the work against membrane potentials of at least 50 mV.

Key words: *Neurospora* — H^+ -ATPase — Reconstitution — Lipid bilayer membranes — Proton pumping

Introduction

Within the past few years the detailed molecular mechanisms of a variety of enzymes have begun to be accessible through techniques of molecular biology. Of especial interest are those enzymes whose function is not to carry out chemical conversions, but rather to transfer individual ions and molecules through space,

from one cell compartment to another or between the cell interior and exterior, thus carrying out osmotic work. In the broadest sense, these "Transport-ases" or "Translocases" are of three classes, depending on the form of energy which is coupled to the osmotic movement. Most general and wide-spread are the ion-coupled co- and counter-transport systems, which drain the chemical concentration gradient (or electrochemical gradient) of one ionic species (usually Na^+ or H^+) in order to build gradients for other species (e.g. sugars, aminoacids, K^+ , phosphate, etc.). The prototype of this class is the lactose permease in the plasma membrane of *Escherichia coli*. The second class comprises redox pumps found in the membranes of energy-conserving organelles and certain bacterial envelope membranes, which couple the movement of protons (and in some cases sodium ions) to electron flow; the best understood example of this class is probably the reaction center protein from photosynthetic bacteria. And finally, the third class comprises a variety of chemically distinct enzymes which couple ion movement to the energy of phosphate anhydride bonds (usually via ATP hydrolysis). These are located in virtually every kind of biological membrane, but are divided chemically into three distinct subclasses, typified by a) the $\text{F}_0\text{-F}_1$ ATPase of the mitochondrial inner membrane, b) the vacuolar ATPase of plant cells, and c) the Na^+ , K^+ -ATPase in the plasma membranes of most animal cells.

With the advent of molecular biological techniques for detailed structural analysis of "Transport-ases", a clear need has also developed to find conditions for studying the physical and physiological function of these enzymes in isolation. At present the most attractive condition is that of incorporation into artificial bilayer lipid membranes, which has been widely used to study ion-channel proteins. In order to develop bilayer incorporation techniques for pump proteins, we have focused on one system which appears simplest among the class of ATP-coupled transporters: the plasma-membrane H^+ -ATPase from the fungus *Neurospora*. This enzyme, whose complete amino-acid sequence has been determined (from the nucleotide sequence of its cloned gene (Hager et al. 1986; Addison 1986)), contains 920 amino acids with a total molecular weight of 99.886 and carries out its major transport function as a single unassociated protein monomer (Goormaghtigh et al. 1986). In terms of both structure and chemical reactivity, this enzyme is closely allied with the Na^+ , K^+ -ATPase of animal cell membranes, but it functions to pump (outward across the cell membranes) only a single H^+ ion for each molecule ATP split. Electrophysiological experiments have shown that *in vivo* this pump can generate currents of approx. $20 \mu\text{A}/\text{cm}^2$ (short-circuit conditions; Gradmann et al. 1978) or can support membrane potentials as large as -350 mV (open circuit conditions; Blatt et al. 1987). Complementary experiments on plasma membrane vesicles (Scarborough 1976) and on enzymes reconstituted into liposomes (Perlin et al. 1984) have shown the enzyme to be electrogenic under those conditions as well.

As compared with channel proteins, pump proteins are difficult to reconstitute

satisfactorily into bilayer membranes, because of their low turnover numbers. The minimal current which can be measured reliably in biological membrane systems are approx. 0.1 pA, which means 6×10^5 charges/s. The estimated turnover number for the *Neurospora* H^+ pump *in vivo* is near 200 ions/s and per molecule of enzyme, meaning that 3×10^3 molecules need to function for minimally detectable reconstitution. (By comparison, channel proteins are clearly measurable at densities of one molecule/bilayer, and bilayer-vesicle fusion experiments normally yield only a few dozen events/bilayer.) Adequate reconstitution of pumps requires, therefore, extreme care in preserving enzymatic activity throughout the steps of purification and incorporation, substantial enhancement of fusion frequencies, and caution to make the measuring amplifiers as quiet and sensitive as possible.

Abbreviations: AO: acridine orange; ATP: adenosine-5'-triphosphate; BTP: 1,3-bis[tris(hydroxymethyl)-methylaminol-propane; CCCP: carbonyl cyanide m-chlorophenyl-hydrazone; DOC: 7-deoxycholic acid, sodium salt; EDTA: ethylenediamine tetra acetic acid; HEPES: N-2-[hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid]

Materials and Methods

Preparation of vesicles

Plasma membrane fragments were isolated from *Neurospora* by the technique of Bowman et al. (1981): partial digestion of the cell walls with snail-gut enzyme, disruption of the cells by homogenization and sonication, fractionation of the membranes by differential centrifugation, resuspension in 1 mmol/l EGTA-Tris at pH 7.2, and storage at -70°C . Reformed vesicles were prepared according to the method of Perlin et al. (1984), by first diluting aliquots of the above suspension (containing ca. 3 mg protein) into HEPES-buffered KCl/asolectin: 2 ml final volume, with 10 mmol/l HEPES-KOH at pH 7.2, 1 mmol/l Na_2EDTA , 150 mmol/l KCl, and 10 mg of acetone-washed asolectin. Purified DOC was added, to a final concentration of 0.6%, and the suspension was clarified by vigorous homogenization. The resultant solution was rapidly applied to a Bio-Gel P-10 column and eluted with HEPES buffer (similar to above, but with 0.1 mmol/l Na_2EDTA and 500 mmol/l KCl) at 1 ml/min. The cloudy fractions were pooled, diluted 40-fold in further HEPES/KCl buffer, and centrifuged at $100,000 \times g$ for 1 hour. The resultant vesicles (pellet) were resuspended in the same buffer and assayed for their ability to generate a pH gradient by means of AO quenching.

Formation of bilayers

Conventional bilayer lipid membranes were formed over a 0.4 mm hole in a teflon septum (0.1 mm thick) which had been pretreated with bilayer-forming solution prior to immersion in the recording medium. The bilayer-forming solution consisted of 45 mg/ml of soybean lecithin (SIGMA, type IV-S) and 5 mg/ml soybean lysolecithin (SIGMA) dissolved in spectroscopic grade decane. One microliter of this solution was pipetted onto the teflon septum and allowed to fill the hole. A black film usually formed within 2–5 min, yielding

a trans-membrane resistance $> 4 \times 10^{10}$ Ohms and a capacitance of ca. $1\text{--}3 \times 10^{-9}$ F. Standard recording medium contained 10 mmol/l HEPES-KOH at pH 7.2 and 10 mmol/l or 100 mmol/l KCl.

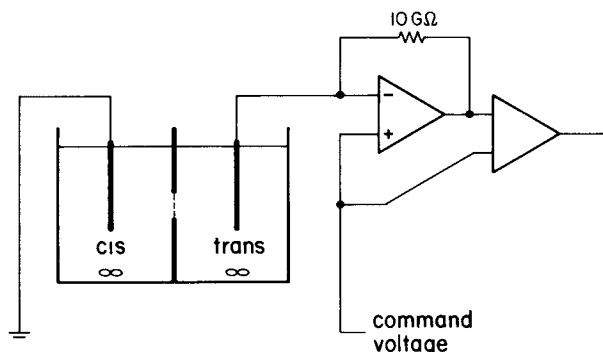


Figure 1. Block diagram of the circuit used to attach the patch-clamp amplifier to the bilayer chamber. The cis compartment of the chamber was grounded, and the trans compartment connected to the head-stage of the amplifier. Membrane potential determined as trans minus cis, with positive clamp current flowing from the trans to the cis compartment. ATP-driven cis-trans current through the pump is thus negative, opposite to the usual *in vivo* electrophysiological convention.

Electrical circuits

A Yale "Mark-V" patch clamp amplifier (design of Dr. D.P. Corey) equipped with a 10 GOhm feed-back resistor was used to measure membrane current. The cis compartment of the recording chamber, to which all additions were made, was grounded; the trans compartment was connected to the high-resistance input of the headstage amplifier, as diagrammed in Fig. 1. Reported voltages therefore represent the potential of the trans compartment relative to that of the cis compartment, and positive clamp current is defined as flowing from the trans to the cis side. Amplifier output was filtered by an 8 pole, low-pass Bessel filter set at 30 Hz for routine work and 1 or 2 Hz for measuring the pump current.

Fusion and testing

Reformed plasma-membrane vesicles were fused to bilayers generally in the manner described by Finkelstein and co-workers (Zimmerberg et al. 1980; Cohen et al. 1980). MgCl_2 (4 mmol/l) was first added to the cis chamber (total volume 3.5 ml) to supply the divalent ions needed for vesicle attachment and the Mg^{2+} needed for ATPase activation. A dense suspension of hyperosmotic vesicles (12 μl volume, containing 500 mmol/l KCl as described above) was lightly sonicated and then injected from a micropipette placed 0.5 mm in front of the lipid membrane. A small DC potential (2–10 mV, usually trans side negative) was imposed on the membrane, as noted in the figure legends below. Fusion

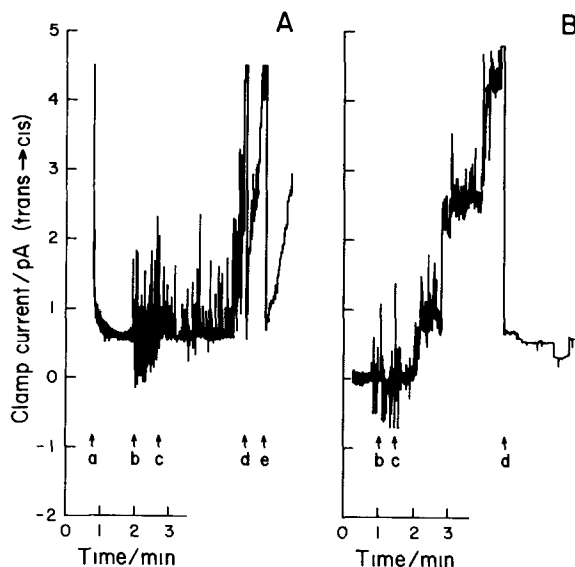


Figure 2. Demonstration of electrical noise believed to be associated with fusion of reformed vesicles into the bilayer membrane. *A* Vesicles prepared by sonicating *Neurospora* membrane vesicles with asolectin in buffer containing 0.5 mol/l sucrose. *B* Standard reformed vesicles made in 0.5 mol/l KCl and sonicated before use to increase leakiness. The membrane potential was clamped to +5 mV at arrow *a* (off scale in part *B*), and 100 μ l of vesicle suspension was injected (cis), with brief stirring, at arrow *b*. Individual fusion events appear as the small (< 2 pA) "spikes", and massed fusion as the step rises of baseline. Current-recording sensitivity was decreased 10-fold at arrow *d* and 10-fold again at arrow *e*. The recording buffers contained 10 mmol/l HEPES-KOH at pH 7.2 and 4 mmol/l MgCl_2 , plus 100 mmol/l KCl. The output filter was set at 30 Hz throughout, the large electrical spikes (> 2 pA) and dense-appearing noise between *b* and *c* artifacts caused by opening the Faraday cage for addition of solutions, etc.

events were indicated by rapid, large current fluctuations (up to ± 50 pA) spanning a period of 0.5–10 minutes after the vesicle addition (see Fig. 2). These events could be abolished by omission of the divalent cations or by use of a quasi-isosmotic vesicle suspensions (i.e., either vesicles prepared in 10–50 mmol/l KCl, or recording solutions with 500 mmol/l KCl). They could be greatly reduced by omitting the vesicle sonication step, or by using pure asolectin vesicles prepared directly by sonication, but lacking protein (channels). They could also be enhanced by using channel-contaminated asolectin (SIGMA type II-S) for vesicle preparation, but this maneuver was tested only as a demonstration of fusion enhancement, not for actual use during the biological experiments.

After the noise associated with vesicle fusion had ceased, stirring of both cis and trans solutions was begun, and the membrane potential was clamped at 0 mV (short circuit). A buffered solution of Na_2ATP (stock containing 10 mmol/l HEPES-KOH at pH 7.2, 100 mmol/l Na_2ATP , 100 mmol/l MgCl_2) was injected to give a final concentration of ca. 2

mmol/l, and the resultant short-circuit current was recorded. In a few experiments ATP solution (4 mmol/l) was injected close to the membrane without stirring, in the same manner as the vesicle suspension was applied. The pump inhibitor orthovanadate (100 μ mol/l) was applied both by addition to the bulk solute and by injection directly at the membrane. Possible binding artifacts associated with ATP/bilayer experiments (see e.g. Hyman 1977) were checked by adding ATP to fusion bilayers formed with ATPase-free liposomes. Such tests generated no significant short-circuit currents.

Results

Demonstration of proton pumping in reformed vesicles

As has already been shown (Perlin et al. 1984), reformed vesicles from *Neurospora* plasma membrane are studded with particles – made visible by freeze-fracture electron microscopy – of appropriate size and density to be the plasma-membrane ATPase. They hydrolyze ATP and accumulate protons at the rate of one H^+ ion pumped per ATP molecule split (Perlin et al. 1986). Vesicles maintained in the presence of other permeant ions (i.e. Cl^-) build steady-state pH differences of 2 units (interior acid), as assayed by quenching of the fluorescent pH-indicator acridine orange (AO; see Fig. 3A), and those maintained in the absence of other

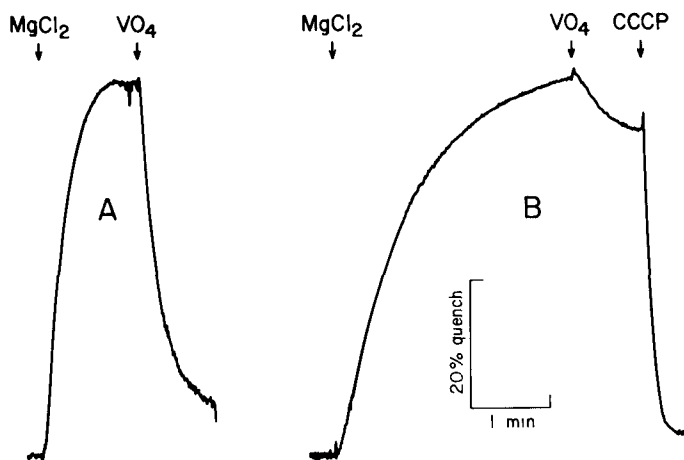


Figure 3. ATP-driven proton uptake by isolated and reformed plasma membrane vesicles from *Neurospora*, assayed by quenching of AO fluorescence, in (A) HEPES buffer + 10 mmol/l KCl, or (B) HEPES buffer + 500 mmol/l KCl. Vesicles were pre-equilibrated with 2 μ mol/l acridine orange; 1.5 mmol/l ATP (final concentration, neutralized with BTP) was added 30–60 s before these tracks commence. ATP hydrolysis was initiated by injecting 1.5 mmol/l $MgCl_2$, and hydrolysis was stopped by addition of 100 μ mol/l orthovanadate. Because the quench was slow to reverse in 500 mmol/l KCl, 20 μ mol/l CCCP was injected to restore the control fluorescence level. 30 mg membrane protein in the vesicles of part A, and 35 mg of protein in part B.

permeant ions (i.e., acetate as the major anion) rapidly develop equivalent membrane potentials (assayed by oxonol-V fluorescence; data not shown).

Fluorescence quenching of AO was checked routinely on all vesicles preparations to be used for bilayer fusion experiments. The rate and extent of quenching varied with the exact preparative procedure, and also with the ionic composition of the test medium. In particular (see Fig. 3B) the initial rate of quenching was 50–65% slower in HEPES buffer containing 500 mmol/l KCl than it was in buffer with 100 mmol/l KCl, but total quenching at steady-state was normally about 15% greater for the higher salt concentration. A simple interpretation of the fluorescence steady-state is that the H^+ difference across vesicle membranes is limited by outward leak of protons (Perlin et al. 1986); and the overall data indicate that elevated KCl can separately reduce both the maximal rate of pumping and the leakiness of the liposomal membrane. (We have so far no explanation of why the leak appears diminished at higher salt (Cl^-) concentrations.)

Occurrence of channels in fused bilayers

In a long series of experiments with variously prepared vesicles, a general correlation was noted between the amount of fusion noise, the ability of post-fusion bilayers to produce a current in response to ATP, and the simultaneous presence of discrete channels in the bilayer. This led to the notion that aqueous permeability of the vesicles might be a controlling factor for fusion events (see also Woodbury and Hall 1987), and also revealed a treacherous experimental problem: to obtain maximal fusion without introducing enough channel proteins to overcome the low-level pump currents. Fig. 4A shows two current traces from a single preparation in which two distinct types of channels were visible shortly after vesicle fusion had occurred. The upper trace, recorded with the membrane potential clamped at -3 mV, shows a large channel (ca. 400 pS, in the HEPES buffer with 10 mmol/l KCl) which was predominantly closed; the lower trace, recorded at $+50$ mV, reveals both the larger channel (now ca. 540 pS), which was mostly open, and a smaller one (here 80 pS), predominantly closed.

The small channels were not seen by themselves in bilayers fused with reformed *Neurospora* membrane vesicles; and neither type of channel was seen at all in a variety of control preparations: simple bilayer made with Sigma type IV-S lecithin plus lysolecithin, bilayers fused to pure asolectin liposomes, or even bilayers treated with 160–260 $\mu\text{g/ml}$ DOC (a very high estimate of residual detergent in *Neurospora* plasma membrane vesicles). The latter type of control, for which a record is illustrated in Fig. 4B (lower trace), did reveal very small channels, 4–6 pS at $+50$ mV, but these were too small to be of concern individually in the present experiments. The obvious conclusion that the “100 pS” channels do in fact originate from the *Neurospora* plasma membrane was further supported by isolation of similar channels in patch bilayers made by equilibrating reformed vesicles at a

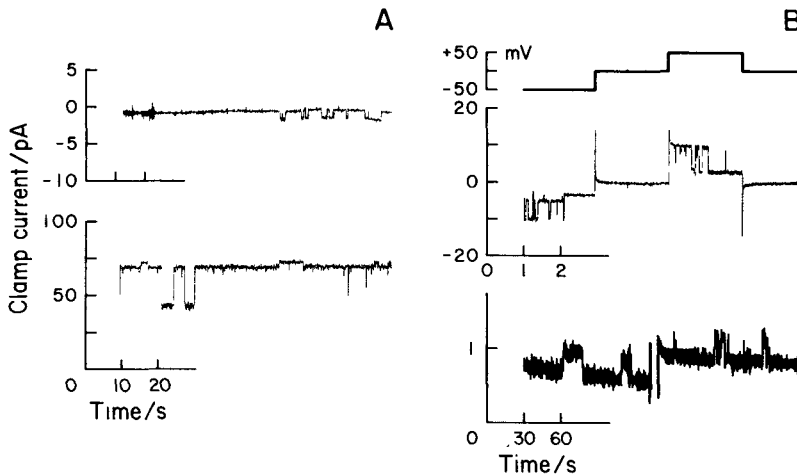


Figure 4. Channel behavior observed in bilayers after fusion with *Neurospora* plasma-membrane vesicles (*A*) or after control treatments (*B*). *A, upper.* Membrane potential clamped at -3 mV; only a large channel (ca. 450 pS) was visible, and was predominantly closed. Denser noise at the record left was residual fusion. *A, lower.* Same preparation, but voltage-clamped at $+50$ mV. Large channel was predominately open, and a small channel (ca. 80 pS) opened occasionally. *B, upper.* Patch record on a double-dip bilayer containing a single 110 pS channels from *Neurospora* plasma membrane. *B, lower.* Tracing of chart record from a simple bilayer, with 160 $\mu\text{g/ml}$ of DOC on the cis side. DOC concentration was raised to 260 $\mu\text{g/ml}$ at the middle of the record. Individual channel conductances are 4 – 6 pS. Clamped potential, $+50$ mV. Buffer composition 10 mmol/l HEPES-KOH (pH 7.2), 4 mmol/l MgCl_2 , 100 mmol/l KCl, for all four traces.

solution-air interface and then double-dipping a 2 μm patch electrode. The record from one such patch experiment is shown in Fig. 4*B* (upper trace), with a single 110 pS channel predominantly closed at -50 mV (pipette inside negative) and initially open (ca. 135 pS) at $+50$ mV. Fig. 5 displays amplitude histograms for both types of *Neurospora* channel, in this case accumulated at a clamped potential of -50 mV. Channel conductances were only slightly sensitive to membrane potential.

Preliminary experiments indicate that 400 to 500 pS channels are anion selective, so they may coincide with channels responsible for action potentials in the *Neurospora* membrane (Slayman et al. 1976; M.R. Blatt, unpublished results), while the 100 pS channels are cation selective and probably contribute to the background conductance of *Neurospora* membranes (Slayman 1965.).

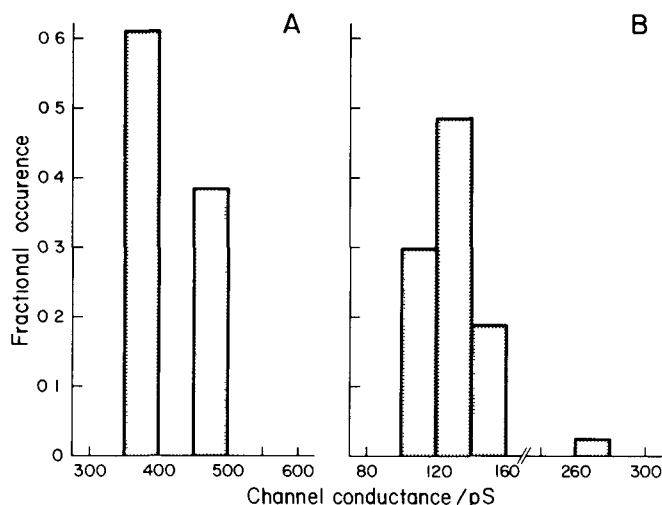


Figure 5. Typical amplitude histograms for the large (*A*) and small (*B*) channels described in Fig. 4A. Data from a single experiment accumulated over about 20 minutes, with membrane potential clamped at -50 mV. The events at 260–280 pS (*B*) were assigned to the small channel since they represented openings, not closings, at -50 mV (see text).

Recording of pump currents

The simplest way to augment fusion of reformed plasma membrane vesicles to bilayer membranes proved to be brief sonication of the vesicle suspension before addition to the bilayer chamber. (Freeze-thawing of the suspension also yielded large fusion noise, but tented to inactivate the enzyme.) In such preparations, ATPase activity remained at 90–95% of the control values, but the initial AO quenching rate was reduced to 50% or less of the control rate, which may be taken as at least a crude measure of the vesicle's increased leakiness.

After fusion with such vesicles, bilayers always displayed channel activity, predominantly of the small variety described in Fig. 4 above. Recording of pump currents required a stable baseline and therefore was possible only when the number of open channels was constant, or nearly so. Fortunately, substitution of the normal buffer solution with 10 mmol/l HEPES containing only 10 mmol/l KCl (plus 4 mmol/l MgCl_2) served both to reduce individual channel conductance and to reduce channel switching frequency.

Fig. 6 shows pump activation in several records from bilayers in which channel behaviour had been nearly suppressed, during short-circuit clamping. In each experiment, ca. 2 mmol/l ATP (added to the cis compartment at arrow *a*) produced a negative current, at steady levels of 0.08, 0.03, and 0.22 pA, respectively

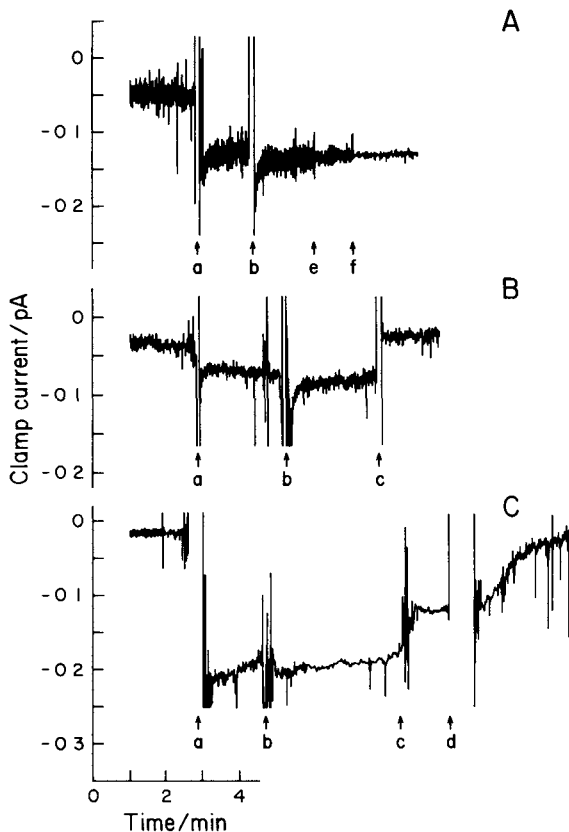
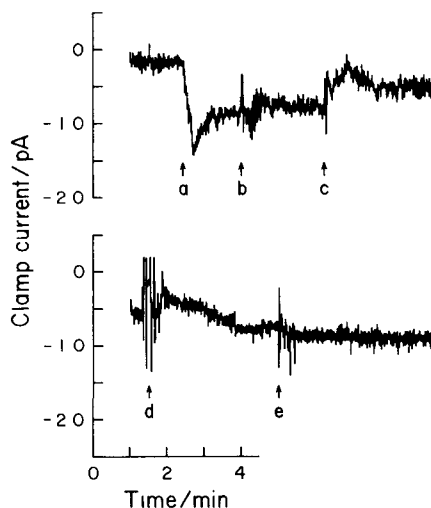


Figure 6. Several records of pump current in bilayer membrane after fusion with reformed vesicles from *Neurospora* plasma membrane. Current initiated by adding ATP to the cis chamber (with stirring) at arrows *a*, and slightly increased by doubled ATP at arrows *b* (traces *A* and *B*, only). 100 $\mu\text{mol/l}$ orthovanadate added at arrows *c*. Voltage clamped at short circuit. Recording solution contained 10 mmol/l HEPES-KOH (pH 7.2), 4 mmol/l MgCl_2 , and 10 mmol/l KCl. *A*: 1.7 mol/l ATP (final concentration) added cis at arrow *a*. *B* and *C*: 2.0 mmol/l ATP at arrow *a*. Large electrical spikes are artifacts associated with cage opening, solution addition, and stirring. Output filter settings: *A*, switched from 30 Hz to 2 Hz at arrow *e*, and to 1 Hz at *f*; *B*, 2 Hz; *C*, 1 Hz.

in traces *A*, *B*, and *C*. Doubling of the ATP concentration (arrow *b* in traces *A* and *B*) increased the current 10–15%, consistent with an apparent K_m of 1 mmol/l for the *Neurospora* proton pump (Bowman and Slayman 1977). These currents were always blocked by the specific pump inhibitor, orthovanadate (100 $\mu\text{mol/l}$), although the time-course of that blockade varied with chamber-mixing conditions. Vanadate was injected at the *c* arrows, but close to the membrane for trace *B* and farther for *C*. It could be shown that in either membrane preparations vanadate alone did not have any effect in the sense of mimicking a membrane current.

A somewhat more elaborate demonstration of ATP-dependence of the pump current is provided in Fig. 7. When 25 μl of 4 mmol/l ATP solution was injected toward the bilayer, clamping current first shifted to 0.76 pA (again the undershoot was an artifact) and then slowly diminished, presumably as ATP dispersed into the chamber volume. That process was accelerated by squirting 25 μl of chamber buffer at the membrane (arrow *c*; again note the overshoot artifact), and was reversed by

Figure 7. Variation of pump current with ATP supply. Continuous record from a single bilayer preparation, to which ATP was added by injection from a micropipette directly in front of the membrane (see Methods). Arrow *a*: 25 μ l of 4 mmol/l ATP added; *b*: pipette removed, with slight stirring; *c*: ATP-free buffer squirted at membrane; *d*: addition of ATP (1.7 mmol/l, final concentration) remote from the membrane, with stirring; *e*: further stirring, which restored pump current to the initial level. The large current undershoot on first injection (*a*) and spikes along the record are all artifacts. Output filter setting, 30 Hz. Recording solution as in Fig. 6.



a second pulse of ATP (arrow *d*).

Bilayers loaded with reformed vesicles of *Neurospora* plasma membrane tented to be fragile under either mechanical or electrical stress, and could not be reliably clamped at potentials larger than about 50 mV. Nevertheless, polarizations up to this level (+/-) could be imposed, and pump activation could be observed when a "bucking" current was applied to the recorder in order to offset the background due to average channel opening. One such recording is illustrated in Fig. 8. Baseline noise in this record represents multiple switching of the 100 pS channels (see Fig. 4).

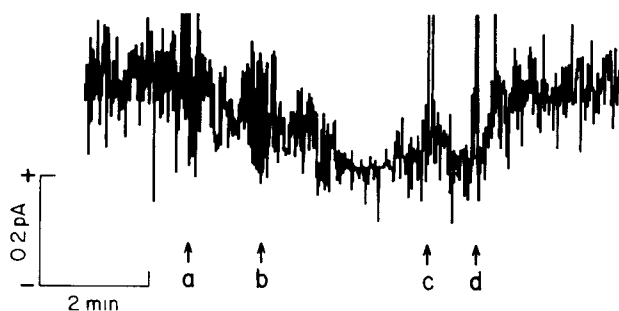


Figure 8. Demonstration of the ability of bilayer-reconstituted pumps to operate against a membrane potential. Membrane potential clamped at +50 mV, with baseline of open channels requiring ca. 50 pA offset current. Arrow *a*: 2 mmol/l ATP added *cis*, with stirring; arrow *c*: 100 μ mol/l orthovanadate added. *b* and *d*: additional stirring. Recording solution as in Fig. 6. Output filter at 1 Hz; baseline noise represents switching of several small (100 pS) channels.

After addition of ATP to the bulk solution in the cis chamber (arrow *a*) and a second stirring (arrow *b*), clamp current increased by -0.17 pA. Addition of $100\text{ }\mu\text{mol/l}$ vanadate to the solution (arrow *c*, with stirring at *d*) abolished the ATP-stimulated current. Although for technical reasons we do not have a direct comparison of ATP effects on a single membrane in the presence and absence of imposed polarization, the result in Fig. 8 makes clear at least that a 50 mV opposing voltage does not suppress the proton pump. This result is in keeping with observations on whole cells of *Neurospora*, which have found the reversal potential for the plasma membrane proton pump to be near -400 mV and pump current to be nearly insensitive to the actual membrane potential, in the range -100 to 0 mV (Gradmann et al. 1978).

Overall, the results described above have shown that protein incorporated into bilayer membranes by fusion with reformed *Neurospora* membrane vesicles can generate transmembrane currents of a magnitude expected for ca. 10^4 functioning enzyme molecules (for numerical estimates see Introduction). Furthermore, the currents are abolished by orthovanadate, a highly-specific inhibitor of "E₁-E₂"-type cation pumps, and they are insensitive to small shifts of membrane potential, as also would be expected for the *Neurospora* plasma membrane proton pump.

Discussion

We regard the data presented above as a minimal demonstration of the feasibility for functional reconstitution of ion pumps into flat bilayer lipid membranes, and the procedures used as a starting point for more effective reconstitution. ATP-stimulated and vanadate-inhibited currents produced by the *Neurospora* plasma membrane ATPase are of the same magnitude as those recently reported in bilayers containing cytochrome *c* oxidase from *E. coli* (Hamamoto et al. 1985) or the F₀-F₁ ATPase from the thermophilic bacterium PS3 (Hirata et al. 1986). However, because steady-state currents obtainable from all three enzymes are near the limit of present recording methods, it is obvious that production-level experiments on these enzymes in bilayers will require much methodological improvement. (Borisova et al. (1984) have also reported currents of 0.2 – 1.0 pA for *Streptococcus fecalis* F₀-F₁ ATPase in artificial bilayer membranes, but serious doubt about that work arises because of contradictions between the conductance and current values given.) As an operational target, we are aiming for currents 10-fold larger than have been obtained thus far, i.e. 1 – 4 pA. This range compares with 10 – 40 pA steady current seen for bacterial rhodopsin incorporated into bilayers (Bamberg et al. 1981), or similar transient currents for Na⁺, K⁺-ATPase attached onto bilayers (Fendler et al. 1985; Nagel et al. 1987).

The most likely route for further enhancing bilayer reconstitution of the *Neurospora* plasma-membrane ATPase is via purified ATPase which has been reincorporated into liposomes. As was noted above, bilayers fused to reformed plasma-

membrane vesicles become fragile, and indeed usually break spontaneously when fusion noise is substantially more intense than that illustrated in Fig. 2. It is reasonable to suppose that fragility arises from protein loading, and since the ATPase is only 5–10% of total protein in the reformed vesicles, a 10–20 fold improvement of current might be achieved with purified enzyme in liposomes. In other words, we might expect incorporation of ca. 10^5 functional pump molecules per bilayer instead 10^4 molecules/bilayer or fewer. An important fringe benefit of purification may be an almost complete abolition of the channel noise observed upon fusion of reformed plasma-membrane vesicles. This in turn might increase measuring sensitivity several-fold.

Several other factors hopefully will also give improvements, albeit smaller than expected for purified enzyme: a) More gentle purification (i.e., with different detergents and lipid protection), since only about 50% of pump molecules in the reformed vesicles are reckoned to be active (K. E. Allen and C. W. Slayman, unpublished experiments). b) Alteration of the osmoticum loaded into the vesicles (by sonication) just before fusion; in preliminary experiments vesicles loaded with 0.25–1.0 mol/l sucrose were able to fuse with bilayers (judged by the recording noise), but did not yield measurable currents. Since the hydrolytic activity of sucrose vesicles was equal to that of control vesicles, the nature of pump inhibition in the bilayers is unclear. It may be related to ionic strength effects of the usual osmoticum (500 mmol/l KCl; see Bowman and Slayman, 1977), and bears serious examination. c) Selection of lipids – in the bilayer and/or the vesicles – with critical phase behaviour as a function of temperature or ambient pH. Such experiments are suggested by burgeoning biochemical work of the past two years (see, e.g., Abstracts of US. Biophysical Society Meeting, New Orleans, March 1987) on the relation of lipid phase transitions to vesicle-vesicle fusion efficiency.

Ideally, of course, we would like to obtain bilayers containing purified pumps at densities similar to those found in normal cell membranes: for the *Neurospora* ATPase ca. $3000 \mu\text{m}^2$, so that comfortable electrical signals could be obtained from cell-sized pieces of membrane (i.e., $100\text{--}1000 \mu\text{m}^2$), which could be electrically isolated in large patch electrodes. Whether such densities can be obtained by physical reconstitution is unclear. Test-tube biosynthesis of single-protein membranes may be a more feasible possibility in the future.

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