Journal of Bioenergetics and Biomembranes, Vol. 19, No. 1, 1987

MINI-REVIEW

The Plasma Membrane ATPase of *Neurospora*: A Proton-Pumping Electroenzyme

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Received May 19, 1986; revised July 23, 1986

Abstract

Probably the best marker enzyme for plasma membranes of eukaryotic cells is a magnesium-dependent, vanadate-inhibited ATPase whose primary function is the transmembrane transport of cations. In animal cells, different species of the enzyme transport different cations: sodium ions released in unequal exchange for potassium ions, calcium ions extruded alone (perhaps), or protons secreted in equal exchange for potassium ions. But in plants and fungi only proton secretion has been clearly demonstrated. A useful model cell for studying the proton-secreting ATPase has been the ascomycete fungus *Neurospora*, in which the enzyme drives an outward current of protons that can exceed 50 $\mu A/cm^2$ and can support membrane potentials greater than 300 mV. Both thermodynamic and kinetic studies have shown that the proton-pumping ATPase of *Neurospora* normally transports only a single proton for each ATP molecule split; and kinetic modelling studies have suggested (contrary to conventional assumptions) that the fast steps in the overall reaction are transmembrane transit of the proton and its dissociation following transport, while the slow steps are the binding of protons and/or ATP. The primary structure of the *Neurospora* enzyme, recently deduced by gene sequencing, is very close to that of the yeast (*Saccharomyces*) enzyme, and the hydropathic patterns for both closely resemble those for the animal-cell plasma-membrane ATPases. All of these enzymes appear to have 6–10 membrane-spanning α-helices, plus a large cytoplasmic headgroup which bears the catalytic nucleotide-binding site. Structural data, taken together with the electrical-kinetic behavior, suggest that the catalytic headgroup functions as an energized gate for protons. From a geometric point of view, action of such a gate would transfer the membrane field across the "transported" ion, rather than vice versa.

Key Words: Membrane ATPase; proton pump; electroenzyme; charge transport; reaction models; fungi.

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Introduction: Classes of Proton ATPases

Over the past 15 years three distinct classes of membrane-bound enzymes have been described which use the energy from hydrolysis of ATP to drive a transmembrane current of protons. The most elaborate of these enzymes structurally are the F_0–F_1 ATPases located in so-called energy-coupling membranes, such as mitochondrial inner membranes, chloroplast thylakoid membranes, bacterial chromatophores, and in the envelope membranes of most bacteria. These enzymes consist of two characteristic components: (i) F_1, a water-soluble assembly of (usually) nine polypeptide subunits having an aggregate molecular weight of 360–400 kD and carrying the catalytic nucleotide-binding sites; and (ii) F_0, a strongly hydrophobic assembly of 9–15 polypeptide subunits having an aggregate molecular weight between 110 and 170 kD and serving (in an as yet undeciphered manner) as a transmembrane channel. In the energy-coupling organelles these enzymes are physiologically regulated to function only as ATP synthetases, fueled by a proton current; but in bacterial plasma membranes they function—under different conditions—either as synthetases or as proton extrusion pumps fueled by ATP hydrolysis (see reviews by McCarty and Carmeli, 1982; Senior and Wise, 1983).

Structurally much simpler than the F_0–F_1 ATPases are the physiological proton pumps located in the plasma membranes of most walled eukaryotic organisms: fungi, algae, and higher plants. These enzymes appear to consist of a single polypeptide chain of molecular weight 95–105 kD. They can be solubilized only with strong detergents (Goffeau and Slayman, 1981), but can be partially digested by exposure to proteolytic enzymes at the membrane inner surface (Addison and Scarborough, 1983). In *Neurospora* the enzyme constitutes approximately 10% of total membrane protein (Bowman *et al.*, 1981a) and is presumed to coincide with ~ 110 Å particles which heavily stud the membrane inner leaflet in freeze-fracture pictures (D. L. Stetson and C. L. Slayman, unpublished experiments; Perlin *et al.*, 1984). In most respects, these enzymes are strongly allied with the cation-pump (Na^+, K^+, Ca^{2+}) enzymes located in the plasma membranes of animal cells, but they have a conspicuous physiological property which commends them to special study: they pump rapidly under cellular steady-state conditions, driving a large proton efflux and creating membrane potentials which often exceed 250 mV (Spanswick, 1972; Blatt and Slayman, 1983).

Finally, a third class of ATPases has emerged recently from convergent investigations on a wide variety of storage organelles: plant and fungal vacuoles, acid-secretory vesicles, chromaffin granules, serotonin granules, lysosomes, endosomes, etc. (Maloney and Wilson, 1985). These enzymes comprise multiple subunits, the largest of which are 70–75 kD, with an

aggregate molecular weight approaching 40 kD. Structurally they are allied with the F_0–F_1 enzymes, but functionally they are strictly pumps, driving protons into the vesicle or vacuole interior. All three classes of ATPases are present within any one eukaryotic cell, and a summary comparison of their properties in *Neurospora* is provided in Table I.

Electrogenesis by the Plasma Membrane ATPase *in vivo*

Massive evidence has accumulated that all three classes of ATPase transport electric charges through their resident membranes. For the *Neurospora* plasma membrane that charge movement can be measured directly, because cell morphology permits easy insertion of microcapillary electrodes. As a necessary background, steady-state membrane potentials of intact cells *lacking pump activity* lie between 0 and −50 mV (cell interior negative) under a variety of conditions (Slayman, 1970; Slayman *et al.*, 1973), and can be conservatively averaged at about −25 mV. During active proton transport, the measurable membrane potential (V_M) becomes sensitive to both ionic and metabolic conditions: it has registered as small as −50 to −60 mV for cells kept in 100 mM KCl without added calcium (Slayman, 1965a), up to −270 mV for metabolically replete cells bathed in 0.1–1.0 mM KCl with 1 mM free Ca^{2+}, and as large as −350 mV for cells deprived of carbon sources and studied in the absence of certain depolarizing anions (e.g., intracellular Cl^-; see Blatt and Slayman, 1983). In the standard recording medium (25 mM K-phosphate or K-dimethylglutarate buffer at pH 5.8, plus 1 mM $CaCl_2$ and 1% glucose), the steady-state membrane potential is about −200 mV.

Membrane conductance, the complementary electrical parameter, varies between 100 μS/cm^2 and 400 μS/cm^2 for potentials near −200 mV. Pump currents, then, have customarily been estimated as the Ohm's-Law product of pump voltage and membrane conductance (Kishimoto *et al.*, 1984), but that trick is illegal either when the transport systems are electrically nonlinear or when a major fraction of membrane conductance is actually in the pump. In such cases it is necessary to map out the dependence of membrane current upon membrane potential, and—by specific manipulation of one or another transport system—to extract components due to ionic leaks and to the proton pump. Applications of this technique to *Neurospora* have been reported elsewhere (Gradmann *et al.*, 1978; Hansen *et al.*, 1981; Slayman and Sanders, 1984), and a typical result is diagrammed in Fig. 1. The middle curve (I_M) represents a smooth fit to stabilized currents flowing through the whole cell membrane when membrane potential is pulse-clamped from the resting value (−200 mV) to 24 other values in the range −300 to +10 mV.

Table I. Physical and Chemical Properties of Different H^+-ATPases in *Neurospora*

	Plasma membrane	Mitochondrial membrane	Vacuolar membrane
Subunit weights (kD)	104	59, 56, 36, 22, 21, 19, 16, 15, 12, 8	70, 62, 15
pH optimum	6.7	8.3	7.5
K_m (ATP), mM	1.8	0.3	0.2
Nucleotide specificity	ATP	ATP > GTP, ITP > UTP > CTP	ATP > GTP, ITP > UTP > CTP
Divalent cation specificity	Mg, Co > Mn	Mn > Mg > Co	Mg, Mn > Co
Inhibitors	DCCD, vanadate	DCCD, oligomycin, azide	DCCD, SCN^-, NO_3^-
References	Bowman *et al.*, 1981 Scarborough, 1977 Bowman and Slayman, 1977 Scarborough and Addison, 1984 Bowman *et al.*, 1978	Mainzer and Slayman, 1978 Bowman, 1983 Jackl and Sebald, 1975 Sebald and Hoppe, 1981	Bowman and Bowman, 1982 Bowman *et al.*, 1986

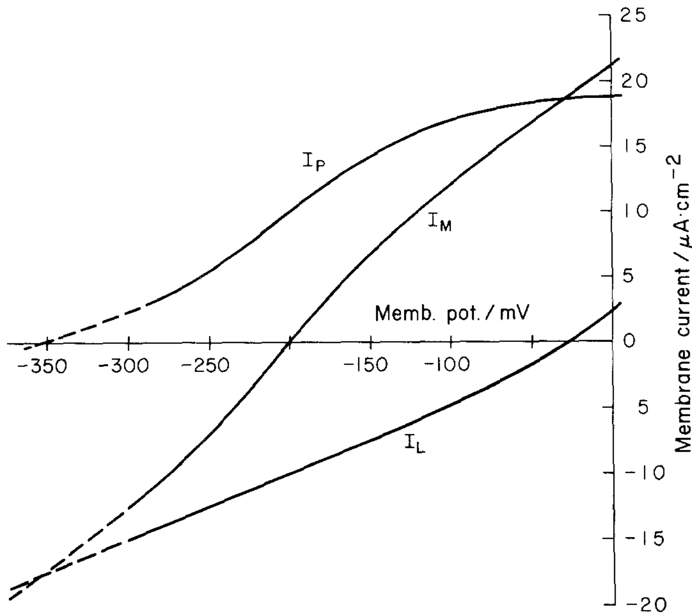

Fig. 1. Current–voltage curves for the plasma membrane of *Neurospora* and its constituent transport systems. I_M: total membrane current plotted against the clamped membrane potential. I_P: current through the proton pump/plasma-membrane ATPase. I_L: current through the ensemble of passive transport systems in the membrane. Slightly idealized curves, based on data in Slayman and Sanders (1984).

This total membrane current–voltage relationship can be represented as the sum of a major current source, designated I_P and assumed to be the proton pump, and a major sink, designated I_L and assumed to be the sum of all passive ion movements through the membrane (i.e., true leaks, ion cotransport systems, possible mediated uniports, and perhaps also some artifactual leak due to damage by the penetrating microelectrodes).

The pump curve, which is characteristically convex upward and usually slightly sigmoidal, has several diagnostic features: (a) its slope, representing the pump's internal conductance (G_P), is about half the total membrane conductance (G_M, slope of the I_M curve) at the normal V_M. [In the case diagrammed, actually $G_P = 95\,\mu S/cm^2$, $G_L = 50\,\mu S/cm^2$, and $G_M = 145\,\mu S/cm^2$.] (b) For membrane potentials positive to about -100 mV, pump current saturates, reaching approximately twice its magnitude at normal V_M. And (c) the stalling voltage (E_P, the so-called reversal potential) of the pump usually lies well negative to -300 mV.

Current–voltage plots for electroenzymes are complementary to the familiar velocity–concentration plots for ordinary enzymes, and yield several

important conclusions when analyzed via simple kinetic models. In the saturating regions, pump current is limited by *voltage-independent* reaction steps: either ion-binding reactions at the membrane surfaces or electroneutral transfer steps; this is contrary to the common assumption that membrane transit itself must be rate-limiting for transport (Turner, 1983). Because most biochemical experiments are carried out essentially under short-circuit conditions (i.e., positive to -100 mV), this effect *cannot be neglected* in proper kinetic analyses. Another major point is the meaning of the gradual rise of pump current, requiring a shift of more than 200 mV for 80% of saturation: such behavior argues strongly that only a single net charge traverses the membrane for each molecular turnover. The same inference can be drawn from the large stalling voltage, which adds to a pH difference of 1.4 units ($pH_i \simeq 7.2$, $pH_o = 5.8$) to give a total opposing potential of 400 mV or greater, for the intrinsic protonmotive force (PMF) of the pump. Since the potential energy available to the pump from ATP hydrolysis is only about 500 mV (Warncke and Slayman, 1980), one charge per ATP molecule split is the largest possible integer stoichiometry. Finally, the actual values of pump current can yield approximate turnover rates, if the density of pumps present is known. In freeze-fracture pictures of normal membranes (D. L. Stetson and C. L. Slayman, unpublished experiments; Perlin *et al.*, 1984), the presumed ATPase particles are present at a density of $2\text{–}3 \times 10^{11}/\text{cm}^2$. If all these are indeed pump molecules, turnover numbers of about 200–300 ions/site · sec would be required for currents of $10\,\mu\text{A/cm}^2$ ($= 6.2 \times 10^{13}$ ions/cm^2 · sec).

Without controlled cell-perfusion techniques, unequivocal *in vivo* identification of substrates for the plasma-membrane current generator is not possible. Nevertheless, electrophysiological experiments first pointed to protons as the pumped ions (Slayman, 1970) and ATP as the energizing substrate (Slayman *et al.*, 1973). Both were implicated by the fact that membrane potential, net H^+ extrusion, and the average intracellular ATP concentration ($[ATP]_i$) all vary in parallel in *Neurospora*, during the sharp metabolic transients which accompany respiratory blockade (e.g., by cyanide). The correspondence between protons and electrical behavior proved only qualitative at the normal extracellular pH of 5.8, where acid release is a small fraction (10–20%) of pump current; but it is quantitative at pH 8–9, where net acid secretion (200 pmol/cm^2 · sec; P. Kaminski and C. L. Slayman, unpublished experiments) can exceed the required pump flux. The parallel variation of membrane potential and $[ATP]_i$ is slightly skewed by a brief delay in the voltage response to respiratory block or release, but such behavior is expected for an enzyme with a Michaelis relationship between reaction velocity and substrate concentration. Plots of voltage versus $[ATP]_i$ give $K_{1/2}$ values of 1.5–2.3 mM (Slayman *et al.*, 1973; Slayman and Sanders, 1985).

Transport Properties of the ATPase in Vesicles

Preparation of plasma membrane vesicles from *Neurospora*—first using concanavalin A-coated membrane sheets from a protoplast mutant (Scarborough, 1976, 1980), and later using broken membrane fragments enzymatically stripped of cell wall material (Bowman *et al.*, 1981b; Perlin *et al.*, 1984)—made possible direct demonstration of the proton-pumping ATPase. The experiments depend on the fact that plasma membrane fragments vesiculate preferentially (85–90%) inside out (Perlin *et al.*, 1984), permitting controlled access of substrate to the catalytic portion of the enzyme regardless of membrane permeability. But since spontaneously formed vesicles (500–5000 Å diameter) are far below the size accessible to microelectrodes, chemical indicators must be used to estimate both membrane potential and pH changes.

In HEPES- or MES-buffer at pH 6.8–7.2, addition of millimolar Mg-ATP causes plasma membrane vesicles to concentrate lipid-soluble anions, whose distribution can be used to estimate membrane potentials. Fluorescent anions such as oxonol V have proven especially convenient for this purpose; rise of membrane potential (vesicle interior positive) is signalled by fluorescence quenching as dye is removed from the medium to the vesicle interior. Calibration of this effect with preset diffusion gradients (e.g., $[K^+]_i/[K^+]_o = 10$, or $pH_i - pH_o = 1$) and specific ionophores (e.g, valinomycin or FCCP) gives pumped membrane potentials near 120 mV. High concentrations (10–100 mM) of thiocyanate (SCN^-) or other permeant anions such as Cl^- or NO_3^- collapse the membrane potential and permit accumulation of protons. Weak base indicators, such as imidazole, quinacrine, and acridine orange, are then accumulated by pH-trapping. In such circumstances, intravesicular pH can fall about two pH units below the extravesicular pH, demonstrating that vesicle membranes sustain the same total PMF, whether primarily as voltage or primarily as ΔpH. Alternate buildup of membrane potential and ΔpH are illustrated in Fig. 2, monitored by fluorescence quenching of oxonol and acridine orange. All of these H^+-pump/ATPase experiments on plasma membrane vesicles can be carried out, with similar results, on the purified enzyme reconstituted into asolectin vesicles (Perlin *et al.*, 1984).

The large discrepancy between the maximal PMF across vesicle membranes (120 mV) and across cell membranes (as large as 350 mV) has been tacitly attributed to abnormal leakiness of the resealed membranes. Presumably because of this leakiness, attempts to demonstrate reversal of the *Neurospora* proton pump—that is, ATP synthesis driven by a large imposed PMF—have failed, although a 10–20% decrease in the rate of ATP hydrolysis does accompany the buildup of intravesicular $[H^+]$ or membrane

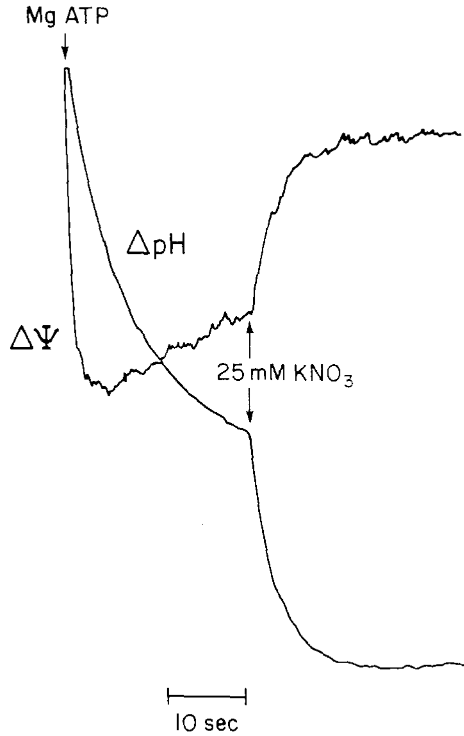

Fig. 2. Superimposed demonstrations of the creation of a pH difference and a membrane potential in everted vesicles of *Neurospora* plasma membrane. ΔpH: pH difference, monitored by quenching of acridine orange fluorescence. $\Delta\psi$: membrane potential (vesicle interior positive) monitored by quenching of oxonol V fluorescence. Vesicles suspended in 50 mM HEPES buffer titrated to pH 7.2 with KOH; fluorescent dyes added at 2.5 μM. Quenching in both cases was initiated by injection of 1.5 mM MgATP. Note that addition of the permeant anion NO_3^- reverses oxonol quenching and enhances acridine orange quenching. Taken (with permission) from Perlin *et al.*, 1984, Fig. 2.

potential (Perlin *et al.*, 1984). Just such a small change (viz., 15% decrement at − 120 mV) is predicted, in fact, by current–voltage analysis of the pump in whole cells (see Fig. 1). Leakiness of the vesicle membrane affords an interesting way to determine the H^+/ATP stoichiometry for the pump, since with steady-state loading of the vesicles, H^+ *leak efflux* equals H^+ *pumped influx*. Then sudden block of the enzyme, as by 10 μM orthovanadate, gives an initial net efflux which measures the steady-state influx of H^+ and can be compared with steady-state ATP hydrolysis. Fifteen measurements on standard vesicle preparations of *Neurospora* have given a ratio of 0.96 ± 0.18, independent of the ATP concentration (0.2–6 mM) or the absolute magnitude of fluxes (Perlin *et al.*, 1986), again confirming the arguments based on electrophysiological data (see above).

Chemical Properties of the Enzyme

Sheets, fragments, or vesicles of *Neurospora* plasma membrane can be scrubbed to less than 1% mitochondrial protein contamination (Bowman *et al.*, 1981b; Brooks *et al.*, 1983) by means of differential centrifugation. Extraction of these isolated membranes with lysolecithin (1 mg/ml) or deoxycholate (0.6 mg/ml DOC in the presence of 45% glycerol), followed by isopycnic centrifugation on glycerol density gradients, yields ATPase preparations containing a single band, of M_r = 104,000–105,000, when run on SDS-polyacrylamide gels (see Fig. 3). As with other ion-pumping ATPases (Dahl and Hokin, 1974; Warren *et al.*, 1974; Dufour and Goffeau, 1980), the *Neurospora* enzyme, too, is inactivated by severe detergent extraction and delipidation, but activity returns upon readdition of acidic phospholipids, among which phosphatidylserine, phosphatidylglycerol, and monophosphoinositides are about equally efficient (Scarborough, 1977). Under optimal

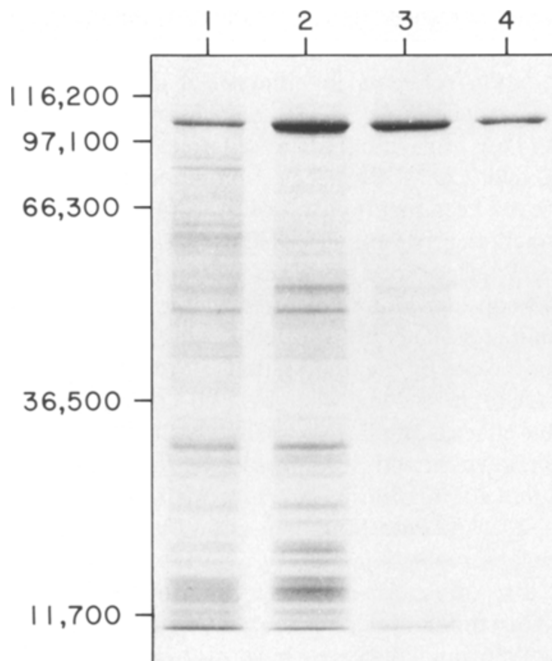

Fig. 3. SDS-polyacrylamide gel electrophoresis of different fractions from purification of the plasma-membrane ATPase of *Neurospora*. Lane 1: Plasma membrane fragments. Lane 2: membranes washed in 0.1% deoxycholate; Lane 3: ATPase solubilized and centrifuged in 0.6% deoxycholate. Lane 4: purified ATPase, the peak fraction from a glycerol gradient. Methods and data from Bowman *et al.*, 1981b; reprinted by permission from Biochemical Society Symposium 50, pp. 11–29, copyright 1985; The Biochemical Society, London.

conditions (pH 6.7 in HEPES or MES buffer, with 5 mM Mg-ATP; see Table I), the peak fraction from the glycerol gradient has a specific activity of about 100 μmol ATP hydrolyzed per min · mg protein (30°C), and an ATPase (104-kD) content of ~70% (Bowman *et al.*, 1981b). If all of the 104-kD protein were catalytically active, the hydrolytic rate would be about 250 phosphoryl residues/sec · molecule, clearly within the same range as the proton turnover number required by the electrical data on the pump *in vivo*.

That catalysis involves formation of a phosphoprotein intermediate was first shown by Dame and Scarborough (1980), who reacted γ-^{32}P-labelled ATP with plasma membrane vesicles and subsequently found ^{32}P covalently bound to the 104-kD band on acid polyacrylamide gels. And, at least under conditions of low turnover (0°C, < 1 phosphoryl residue/sec · molecule of enzyme), the rate of phosphate binding was more than sufficient to account for the observed hydrolytic rate. The phosphate binding site was identified as an aspartyl residue, by its reactivity with hydroxylamine and with tritium-labelled (Na) borohydride (Dame and Scarborough, 1981). These facts, together with molecular weight and (high-affinity) inhibition by orthovanadate ($K_i \lesssim 1\ \mu$M; Bowman and Slayman, 1979), clearly ally the H^+-ATPase with various metal cation ATPases in animal cell membranes: ($Na^+ + K^+$)-ATPase in plasma membranes (Dahl and Hokin, 1974; Jørgensen, 1982), ($H^+ + K^+$)-ATPase in gastric parietal cell apical membranes (DePont and Bonting, 1981), and (Ca^{2+})-ATPase in sarcoplasmic reticulum (Inesi, 1981).

As has already been mentioned, however, in at least one major respect —its charge stoichiometry—the H^+-ATPase clearly differs from the metal cation ATPases. Transport of a single charge (proton) for each ATP molecule split, along with consideration of the free energy involved, requires that no other ions be unidirectionally transported within the pump cycle. This prediction has been borne out by the finding that, although NH_4^+ and alkali metal cations do stimulate the enzyme when assayed in plasma membrane vesicles or micelles, their effect is small (maximally about 60%) and neither specific nor synergistic (Bowman and Slayman, 1977), thus hardly comparing with the 10- to 100-*fold* stimulation by K^+ seen with the ($Na^+ + K^+$)-ATPase and the ($H^+ + K^+$)-ATPase (Dunham and Glynn, 1961; Wallmark *et al.*, 1980). That the observable stimulation of ATP hydrolysis by alkali metal ions is unrelated to transport of those ions is further indicated by the fact that K^+ does not stimulate dephosphorylation of the *Neurospora* enzyme (Kasher *et al.*, 1986) and does not have an appropriate kinetic effect on ^{18}O-exchange in the H^+-ATPase of *Schizosaccharomyces pombe* (Amory *et al.*, 1982).

The reaction specificity of cation ATPases varies widely, both among the different (cation) types of ATPase, and among ATPases of the same type isolated from different cells and tissues (i.e., from erythrocytes versus

kidney); it also varies depending on the mode of assessment: i.e., whether enzyme phosphorylation or ATP hydrolysis is measured. From measurements of hydrolysis, the *Neurospora* enzyme is strongly specific for ATP (Bowman and Slayman, 1977), having relative hydrolytic rates for ATP : dATP : UTP : ITP (all at 5 mM) of 100 : 22 : 6 : 3. And along with other fungal and plant plasma membrane ATPases (Goffeau and Slayman, 1981), the *Neurospora* enzyme has a rather high apparent $K_{1/2}$ for ATP: 1.3 mM in vesicle or micelle preparations (Bowman *et al.*, 1981), and 1.5–2.3 mM in intact cells (Slayman *et al.*, 1973; Slayman and Sanders, 1985), compared with micromolar values for the metal cation ATPases.

Potentially the most important line of reactivity studies on the *Neurospora* plasma membrane ATPase has been examination of site-specific reagents whose action is modified by the presence of substrate. The arginine reagents phenylglyoxal and butanedione, and the –SH reagent N-ethylmaleimide (NEM), for example, bind to and inhibit the enzyme in the absence of nucleotides, but are blocked by either MgATP or MgADP (Brooker and Slayman, 1982; Kasher and Slayman, 1985; Kasher *et al.*, 1986). Detailed experiments with NEM have shown that the apparent K_D for MgATP protection (1.5 mM at 0°C) is essentially identical to the $K_{1/2}$ for ATP activation of the enzyme; and the K_D for MgADP protection (0.1 mM) is close to the K_i for inhibition of ATP hydrolysis by MgADP. Both binding sites (cysteine for NEM, and a single arginine for phenylglyoxal and butanedione) are presumed to lie within the active site cavity.

The proteolytic enzyme trypsin has also given useful information about ATPase conformations (Addison and Scarborough, 1983; Scarborough and Addison, 1984). Used alone, it inactivates the enzyme in plasma-membrane vesicles and degrades it to small fragments, with a half-time of 1–2 min (ca. 50 μg trypsin/200 μg membrane protein; 20°C). But when used in combination with nucleotides or orthovanadate, it produces two-step degradation, first (1–3 min) to a hyperactive ATPase of M_r = 95,000, and later to an inactive piece of M_r = 88,000. MgATP accelerates formation of the 95-kD species, while added vanadate retards its breakdown; MgADP accelerates formation of the 88-kD piece, and one nonhydrolyzable ATP analogue (Mg-β,γ-methylene ATP) acts like MgATP + vanadate. Addison and Scarborough (1983) have argued that the 95-kD and 88-kD pieces may be conformationally related to the high-energy ($E_1 \sim P$) and low-energy ($E_2 \cdot P$) phosphoenzyme states of the (Na^+ + K^+)-ATPase (Post *et al.*, 1969) and the H^+-ATPase of the fission yeast (Amory *et al.*, 1982), but this view has not been reconciled with the E_2P-binding action of vanadate (Smith *et al.*, 1980), nor with the fact that NEM fails to trap the *Neurospora* ATPase (R. J. Brooker and C. W. Slayman, unpublished experiments) in phosphorylated form, as $E_1 \sim P$ (Post *et al.*, 1969).

Although the stoichiometry of protons moved and ATP molecules hydrolyzed in a single transport cycle is well established (1 : 1), the number of enzyme monomers participating in a single reaction cycle is unsettled. Four lines of evidence suggest that the functioning enzyme might be a dimer of two M_r = 104,000 chains. First, both hydrolysis data (Bowman *et al.*, 1981b) and current–voltage data (Slayman *et al.*, 1973) indicate cooperativity in activation of the enzyme by ATP. Hill numbers for velocity vs. concentration plots lie between 1.5 and 2.1. Second, the inhibitor orthovanadate—which because of its chemical homology with phosphate is presumed to bind within the active site—actually *stimulates* ATP hydrolysis at low ATP concentrations. Such could happen if positive interaction of two enzyme monomers were required for catalysis. Third, it was surprising to find that DCCD blocks enzyme activity upon binding of 0.4–0.5 diimide residues per 104 kD chain (Sussman and Slayman, 1983), as if only one chain in two need be blocked to inhibit the total enzyme. And finally, radiation-inactivation studies on the hydrolytic activity of the enzyme (Bowman, 1985) confirm that a single "hit" inactivates a unit of M_r = 219,000. These results and arguments resemble ones aired a decade ago for "alternating-sites" (half-of-sites) reactivity in the (Na^+ + K^+)-ATPase (Schön *et al.*, 1974; Cantley *et al.*, 1979). Subsequent studies made clear, however, that the preferred state for (Na^+ + K^+)-ATPase is a simple heterodimer (one α chain and one β chain), in which all of the catalytic activity resides in the single α subunit (Craig and Kyte, 1980; Kyte, 1981). Functional significance for cooperative phenomena in the proton ATPase, therefore, would be novel and remains to be proven.

The most exciting recent development in the chemistry of fungal plasma-membrane ATPases has been solving the primary structure, for both the *Saccharomyces* and *Neurospora* enzymes (Serrano *et al.*, 1986; Hager and Slayman, 1985; Hager *et al.*, 1986). As had been indicated by strong immunological cross-reactivity—between polyclonal antibodies made to denatured *Neurospora* enzyme and tested against denatured yeast enzyme (K. Hager and C. W. Slayman, manuscript in preparation)—sequence coincidence between the two ATPases is very high: nearly 75% (K. Hager and C. W. Slayman, manuscript in preparation). Sequence coincidence between the *Neurospora* enzyme and the (Na^+ + K^+)-ATPase (Shull *et al.*, 1985; Kawakami *et al.*, 1985) or Ca^{2+}-ATPase (MacLennan *et al.*, 1985) is significant, too, but much lower: probably no more than 25%, with the longest and most conspicuous common sequences lying in the long central region of hydrophilic amino acids which contain the nucleotide binding sites. Despite the difference of primary structure between fungal ATPases and the animal-cell cation ATPases, hydropathic analysis of all four enzymes thus far sequenced indicates a strong similarity of overall morphology and distribution with respect to the membrane. This point will be discussed further in the section below.

Kinetic and Structural Models

On balance, it seems clear that the known chemical and structural similarities between the H^+-ATPase of *Neurospora* and the cation ATPases of animal cells far outweigh their differences. Therefore, even in the absence of detailed knowledge about partial reactions of the *Neurospora* enzyme, it should nevertheless be possible to model the transport process by analogy with the better-understood cation transport enzymes, particularly the $(Na^+ + K^+)$-ATPase. That enzyme requires about 20 discrete intermediates to accommodate all known reaction steps (Karlish *et al.*, 1978). If a similar cycle occurs in the *Neurospora* enzyme, but simplified by binding of only a single H^+ ion (rather than 3 Na^+ and 2 K^+), a diagram like that in Fig. 4 can be drawn. Such large kinetic models have too many degrees of freedom to be studied usefully by means of single substrate changes, and in order to simplify the models *without physical bias*, Hansen and his collaborators (Hansen *et al.*, 1981; Sanders *et al.*, 1984) developed systematic, matrix algebraic methods for lumping inaccessible parameters together. These methods have been applied to current–voltage data (see Fig. 1), as modulated by changes of substrate concentration ($[H^+]_i$, $[H^+]_o$, and $[ATP]_i$), to give information

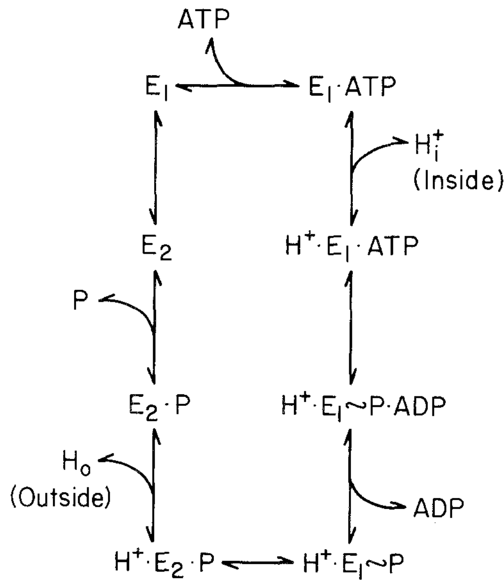

Fig. 4. Hypothetical reaction scheme for the proton-pumping ATPase of *Neurospora* plasma membrane. Simplified from the scheme of Karlish *et al.* (1978) for the $(Na^+ + K^+)$-ATPase, so that only a single ion is bound, transported, and released, for each ATP molecule hydrolyzed.

about the relative magnitudes of selected reaction constants and a picture of how their poise affects the overall transport process.

The analysis has yielded crisp explanations for several long-standing puzzles in the behavior of the *Neurospora* proton pump. For example, how can complete respiratory blockade, which depletes cytoplasmic ATP about 10-fold (from ~3 to ~0.3 mM; Slayman, 1973), diminish the cell membrane potential by 150–200 mV (Slayman, 1965) while reducing the driving potential to the pump by only ~60 mV? According to the model, this can happen when initial binding of ATP ($E_1 \rightleftharpoons E_1 \cdot ATP$) rate-limits the pump for clockwise cycling (Fig. 4), so that pump current diminishes almost in proportion to the decline of $[ATP]_i$ (Gradmann *et al.*, 1982a). And it is the decrement of pump current re-entering through the membrane resistance, not the thermodynamic effect of reduced ATP, which diminishes the membrane potential. Or what is the meaning of the fact that pump velocity is highly sensitive to intracellular pH, but nearly insensitive to extracellular pH (Slayman and Sanders, 1984)? Modelling shows this will happen when the proton-binding/release steps are strongly *dissociated*, with apparent acid pK's 2–3 pH units below the solution pH's: $(pK_a)_{out} \simeq 2.9$, compared with $pH_{out} = 5.8$; and $(pK_a)_{in} \simeq 5.4$, compared with $pH_{in} = 7.2$. The direction of asymmetry to substrate (product) variations then depends on the direction of net reaction flow; i.e., clockwise flow (Fig. 4) results in insensitivity to $[H^+]_o$. The same condition can also produce *kinetic* interchangeability for *thermodynamically* equivalent changes of membrane potential and transmembrane pH difference (Hansen *et al.*, 1981), a much discussed physiological property of mitochondrial and bacterial transport systems (Maloney, 1982; Kaback, 1983; Fillingame, 1980).

Most data thus far available on vanadate-sensitive ion-pump/ATPases —including the H^+ pumps in the *Neurospora* (Slayman and Sanders, 1984) and *Chara* (Smith and Walker, 1981) plasma membranes, the ($Na^+ + K^+$)-pump in a variety of nerve and muscle preparations (DeWeer and Geduldig, 1978; Daut and Rüdel, 1982; Gadsby, 1982; Marmor, 1971), and the chloride pump in *Acetabularia* (Mummert *et al.*, 1981)—require only a single voltage-dependent step (the charge-transport step). Current–voltage data on *Neurospora* (Gradmann *et al.*, 1982) constrain that step to be the transition between high-energy phosphorylated intermediate ($E_1 \sim P$) and low-energy phosphorylated intermediate ($E_2 \cdot P$), as shown in the reaction diagram of Fig. 4. However, considerations of ATPase morphology, along with a variety of physical transport mechanisms, suggest that in reality more than one transition within the enzyme may be voltage-dependent, or—in other words —that charge transfer across the membrane occurs in two or more discrete steps which interact with the membrane electric field. One might imagine, for example, that each of the transitions between $H^+ \cdot E_1 \cdot ATP$ and $H^+ \cdot E_2 \cdot P$,

clockwise, in Fig. 4, involves charge displacement partway across the membrane. Indeed, the likely involvement of two or more voltage-dependent steps is testified by recent experiments identifying regions of negative conductance in I–V curves for the sodium pump (DeWeer and Rakowski, 1984; Eisner and Lederer, 1980; LaFaire and Schwarz, 1985) and for chloride pumps (Gradmann *et al.*, 1982; Graves and Gutknecht, 1977). Such regions, if they are not methodological artifacts (Chapman *et al.*, 1983; Blatt, 1986), demand at least two voltage-dependent steps in any reaction-kinetic model.

For morphological data, as for partial reaction chemistry, the most complete information available on a single pump-ATPase is that on the α-subunit of the ($Na^+ + K^+$)-ATPase. Overall, it resembles the mitochondrial ATPase except for being much smaller: comprised of a single polypeptide embedded in and protruding from the membrane. This view, which has recently been summarized by Nicholas (1984), is supported by specific labelling studies and by examination of proteolytic digestion fragments from membrane-bound enzyme (Nicholas, 1984; Jørgensen, 1982), by electron microscopy (Deguchi *et al.*, 1977), and more recently by hydropathic analysis of the amino acid sequence (Shull *et al.*, 1985; Kawakami *et al.*, 1985) deduced from the *c*DNA sequence for *Torpedo* and sheep-kidney enzyme. The membrane-embedded portion contains 6–10 α-helical stretches which (are presumed to) lie approximately parallel to each other forming a channel which spans most of the membrane thickness, and which comprise 30–40% of the total molecule. Another ~5% lies at the membrane–water interface on the cell exterior; and the largest part of the molecule forms a globular headpiece extending into the cytoplasm. Such a structure is both too massive and too well anchored to act as a classical diffusible carrier, but must instead function as an "activated" channel for the transported ion(s).

Several distinct notions of the activation/transport process have been suggested in recent years, including (i) *ligand conduction*, in which the transported ion would hop along an internal surface or array of sites through the protein (Mitchell, 1979); (ii) *channel peristalsis*, in which a directed and progressive conformational change or energy wall would push the ion through a defined pore in the protein (Laüger, 1984); and (iii) *double-gating* of a channel, in which the ion would be gated into the membrane from one side, trapped or occluded with both gates closed (Glynn *et al.*, 1985), and then gated out on the opposite side of the membrane. Although each of these models is mechanistically distinct, from a kinetic point of view all three can be formulated in similar fashion. Double gating is the easiest to visualize in mechanical terms, and permits the reaction cycle of Fig. 4 to be transformed, with some lumping of steps, into the cartoon cycle of Fig. 5, which derives from a suggestion made by Jardetzky (1966).

The uncomplexed enzyme is viewed as comprising two cavities: one being a channel through the membrane; and the other, located near the catalytic site, being a space in which ATP and the transported proton can bind. In the normal nontransporting condition of the enzyme, the two cavities would be separated by a gate or constriction which forms the principal dielectric barrier through the protein and sustains essentially the entire membrane potential. Once both ATP and H^+ have bound, catalysis (clockwise) would transfer the γ-phosphate from ATP to the aspartyl group, allowing ADP to be released and gating the catalytic space shut from the cytoplasm (lower right, Fig. 5). This gating step would necessarily consume energy, since

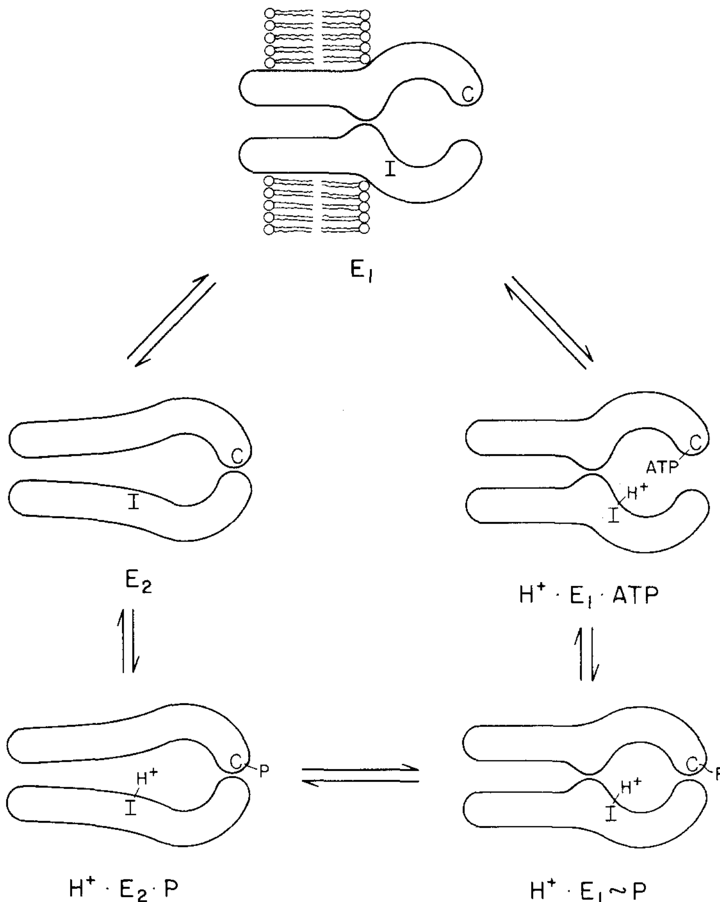

Fig. 5. Hypothetical double-gated mechanism to pump protons. The model is a variation of the flip-flop (Jardetzky, 1966) or peristaltic channel (Laüger, 1984), postulated previously. Reprinted by permission from Biochemical Society Symposium 50, pp. 11–29, copyright 1985; The Biochemical Society, London.

the dielectric barrier within the protein would be split, with part of the electric field having moved across the occluded ion. Subsequently, the left-hand gate would open (lower left, Fig. 5), thus moving the second part of the field across the bound proton to the right-hand gate. This step would consume the remainder of the phosphate-bond energy in the ATPase. Once the proton —now exposed to the cell exterior—has dissociated and diffused away, the protein would be free to relax back to its initial state (E_1), releasing inorganic phosphate and transferring the field back to the left-hand gate but *without crossing any occluded ions*.

This kind of model is compatible with two other current mechanistic notions about transport processes and ATPases. The first mechanistic notion is that the major energetic transition in synthesis (or hydrolysis) of ATP occurs during release (or binding) of the ATP, not during its actual formation (or splitting) (Boyer *et al.*, 1985). In other words, the major energy-requiring steps are alternate admission and exclusion of water from the catalytic site. In the case of phosphoglycerate kinase, this process can be described as opening and closing of a cavity within the protein, visualized by X-ray diffraction studies (Pickover *et al.*, 1979). Evidence that it is a general phenomenon has come from ^{18}O-exchange studies on myosin ATPase (Bagshaw *et al.*, 1975), on mitochondrial ATPase (Cross and Boyer, 1975), on the plasma-membrane H^+-ATPase from *Shizosaccharomyces pombe* (Amory *et al.*, 1982), and on Ca^{2+}-ATPase from cardiac muscle (de Meis, 1985). The second mechanistic notion is that the α-helical "bars" of protein play a crucial role in transport (Edmonds, 1985), forming static or elastic (perhaps peristaltic) channels. This notion seems required by the simple observation that certain "active" transport systems, such as bacteriorhodopsin (Ovchinnikov *et al.*, 1978; Henderson and Unwin, 1975) and the lactose transport system of *Escherichia coli* (Büchel *et al.*, 1980; Foster *et al.*, 1983) consist of almost nothing but α-helical, membrane-embedded regions.

The double-gating model in Fig. 5 is in fact only one simple way to combine functions of a globular-protein catalytic region lying structurally in series with an array of coils which form a presumptive channel. The model contains two discrete voltage-dependent steps, as was indicated at the outset of this discussion; and it would involve large electric fields across the gating regions, if they were much thinner than the membrane proper. Finally, an important and novel feature of the model, to which attention has only recently been drawn (Slayman and Sanders, 1985), is that from a physical point of view the ion is not pumped through the membrane. Rather, the membrane or the membrane electric field is moved past the occluded ion. Only the unoccluded ion need move, and it could do so strictly by diffusion.

Acknowledgements

Preparation of this review was supported by Research Grant GM-15858 from the National Institute of General Medical Sciences. The author is indebted to Dr. Carolyn Slayman, of the Yale Department of Human Genetics, for many helpful discussions, for permission to cite unpublished data, and for permission to use Table 1 and Figs. 2 and 3.

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