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SHORT REVIEW

The Proton Pumps of the Plasmalemma and the Tonoplast of Higher Plants

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Abstract

Studies on intact cells, membrane vesicles, and reconstituted proteoliposomes have demonstrated in higher plants the existence of an ATP-driven electrogenic proton pump operating at the plasmalemma. There is also evidence of a second ATP-driven H^+ pump localized at the tonoplast. The characteristics of both these ATP-driven pumps closely correspond to those of the plasmalemma and tonoplast proton pumps of *Neurospora* and yeasts.

Key Words: H^+ pumps; higher plants; ATPase; plasmalemma; tonoplast.

Introduction

According to a definition accepted by most physiologists and adopted in this article, a proton pump is defined as a biochemical mechanism that mediates the obligatory coupling of an exergonic chemical process to the eventually endergonic transport of protons across a biological membrane, the overall thermodynamics of the whole process resulting in a decrease in free energy. Different forms of chemical energy can be utilized by the driving process: oxidation–reduction (in chloroplasts, mitochondria, erythrocyte, and bacterial plasma membranes) or group transfer potential (phosphate transport potential in the reversal of photosynthetic and mitochondrial phosphorylation, and in the bacterial and plant plasma membrane).

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Endergonic transport of protons is taken in a broad sense, indicating a process leading to a net decrease in H^+ concentration on one side of the membrane, and in increase on the other side. This transformation involves an increase in the electrochemical potential of the proton gradient across the membrane. Such a definition of a proton pump does not specify whether the changes in proton concentration on the two sides of the membrane are due to actual transport of the individual protons as such, or to their association or dissociation, such as might result from the transport of hydroxyl ions. At the present state of knowledge, no formal differentiation can thus be established, in most cases, between "true," direct H^+ transport and "apparent," indirect H^+ transport consequent to "true," direct OH^- transport. However, the term "H^+ transport" is not usually used to describe the H^+ gradient changes dependent on the movement of H^+ together with specific anions (whether in the associated or dissociated form) or of CO_2. On the other hand, the maintenance of electroneutrality at the two sides of the membrane requires the ("true" or "apparent") H^+ flux to be electrically compensated by the movement of other charges usually belonging to inorganic cations (preferentially although not necessarily, K^+). The coupling between the opposite fluxes of H^+ and of the compensating cations can be either strictly specific and obligatory, with the transport of protons occurring at the level of the same catalytic mechanism involved in the transport of the cations (chemical coupling), or loose, nonspecific, and driven by the transmembrane electrical potential change induced by the electrogenic H^+ transport (electrical coupling). Finally, whereas a pump transporting only H^+ must be clearly electrogenic, in the chemical coupling the opposite coupled fluxes of protons and other cations can be either electroneutral or electrogenic, depending on the charge stoichiometry.

Therefore, a description of any particular H^+ pump should take into consideration: (a) the localization of the mechanism in a particular cell membrane; (b) the nature of the process used as energy source and the characterization of its biochemical (enzymatic) basis; (c) the identification of the ion species eventually transported simultaneously with H^+; (d) the definition of the coupling between the energy-providing process and the H^+ (eventually other ions) transport; (e) the electrophysiological consequences of the whole process; (f) the overall stoichiometry and thermodynamics of the whole process.

Extensive information is available concerning these aspects of H^+ pumps in animal and in bacterial cells. As far as higher plants are concerned, most of the work carried out in the last decades concern the redox-driven H^+ transporting mechanisms present in the chloroplast thylakoids and in the mitochondrial cristae, as well as the potentially reversible coupling between H^+ transport and ATP synthesis or hydrolysis at the level of these

membranes. Investigation of the mechanisms of proton transport at the plasmalemma and the tonoplast is still in an initial phase.

Here we present the relevant data and ideas concerning the existence and the nature of proton pumps at the plasmalemma and the tonoplast of higher plants. The more complete information available for fungi has been extensively reviewed (Goffeau and Slayman, 1981; Eddy, 1982) and will be dealt with here very synthetically, and principally for a comparison with the situation in higher plants.

Proton Pumps of Fungi and Algae

The Plasmalemma Pump of Neurospora and Yeasts

The capacity of both *Neurospora* and yeast cells to acidify the medium by extruding protons in exchange with other cations (preferentially K^+) was shown in the late sixties to be associated with a marked hyperpolarization of the membrane electrical potential and to be strongly inhibited by respiration inhibitors and by protonophore uncouplers (Slayman, 1965a, b). The correlation between ATP contents, H^+ efflux, and value of the electrical potential, as well as experiments with ATPase inhibitors, suggested that ATP hydrolysis provided the energy required to move H^+ against a very steep electrochemical gradient (Slayman, 1970; Slayman *et al.*, 1973).

This view was confirmed by a series of studies utilizing plasmalemma vesicles, and by studies leading to the isolation and characterization of an ATPase present in membrane preparations which had been efficiently enriched in plasmalemma (see, for *Neurospora*, Scarborough, 1975, 1977, 1980); Bowman and Slayman, 1977; and, for yeasts, Santos *et al.*, 1978; Bussey *et al.*, 1979). The sealed native vesicles thus obtained were able to effect strictly ATP-dependent, electrogenic H^+ transport (Scarborough, 1976, 1980).

From these or similar preparations from *Neurospora*, as well as from various yeasts (Dufour and Goffeau, 1978; Blasco and Chappuis, 1981), an ATP hydrolyzing enzyme was extensively purified that showed the following characteristics; (a) a marked affinity for lipids, preferentially lysolecithin, which required a rather sophisticated use of detergents for solubilization; (b) a very high specificity for $ATP \cdot Mg^{2+}$, considered to be the true physiological substrate (with CO^{2+}, Mn^{2+}, Ni^{2+}, and Zn^{2+}, in decreasing order of efficiency, but not Ca^{2+} and Sr^{2+}, substituting for Mg^{2+}); (c) a relatively high K_m for $ATP \cdot Mg$ (1–3 mM); (d) an optimal activity at slightly acid pH (between 5.5 and 6.5); (e) a slight activation by monovalent cations, rather nonspecific but with some preference for K^+ and Rb^+; (f) a strong

inhibition by vanadate, DES,[2] and DCCD, but no sensitivity to azide, oligomycin, ammonium molybdate and nitrate, or other anions; (g) the formation of a rapidly turning over enzyme-phosphate intermediate during ATP hydrolysis; (h) a molecular weight of the catalytically active polypeptide of about 100,000 daltons (for a detailed presentation of these characteristics, together with the relative references, see the recent review by Gouffeau and Slayman, 1981).

The capability of this ATPase to catalyze ATP-dependent electrogenic H^+ transport has been convincingly demonstrated in proteoliposomes obtained by recombination of the purified enzyme with phospholipids (Villalobo *et al.*, 1981; Dufour *et al.*, 1982).

The stoichiometry of the ATP-dependent proton transport process catalyzed by this plasmalemma enzyme has not yet been clarified. In *Neurospora*, electrophysiological experiments (current–voltage curves) combined with determinations of the values of the H^+ electrochemical gradient and of the free energy change of ATP hydrolysis led Warncke and Slayman to arrive at a transported H^+/ATP ratio of 1 in the normal cell, which, however, possibly shifted to a ratio of 2 under energy shortage conditions (Warncke and Slayman, 1980).

A second important point concerning the stoichiometry of the process is whether H^+ efflux is obligatory (chemically) coupled to the transport of other ions, and, in particular, to the influx of monovalent cations (K^+ or Rb^+ or Na^+). The available data, which show that in plasmalemma vesicles ATP-driven H^+ transport is not influenced by the omission of either Cl^- or Rb^+ or Na^+, contradict this hypothesis and are thus in favor of a model in which H^+ is the only ion directly transported by the pump (Scarborough, 1980). The influx of K^+ (or other cations) usually associated with H^+ efflux (H^+/K^+ exchange) would thus be a nonspecific consequence of the electrogenicity of H^+ extrusion (electrical coupling).

The Plasmalemma Proton Pump of Chara

The presence in higher algae of an ATP-driven plasmalemma proton pump, already indicated by observations in intact cells (Walker and Smith, 1977), has been recently confirmed by experiments in which the plasmalemma of the large internodal cells of *Chara* was perfused with an artificial medium after removal of the cytoplasm (Tazawa *et al.*, 1976). Experiments of this type demonstrate both the electrogenic nature and the ATP dependence of the

[2] Abbreviations: DES, diethylstilbestrol; DCCD, *N,N'*-dicyclohexylcarbodiimide; PD, transmembrane electrical potential difference; FC, fusicoccin; FCCP, (*p*-trifluoromethoxy)-carbonylcyanide-phenylhydrazone; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DNP, 2-4-dinitrophenol; $\Delta\psi$, electrical potential difference; TBBA, tributylbenzylammonium chloride; ABA, abscisic acid.

process, whereas its sensibility to vanadate suggests the involvement of an ATPase similar to that operating at the plasmalemma of *Neurospora* and yeasts (Walker, 1980; Tazawa and Shimmen, 1980; Shimmen and Tazawa, 1982).

The Tonoplast Proton Pump of Neurospora and Yeasts

The presence of an active H^+ translocating mechanism at the tonoplast of the fungal cells was suggested by the observation of an often steep H^+ gradient across this membrane as well as by data indicating that the energy of this gradient could be used for the accumulation of solutes in the vacuole (see, for example, arginine in Bowman and Bowman, 1982). This view was confirmed by the recent work on purified vacuolar membrane vesicles from yeast, which showed that an ATPase, clearly distinct from that isolated from plasma membrane preparations, is present in the tonoplast, where it couples ATP hydrolysis with the electrogenic transport of protons (Bowman and Bowman, 1982; Okorokov and Lichko, 1983; Kakinuma *et al.* 1981).

The general characteristics of this tonoplast ATPase in *Neurospora* and in yeast appear very similar: preference for ATP, but significant activity with other nucleoside triphosphates; requirement for Mg^{2+}; inhibition by DCCD but not by vanadate, oligomycin, or azide. Interestingly, the enzyme (at least in *Neurospora*, Bowman and Bowman, 1982) is markedly inhibited by NO_3^-, which, together with the lack of sensitivity to vanadate, seems to indicate a relationship with the enzyme present in higher plant membrane preparations presumably enriched in tonoplast.

Several important characteristics of this tonoplast system, including the stoichiometry of the process and its regulation, are still open to investigation. However, the available data seem sufficient to demonstrate the presence of an electrogenic, ATP-driven proton pump in the tonoplast of fungal cells.

The Plasmalemma and Tonoplast Proton Pumps of Higher Plants

Evidence In Vivo

Extensive research on the mechanism of salt uptake in plants has shown that K^+ and in general, cation uptake is usually associated with an efflux of H^+ and is stimulated by high external pH (see, for example, Rothstein and Enns, 1946; Jacobsen *et al.*, 1950; Hurd and Sutcliffe, 1957; Jackson and Adams, 1963; Poole, 1966; Hodges, 1973). The linkage of H^+ efflux with the influx of K^+ or other cations and its relation with dark CO_2 fixation and thus with malate accumulation, which balances the excess of cation over anion

uptake, has been clearly demonstrated by Jacoby and Laties (1971) and confirmed by several papers (Raven and Smith, 1974; Clarkson and Hanson, 1980). Thus Pitman (1970) proposed that the H^+ efflux associated with cation uptake depended on the outward transport of protons rather than increased respiration or sugar breakdown, whereas a major role of the transport of HCO_3^-, rather than of H^+, seemed to be ruled out by the finding that K^+ uptake was influenced by extracellular pH even at constant bicarbonate concentration (Poole, 1974). Important progress in understanding the nature of H^+ and, in general, of ion movement in higher plants came from the electrophysiological approach, which already has been successfully used in animal physiology. It thus became increasingly clear that the electrical potential difference (PD) across the plant plasmalemma could not be explained simply by passive ion diffusion, as predicted by the Goldman equation, and that the intracellular–extracellular distribution of several ions was often very far from their electrochemical equilibria. In particular, the intracellular concentration of K^+ (at very low, but not at high, extracellular concentrations of this ion) was much higher, whereas that of H^+ was lower by some orders of magnitude (up to 5) than the values predicted by the equilibrium Nernst equation. These findings, together with the dependence of a large fraction of PD on the integrity of respiratory metabolism and temperature, suggested that metabolic energy-dependent mechanisms (pumps) were involved in the active transport of at least some ions across the plasmalemma. In particular, the large PD hyperpolarization induced by treatments that increased the rate of the thermodynamically uphill H^+ efflux appeared to be a clear indication of an electrogenic, active proton extrusion mechanism located at the plasmalemma of higher plants and similar to the proton pump operating in fungi and in algae (Higinbotham and Anderson, 1974; Poole, 1974; Mercier and Poole, 1980; see also reviews by Poole, 1978, and Spanswick, 1981).

The hypothesis of the existence of such an electrogenic proton pump was further supported by *in vivo* studies using a new, interesting tool, namely the discovery of the peculiar capacity of fusicoccin (FC) (a growth fungal toxin isolated by Graniti, 1964, and chemically characterized by Ballio *et al.*, 1968) to induce in a number of plant tissues a strong stimulation of H^+ extrusion coupled to K^+ (or other cation) uptake, and, simultaneously, a marked hyperpolization of PD (Marrè *et al.*, 1974; Pitman *et al.*, 1975, see also the review by Marrè, 1979).

The effects of FC on electrogenic H^+ extrusion is strongly metabolism-dependent, is suppressed by protonophore uncouplers (Cocucci *et al.*, 1976; Felle and Bentrup, 1977), and apparently does not involve changes in plasmalemma permeability to H^+ (Pitman *et al.*, 1977; Felle, 1982). Maximal FC-induced PD hyperpolization has been observed in the

absence of K^+ from the medium (Cocucci *et al.*, 1976; Felle, 1982; Marrè *et al.*, 1982), whereas maximal rate of H^+ efflux required high extracellular K^+ concentrations. Conversely, the uptake of K^+ (and also other cations) was stimulated by FC in parallel with H^+ extrusion (see the review by Marrè, 1979). The requirement of FC-induced H^+ extrusion for extracellular K^+ can be fulfilled by other cations, and even by divalent Ni^{2+}, Co^{2+}, Mn^{2+}, and Zn^{2+} (Marrè *et al.*, 1982). In the absence of K^+ or other permeant cations, FC-induced H^+ extrusion can still be induced by passively permeant lipophilic organic cations such as TBBA, etc. (Bellando *et al.*, 1979). This effect apparently depends on the capacity of the lipophilic cations to increase the passive efflux of Cl^-, and thus to depolarize PD (Cerana *et al.*, 1981).

On the other hand, the finding that FC-induced PD hyperpolarization occurs also in the absence of any electrolyte in the medium and is not accompanied by conductance changes suggests that the toxin directly influences a H^+ pump without affecting passive ion transport (Felle, 1982). These combined data thus indicate that the coupling between H^+ efflux and K^+ influx is not obligatory and depends on the hyperpolarization associated with H^+ extrusion (electrical rather than chemical coupling of the opposite H^+ and K^+ fluxes).

The location of the FC-sensitive electrogenic H^+ pump at the plasmalemma is strongly suggested by: (a) the demonstration of a high-affinity FC-binding factor in plasmalemma-enriched membrane preparations (Dohrmann *et al.*, 1977; Pesci *et al.*, 1979a, b; Ballio *et al.*, 1980; Radice *et al.*, 1981; Aducci *et al.*, 1982a, b); (b) the finding that FC hyperpolarizes the transmembrane potential difference across the plasmalemma, rather than that across the tonoplast (Goldsmith and Cleland, 1978); (c) circumstantial evidence which suggests that FC basifies the cycloplasm, as deduced by its effects in increasing the cell sap pH (Guern *et al.*, 1982; Kurkdjian *et al.*, 1981; Romani *et al.*, 1983a) and in enhancing dark CO_2 fixation into malate (Johnson and Rayle, 1976; Romani *et al.*, 1983a).

Finally, the strong link between the effects of FC in hyperpolarizing the PD and in stimulating H^+ extrusion is confirmed by the finding that both effects are simultaneously suppressed by protonophore uncouplers such as FCCP, CCCP, and DNP. Kinetic measurements have shown that this action depends primarily on the permeabilization of the plasma membrane to protons, rather than on the (delayed in time) effect of these uncouplers on mitochondrial phosphorylations (Felle and Bentrup, 1977; Mercier and Poole, 1980).

In vivo studies have also indicated that ATP hydrolysis is the energy source for electrogenic H^+ extrusion in intact plant cells. In fact, practically all of the inhibitors of respiratory metabolism (azide, cyanide, CO, DCCD, arsenate, as well as anaerobiosis (Higinbotham and Anderson, 1974; Marrè

et al., 1973b; Rasi-Caldogno *et al.*, 1978; Mercier and Poole, 1980, etc.) also markedly inhibit electrogenic H^+ extrusion (basal as well as FC-stimulated).

This first indication is supported by two lines of evidence: (a) the activity of the H^+ pump is also repressed by some inhibitors of membrane ATPases that are inactive on the mitochondria or chloroplast ATPases and do not influence the ATP level *in vivo*, such as vanadate (Cocucci *et al.*, 1980; Colombo *et al.*, 1981); (b) the strong stimulation of the pump by FC is accompanied by a slight but significant decrease in ATP level (Rasi-Caldogno *et al.*, 1980; Guern *et al.*, 1982), which has been interpreted as due to its use for H^+ extrusion.

In conclusion, the results cited in this section indicate the presence, at the plasmalemma of the higher plant cell, of a mechanism that uses the energy of ATP hydrolysis for the electrogenic extrusion of H^+, which is electrically coupled with K^+ (or other cations) uptake. This mechanism seems specifically stimulated by FC. Even if the stoichiometry of the process in physiological conditions is still open to investigation, an ATP hydrolyzed/H^+ extruded ratio of 1/1 is suggested by the very steep electrochemical proton gradient reached in the condition of stimulation by FC (ΔpH of more than 3 pH units, PD of -120 mV in maize roots; Cocucci *et al.*, 1976; see also Felle, 1982).

Evidence for ATP-Driven H^+ Pumps in Sealed Vesicles from Membrane Preparations

Much evidence has been accumulated since 1980 to demonstrate the presence of ATP-dependent mechanisms operating the electrogenic transport of protons across nonmitochondrial sealed membrane vesicles present in the so-called "microsomal" (namely, sedimenting between about 8000–80,000 $\times$ *g*) preparations from several higher plants. In spite of the most disturbing heterogeneity of these preparations, studies of density gradient fractionation have allowed a partial separation of the membranes of different origin, or at least some differential enrichment of the various activities present. The results obtained with this method, combined with those of marker enzyme activities and of electron microscope techniques, indicate that the membrane vesicles thus obtained belong mainly to plasmalemma, tonoplast, endoplasmic reticulum, and the Golgi apparatus, with minor contaminations from other organelles and a small amount of mitochondria and plastid fragments (see, for example, Hodges and Leonard, 1974; Hodges, 1976).

A first indication of the capacity of these "sealed" (namely, relatively little permeable to H^+, K^+, and other ions) vesicles to use ATP for H^+ transport came from the observation that the nonmitochondrial (oligomycin- and azide-insensitive, relatively low pH optimum) ATPase activity of these

preparations could be markedly stimulated by protonophores (CCCP, FCCP, gramicidin, etc.) and by ionophores inducing either H^+/K^+ exchange or a depolarization of the transmembrane potential, such as nigericin and valinomycin (Rungie and Wiskich, 1973; Sze, 1980). Further work on membrane preparations from various materials, including tobacco callus, oat and corn roots, pea internodes, corn coleoptiles, and radish seedlings, showed that the vesicles were able to build up a strictly ATP-dependent electrical PD, inside positive (Rasi-Caldogno *et al.*, 1981; Sze and Churchill, 1981; Stout and Cleland, 1982; Mandala *et al.*, 1982; Bennett and Spanswick, 1983a), associated with (and presumably dependent on) an accumulation of H^+ and thus with the formation of a proton gradient (Dupont *et al.*, 1982a, b; Macrì *et al.*, 1982; Bennett and Spanswick, 1983; Churchill and Sze, 1983; Stout and Cleland, 1982; De Michelis *et al.*, 1983).

The electrogenic H^+ transport thus demonstrated was clearly and specifically ATP-dependent, and influenced by the protonophores CCCP and FCCP, by gramicidin, and by nigericin and other PD-depolarizing agents such as valinomycin-K^+ and Cl^-. These characteristics, together with the optimum pH between 6 and 7.5, the insensitivity to oligomycin, and the very low activity of mitochondrial marker enzymes in these preparations clearly demonstrated the presence of proton transporting mechanisms different from that of mitochondria.

On the other hand, it soon became apparent from experiments with inhibitors and fractionation by density gradient that at least two different ATP-driven H^+ pumping systems, involving two different ATPases, coexisted in most of these preparations; a first system strongly inhibited by vanadate and insensitive to nitrate, and a second system, vanadate-insensitive and inhibited by nitrate at millimolar concentrations. The characteristics of the vanadate-sensitive ATPase involved in the first system appeared very similar to those of the plasmalemma enzyme of fungi, whereas the vanadate-insensitive, Cl^--activated and nitrate-inhibited ATPase involved in the system system seemed similar to the enzyme found in isolated vacuoles and in tonoplast preparations from *Neurospora* and yeasts (De Michelis *et al.*, 1983; Churchill and Sze, 1983). The conclusion that the first ATPase belonged to the plasma membrane, and the second to the tonoplast, was also supported by their distribution, together with plasmalemma and tonoplast marker enzymes, respectively in the light and heavy regions of the density gradients (De Michelis *et al.*, 1983). These results support the *in vivo* evidence that ATP hydrolysis is the energy source for at least one type of plasmalemma proton pump. At the same time, they also support other evidence of a second electrogenic H^+ pump, distinct from that of the plasmalemma, at the tonoplast.

The relevant data in this regard can be summarized as follows:

1. The differences in pH and electrical potential between cytoplasm and vacuole and studies on sensitivity to protonophore uncouplers suggest the existence of active H^+ mechanisms of ion transport at the tonoplast (in *Kalankoe* leaf cells, Rona *et al.* 1980; in sugar cane cell cultures, Komor *et al.*, 1982).

2. ATP-stimulated accumulation of H^+ associated with some shift of $\Delta\psi$ toward positivity has been observed in isolated vacuoles from *Tulipa* petal cells (Wagner and Lin, 1982) (see, however, the opposite effect of ATP on $\Delta\psi$ reported by Doll and Haver, 1981 in isolated vacuoles from red beet root cells).

3. Tonoplast preparations from isolated vacuoles contain a vanadate- and oligomycin-insensitive, Cl^--stimulated ATPase (Lin *et al.*, 1977, in *Tulipa* petal vacuoles; Leigh and Walker, 1980; Walker and Leigh, 1981, and Admon *et al.*, 1981, in red beet root vacuoles). In the more thoroughly investigated red beet tonoplast preparations, the vacuolar ATPase is strongly inhibited by nitrate (Walker and Leigh, 1981). The general characteristics of the vacuolar ATPase thus appears similar to those of the vanadate-insensitive, nitrate-inhibited, Cl^--stimulated ATPase involved in H^+ transport in membrane vesicles that migrate in the light region of the density gradient and whose preparations are presumably enriched in tonoplast (Churchill and Sze, 1983; De Michelis *et al.*, 1983; O'Neill *et al.*, 1983; Mandala *et al.*, 1982).

4. The lutoids, a specialized type of vacuoles easily isolated from *Hevea brasilensis* latex, are able to effect, thermodynamically uphill, ATP-dependent H^+ accumulation associated with a shift of $\Delta\psi$ toward positivity (Marin, 1980; Marin *et al.*, 1981, 1982; Cretin, 1982; D'Auzac *et al.*, 1982). The same organelles also contain a membrane-bound ATPase with an optimum pH of 6.5–7.5, activated by Cl^- and various organic anions, inhibited by DCCD and by nitrate, but insensitive to oligomycin and vanadate (Cretin, 1982; Marin *et al.*, 1981; D'Auzac, 1977) and thus similar to the ATPase involved in the proton pump of normal vacuoles and of tonoplast-enriched vesicle preparations.

In conclusion, these data provide convincing, although still largely circumstantial, evidence for the existence in higher plant cells of two distinct ATP-driven proton pumps, one of which belongs to the plasmalemma and the other to the tonoplast.

Isolation and Purification of the ATPases Presumably Involved in the Plasmalemma and in the Tonoplast Proton Pumps

In the previously mentioned investigations, the ATPase activities involved in the postulated plasmalemma and tonoplast proton pumps have

been studied in the membrane-bound state, with attention mainly on the characterization of electrogenic H^+ transport. Extensive parallel work aimed to isolate and characterize the involved ATPases has been carried out in the last decade. Thus, nonmitochondrial ATP hydrolyzing enzymes present in higher plant membrane preparations have been described in several laboratories (see, for example, Hodges and Leonard, 1974; Hodges, 1976; Tipton *et al.*, 1975; Marrè *et al.*, 1980; Cocucci and Ballarin-Denti, 1981; Vara and Serrano, 1981; Tognoli and Marrè, 1981; Gallagher and Leonard, 1982). The most recent development has led to the recognition and the partial purification of ATPases endowed with characteristics closely corresponding to those of the ATP-driven electrogenic proton transport in membrane vesicles. At present, it seems that these ATPases can be classified into two classes: vanadate-inhibited, anion-insensitive, presumably plasmalemma-bound ATPases, and vanadate-insensitive, chloride-stimulated, nitrate-inhibited, presumably tonoplast-bound ATPases. Below we list some of the characteristics shown by these enzymes in the most active partially purified preparations available.

1. Vanadate-inhibited ATPases (presumably plasmalemma-bound). Enzymes of this class have been partially purified from plasmalemma-enriched membrane preparations from maize roots (Dupont *et al.*, 1981; Briskin and Leonard, 1982; Scalla *et al.*, 1983), oat roots (Vara and Serrano, 1982, 1983), red beet root cells (Briskin and Poole, 1983a, b, c), and radish seedlings (Cocucci and Marrè, 1984). The specific activities reached by purification were $2\,\mu mol \times mg^{-1} \times min^{-1}$ for the enzyme from maize, $1\,\mu mol \times mg^{-1} \times min^{-1}$ for that from red beet roots, $2.7\,\mu mol \times mg^{-1} \times min^{-1}$ for that from oats, and $13\,\mu mol \times mg\ protein^{-1} \times min^{-1}$ for that from radish seedlings. These values can be compared with those obtained for the similar *Neurospora* and *Saccharomyces pombe* plasmalemma enzymes (respectively 97 and $35\,\mu mol \times mg\,protein^{-1} \times min^{-1}$). Common features for the vanadate-sensitive ATPases partially purified from the four above-mentioned plant species are: a high specificity for ATP · Mg as substrate; the requirement for Mg^{2+}, Mn^{2+}, or Co^{2+}, whereas Ca^{2+} is inhibitory; a simple Michaelis–Menten kinetics and a relatively high (0.5–2 mM) K_m for ATP · Mg; an optimum pH between 6 and 6.7; some activation by monovalent cations (preferentially, $K^+ > Rb^+ > Na^+$); a marked inhibition by vanadate, diethylstilbestrol, and DCCD, but not by oligomycin, azide, molybdate, or ouabain.

Other important information available for some of these enzymes are the insensitivity to nitrate (ATPases from radish seedlings and red beet roots); the formation of a rapidly turning over phosphorylated intermediate during the enzyme action, and a molecular weight of about 100,000 daltons for the catalytic subunit (ATPases from maize, red beet, and oat roots); and a

strong requirement for phospholipid (observed in the preparations endowed with the higher specific activity and obtained from oat roots and radish seedlings). The capacity of the enzymes of this group to carry out H^+ transport in reconstituted proteoliposomes has been demonstrated for the oat root (Vara and Serrano, 1982) and for the radish ATPase (Cocucci, De Michelis, Pugliarello, and Rasi-Caldogno, unpublished data). All of the above-mentioned features are also characteristic of the plasmalemma ATPases of *Neurospora* and yeasts, as described in a previous section.

2. Nitrate-inhibited, vanadate-insensitive ATPases (presumably tonoplast-bound). Due to the difficulty of obtaining reasonable amounts of isolated vacuolar membranes, the purification and characterization of ATPases of this group is still in an initial phase. The solubilization and the characterization of a presumably tonoplast-bound H^+-transporting ATPase from a maize root tonoplast-enriched membrane preparation have been recently reported by Bennett and Spanswick (1983b). The solubilized enzyme (specific activity about 0.1 μmol $\times$ mg^{-1} protein $\times$ min^{-1}) required Mg^{2+} for activity, was stimulated by Cl^- but not by K^+ or other monovalent cations, and was strongly inhibited by nitrate, DCCD, and diethylstilbestrol, whereas it was insensitive to vanadate, azide, and oligomycin, thus showing the characteristics of the ATPase activity involved in electrogenic H^+ transport in the tonoplast-enriched vesicle systems. Reconstitution of the enzyme into proteoliposomes by phospholipid addition led to the formation of vesicles able to carry out ATP-dependent proton transport.

Indications for a Redox Energy-Driven Plasmalemma Proton Pump

The results mentioned in the preceding sections indicate that at least a large fraction of the active, electrogenic H^+ transport at the plant cell plasmalemma depends on ATP hydrolysis for energy. However, from recent results there is some suggestion of the participation of a transplasmalemma electron transport system in this process.

The capacity to carry out an FC-stimulated reduction of exogenous ferricyanide associated with an increased H^+ extrusion has been reported for intact carrot cells by Craig and Crane (1981, 1982) and for maize roots by Federico and Giartosio (1983), who also demonstrated the presence of a cyanide-insensitive NADH-ferricyanide oxidoreductase activity in membrane preparations from the same material. The operation of an exogeneous NADH-oxidizing system (O_2 as final acceptor) at the plasmalemma of maize root protoplasts, with NADH oxidation associated with an increased rate of H^+/K^+ exchange, has been reported by Lin (1982), who was also able to isolate from the intact protoplasts the plasmalemma-bound NADH-oxidizing activity by means of a mild treatment with trypsin.

These results are consistent with the view that a transmembrane redox system transferring electrons from NADH to O_2 (or eventually to an artificial acceptor such as ferricyanide) can provide the energy for electrogenic proton extrusion at the plasmalemma (redox-driven H^+ pump). The relationship between this sytem and the ATP-driven H^+ pump, as well as its relative importance, are open to further investigation.

Physiological Role of the Plasmalemma and the Tonoplast Proton Pumps of Higher Plants

A detailed treatment of this topic is outside the scope of this article. However, some general considerations may help to identify the relevant aspects concerning the regulation of the activity of the plasmalemma and tonoplast proton pumps and thus their integration within the economy of the plant cell and organism.

The physiological "product" of the activity of these pumps is the building up of electrochemical H^+ gradients at the plasmalemma and the tonoplast. The use of these gradients (protonmotive force) for a number of otherwise endergonic processes of solute transport by means of either PD-driven electrogenic uniports or specific mechanisms coupling the reflux of H^+ to the movement of ions or nonelectrolytes (H^+ symports or antiports of amino acids, cations such as Na^+ or Ca^+, anions such as Cl^-, HPO_4^{2-}, and NO_3^-, and sugars and amino acids) is now well established (see, for example, Ratner and Jacoby, 1976; Komor and Tanner, 1980; Novacky *et al.*, 1980; Lüttge *et al.*, 1981; Novacky and Ullrich-Eberius, 1983). It should be noted that the values of the H^+ gradient and of PD can only unspecifically influence the thermodynamic feasibility of these processes, and that the rate of transport of each particular solute must be specifically regulated by the concentration and the activation state of each of the permeases or carriers involved in the particular uniports, symports, or antiports. However, experiments with intact cells or tissues have shown that treatments influencing the activity of the proton pump, and thus the intensity of the protonmotive force, can significantly modify the rate of transport of several solutes, including various ions, amino acids, and sugars (Malek and Baker, 1978; Colombo *et al.*, 1978; Marrè, 1979; Lüttge *et al.*, 1981). It thus appears clear that the rate of operation of the H^+ pumps at both the plasmalemma and the tonoplast can influence the solute composition (and thus the metabolic behavior) of the three main compartments of the plants, namely, the intercellular free space (wall space), the cytoplasma, and the vacuole. For the sake of brevity, the discussion of the long-reaching effects of changes in the activity of the H^+ pumps on the levels and distribution of the individual

solutes is limited here to the case of H^+. The observed effects of changes in pH in the free space and in the cytoplasma can be summarized as follows:

a. In the free space. Changes in pH induced by stimulation of the H^+ pump with FC or by its inhibition with ATPases inhibitors (for example, vanadate; see Colombo *et al.*, 1981) have been shown to markedly influence the plastic extensibility of the cell wall (Cleland, 1976; Marrè, 1979; Gabella and Pilet, 1978; Sakurai *et al.*, 1977; Yamagata and Masuda, 1975), thus explaining the strong effects of these treatments on growth by cell expansion (acid growth theory: see Rayle and Cleland, 1977; Hanson and Trewavas, 1982). A second important process influenced by changes in pH in the free space is the absorption of weak acids, including some important hormones such as auxin (Guern *et al.*, 1982) and abscisic acid (Astle and Rubery, 1983; Kaiser and Hartung, 1981; Kurkdjian *et al.*, 1979), where the effect of low pH in the free space in increasing the uptake of weak acids or hormones can be explained by their symport with protons or by the much higher permeability of the plasmalemma to the undissociated acid. Consequently, the extracellular pH values characteristic of the various tissues would control the concentration of various solutes and hormones in the free space (and thus also their movement by diffusion in the apoplast).

b. In the Cytoplasm. A change in activity of the proton pumps of either the plasmalemma or the tonoplast would influence the intracellular H^+ concentration, with consequent homeostatic responses consisting mainly, on the basis of available evidence, of rapid changes in the rate of (H^+ releasing) malate synthesis by PEP carboxylation (biochemical pH stat theory of Davies, 1973, and Raven and Smith, 1974; see also Osmond, 1976, and Guern *et al.*, 1982). In fact, the stimulation of electrogenic H^+ extrusion by either FC or natural hormones in shoots and by FC or brassinolide in roots has been shown to be accompanied by an increase in dark CO_2 fixation and malate level (Johnson and Rayle, 1976; Marrè, 1979; Gabella and Pilet, 1980; Romani *et al.*, 1983b). Similarly, in the stomata guard cells, where FC stimulates and ABA inhibits the proton pump, the increase in malate level associated with that of K^+ uptake appears to be responsible for the increase in water uptake and cell turgor, which leads to the opening of stomata (Van Kirk and Raschke, 1978).

The pH-induced shift of PEP metabolism from normal glycolysis to the PEP + HCO_3^- → oxalacetate → malate pathway quite probably involves other important metabolic consequences, and it has been proposed as a cause of the inhibition of the Gl_6P oxidation pathway observed in FC as well as in the auxin-treated tissues (Marrè, 1979). Thus, changes in the metabolic pattern induced by variations in intracellular pH may play an important role in the gross physiological responses associated with experimental treatments

that influence electrogenic H^+ extrusion. In particular, the increase in the capacity of solute uptake due to the stimulation of the proton pump, the intracellular pH-dependent control of PEP malate metabolism, and also the changes in intracellular levels of hormones such as ABA and/or gibberellic acid seem to converge in the striking effect of FC (presumably directly acting on electrogenic H^+ extrusion only) to promote germination and thus the activation of all aspects of growth in dormant seeds (Lado *et al.*, 1975; Cocucci *et al.*, 1981; for a comprehensive interpretation, see Marrè, 1979).

Regulation of the Activity of the Plasmalemma Proton Pump

Physiological considerations suggest, and experimental results demonstrate, that in the intact plant cell the activity of the proton pumps is regulated by a number of factors, such as the transmembrane electrical potential, the intracellular and extracellular pH values, various plant hormones, and the ionic composition of the extracellular medium.

Changes in transmembrane potential and pH in the free space and the cytosol are the direct products of electrogenic H^+ extrusion, and this suggests some kind of negative feedback regulation of the proton pump by these factors. This hypothesis is confirmed by the finding that H^+ extrusion is stimulated by PD-depolarizing treatments, such as with K^+ or other permeant cations that presumably enter the cell by passive electrogenic uniport, or with lipophilic cations such as TBBA, the depolarizing activity of which can be explained by an increase in Cl^- efflux (Cerana *et al.*, 1981). The activation of H^+ secretion by these depolarizing treatments appears much greater in cells previously hyperpolarized by treatment with FC; moreover, in the case of the treatment with permeant divalent cations, their influx is electrically compensated by H^+ efflux only at the high (FC-induced) PD values, whereas at lower values it is mainly accounted for by exchange with K^+ (Marrè *et al.*, 1982). These results, together with data and calculations which indicate that ATP-driven H^+ secretion in plants is largely exergonic (see Felle, 1982), suggests that the effect of PD on proton secretion depends on a modification of the catalytic activity of the pump rather than on the change in the thermodynamic balance of the process (see Komor *et al.*, 1979, for similar considerations on the effect of PD on the H^+/glucose symport mechanism in *Chlorella*).

An important role of pH in the regulation of the activity of the proton pumps is obviously suggested by their capacity to influence H^+ concentration in the free space as well as in the cytoplasm. In fact, an increase in acidity of the medium is known to induce a marked decrease in proton secretion (see, for example, Lado *et al.*, 1976). In maize roots this decrease is not associated with marked changes in PD (Cocucci *et al.*, 1976), and appears to be due to

an effect on the pump activity rather than the passive reflux of H^+ into the cell.

An effect of intracellular pH in controlling the activity of the plasmalemma proton pump of higher plants is indicated by recent experiments in which acidification of maize root cytoplasm by treatment with permeant weak acids has been shown to induce an increase in the active component of PD and K^+ uptake (in maize roots; Marrè *et al.*, 1983, and Romani *et al.*, 1983a; in wheat roots: Bellando, unpublished data; see also Sanders *et al.*, 1981; and Tromballa, 1978, for similar results in lower plants). These results should be considered in relation to the finding that the activity of the vanadate-sensitive ATPase presumably involved in the plasmalemma pump is markedly stimulated by an increase in acidity in the pH range 6.5–7.5.

Besides the exogenous toxin FC, various natural plant hormones appear able to regulate the activity of the plasmalemma proton pump. Thus, electrogenic H^+ extrusion is stimulated in stems and coleoptile segments by natural and synthetic auxins (Marrè *et al.*, 1973a, b, 1974; Cleland, 1973; Cleland *et al.*, 1977), in stems and in roots by the pollen hormone brassinolide (Cerana *et al.*, 1983; Romani *et al.*, 1983b), and in germinating seeds by gibberellin A_3 (Lado *et al.*, 1975; Cocucci *et al.*, 1981), whereas it is inhibited in roots by auxin, at concentrations which are stimulatory in stems (Pilet *et al.*, 1979; Moloney *et al.*, 1981), and in germinating seeds and stomatal guard cells by abscisic acid (Ballarin-Denti and Cocucci, 1979; Van Kirk and Raschke, 1978). In all cases, these effects of the hormones on acid secretion and electrogenesis appear important in mediating at least part of their physiological action on general processes such as growth, germination, transpiration, and gas exchanges through the stomata. On the other hand, although it appears reasonable to assume that hormones influence electrogenic H^+ extrusion by acting on the proton pump, the mechanism of this action is still unknown.

Conclusions

The aforementioned information allows us to make some observations.

1. Proton pumps, namely mechanisms utilizing metabolic energy for the electrogenic extrusion of H^+ against an electrochemical gradient, are operating at the plasmalemma and at the tonoplast of higher plants, where they are responsible for a large fraction of the active component of the transmembrane electrical potential and of the building up of electrochemical proton gradient energy (protonmotive force).

2. The electrogenic extrusion of protons by these pumps appears to be

electrically rather than chemically coupled with the influx of cations (preferentially but not obligatorily, K^+) required for electroneutrality.

3. The evidence *in vivo* together with that obtained from native membrane vesicles and reconstructed proteoliposomes indicate ATP hydrolysis as the energy source for H^+ extrusion for at least two distinct proton pumps, one operating at the plasmalemma and the other at the tonoplast. The characteristics of these two ATP-driven H^+ pumps are strikingly similar to those of the ATP-driven proton pumps operating at the plasmalemma and at the tonoplast of *Neurospora* and yeasts.

4. There are some reports which could be considered to indicate the existence of a redox-driven proton pump at the plasmalemma, in which a transmembrane NADH-oxidizing system could provide the energy for H^+ efflux. The characterization of this interesting system (possibly homologous to the better known NADPH oxidation-driven H^+ pump of erythrocytes; Goldenberg, 1982) is still incomplete.

5. The activity of the plasmalemma proton pump is controlled by PD and by extracellular and intracellular pH. Also, changes in the levels of the plant hormones auxin, gibberellin, and abscisic acid can influence the rate of operation of the pump, and this effect is important in determining the final physiological responses to these hormones.

6. The physiological roles of the H^+ pumps derive from their capacity to: (a) transform metabolic energy into protonmotive force at the plasmalemma and the tonoplast; (b) influence H^+ concentration in the free space, in the cytoplasm, and in the vacuole. The mode of utilization of protonmotive force for the transport of many solutes to or from the cytoplasm and the vacuole, and the control of cytoplasmic and vacuolar pH, are essentially similar in higher plant cells and in yeasts, fungi, and bacteria. In higher plants, however, the multicellular organization and thus the appearance of an intercellular space introduces a new function: that of the regulation of the concentration of H^+ and other solutes in the free space, or apoplast. Ions, nutrients, and hormones are normally present in this compartment, together with various enzymes and the mechanical constituents of the cell wall, the characteristics of which control growth by cell enlargement. Regulation of pH in this compartment is thus an obvious necessity, and the plasmalemma H^+ pump appears to be the main mechanism specifically involved in this role.

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