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The plasma membrane H^+-ATPase of higher plant cells: biochemistry and transport function

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Contents

I. Introduction

The plasma membrane H^+-ATPase has a primary role in the coupling of metabolic energy to solute transport at the plasma membrane of plant cells (Ref. 1 and references therein). This membrane-associated enzyme provides the protein machinery whereby ATP hydrolysis can be coupled to the translocation of H^+ to the cell exterior. Through this activity, the H^+-ATPase functions to produce an inwardly-directed proton electrochemical gradient across the plasma membrane which consists of an acid-exterior pH difference and negative-

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interior membrane electrical potential difference [2,3]. Coupling of the potential energy conserved in this proton electrochemical gradient established by the H^+-ATPase to the uphill movement of other solutes is then proposed to be mediated by other secondary transport systems associated with the plasma membrane, which can act as H^+/solute symports, antiports or electrically-driven uniports and channels [1–4]. This mode of energy coupling to solute transport, where the plasma membrane H^+-ATPase serves as the primary transducer between chemical energy in the form of ATP and the generation of potential energy useful for driving transport, is thought to be central to a number of important physiological processes in plants including nutrient uptake by plant roots from the soil (Ref. 1 and references therein), nutrient allocation on a whole plant basis [5], stomatal movements [6] and cell elongation [7–9].

Although its action was recognized in early studies on plant tissues involving measurements of external acidification [10] or hyperpolarization of the membrane potential [10,11], it was the pioneering studies of Hodges and colleagues (Ref. 12 and references therein) that clearly demonstrated the existence of plasma membrane associated ATPase activity with characteristic properties and promoted the concept that this activity was involved in the energization of ion transport. Later studies involving transport-competent plasma membrane vesicles [13–16] and reconstituted preparations of the enzyme [17–20] confirmed that this enzyme activity initially characterized by Hodges and co-workers [12] did in fact function to couple ATP hydrolysis to H^+ transport. Detailed biochemical characterizations of the plant plasma membrane H^+-ATPase have been carried out with the enzyme either associated with native membrane vesicles [13–16] or in partially purified enzyme preparations following detergent solubilization of the plasma membrane [21–24]. Recent efforts by several groups [25–27] have resulted in the successful cloning of the plasma membrane H^+-ATPase gene and the deduction of the primary amino acid sequence for this protein. The information provided by these studies will greatly facilitate efforts to understand the relationship of the structure of this enzyme to its function in coupling ATP hydrolysis to H^+ translocation.

This review will focus on the biochemical characteristics of the plant plasma membrane H^+-ATPase as related to the mechanism by which ATP hydrolysis is coupled to H^+ translocation across the plasma membrane. This will be addressed primarily from the perspective of enzyme kinetics, reaction mechanism and protein structure. The molecular biology of this enzyme will not be discussed in great detail and readers are advised to consult several recent reviews on this topic [29–32]. Although there is often a tendency in discussions of the plant plasma membrane H^+-ATPase to group together work conducted on fungal as well as higher plant systems, this review will emphasize the latter.

II. Biochemical characteristics and transport function of the plasma membrane H^+-ATPase

II-A. General characteristics of ATP hydrolytic activity associated with plasma membrane fractions

Initial studies on the plasma membrane H^+-ATPase were conducted with plasma membrane fractions produced by cell fractionation of plant tissues such as roots (Ref. 33 and references therein). Although these membrane fractions were enriched for plasma membrane vesicles, contamination by vesicles from other membrane components occurred to some extent [34]. However, it was apparent that enrichment for plasma membrane vesicles was correlated with the presence of ATP hydrolytic activity which demonstrated characteristic properties (i.e., substrate specificity for ATP, pH optimum, inhibitor sensitivity, etc.) that allowed it to be distinguished from other phosphohydrolyases associated with membrane fractions [12,33,35]. These other phosphohydrolyases could include non-specific acid phosphatases (Ref. 33 and references therein) and a Ca^{2+}-dependent ATPase which has recently been shown to be involved in mediating Ca^{2+} transport [36,37]. These characteristics unique to the plasma membrane H^+-ATPase are also observed for activity present in highly purified plasma membrane fractions produced using the aqueous polymer phase partitioning method (Ref. 38 and references therein). This approach has the advantage that plasma membrane fractions can be produced from green tissue such as leaves where thylakoid membrane contamination has precluded the use of sucrose gradients (Ref. 34 for discussion). Although this ATPase activity associated with isolated membrane fractions appeared to correlate with ion transport properties of the tissue from which they were isolated (i.e., level of K^+ stimulation vs. level of K^+ uptake; kinetics of K^+ stimulation of ATPase and kinetics of K^+ uptake, etc.), it was not until the enzyme was studied in transport-competent native vesicles (Ref. 39 for review) or reconstituted preparations (Ref. 29 and references therein) that a transport function for this enzyme activity could be confirmed.

When examined in membrane preparations, ATP hydrolytic activity is magnesium-dependent and further stimulated by KCl [33,40–42]. Although the dependence on magnesium is most likely related to Mg : ATP being the true substrate for the enzyme [43], a specific role for this divalent cation in the reaction mechanism of ATP hydrolysis was shown from subsequent kinetic studies (see subsection III-C). In non-sealed membrane fractions, stimulation by KCl is related to a direct cation effect upon the enzyme and when enzyme activity is

assayed in the presence of different monovalent cations present as chloride salts, the sequence of stimulation: $K^+ > NH_4^+ > Rb^+ > Na^+ > Cs^+ > Li^+$ is often observed [44]. Enzyme activity is optimal at about pH 6.5 when assayed in the presence of magnesium and KCl and displays a strong substrate specificity for ATP over other phosphorylated compounds [1,35,40–42,45]. The enzyme displays simple Michaelis-Menten kinetics for substrate dependence (Mg:ATP) of ATPase activity [12,35,40,42] and a complex, non-Michaelis-Menten type kinetic relationship between potassium concentration and the stimulation of ATPase activity by this monovalent cation [35,40–42,45]. This complex kinetic relationship has been interpreted as representing either a negative cooperative [35,45] or a 'multiphasic' response of the enzyme to increasing potassium concentration (Ref. 46 and references therein). In this latter interpretation, potassium stimulation with increasing potassium concentration is considered as a multiple phase, discontinous function (see Ref. 46 for extensive discussion). Although the significance of this complex kinetic relationship for potassium stimulation of ATPase activity is unknown, a similar complex kinetic relationship for K^+ ($^{86}Rb^+$) uptake has been observed [35,45]. This similarity in the kinetic dependence of these two processes on potassium concentration has been suggested as evidence for direct involvement of the plasma membrane H^+-ATPase in mediating potassium transport [35,45,47]. However, this topic remains controversial (see Ref. 47 for discussion) and further work will need to be done to resolve this question (see subsection II-E).

A number of chemical compounds have proven useful in the study of the plasma membrane H^+-ATPase present in membrane fractions. In terms of inhibitors, the plasma membrane H^+-ATPase is sensitive to *N,N'*-dicyclohexylcarbodiimide (DCCD), sulfhydryl reagents, octylguanidine, diethylstilbestrol (DES) and orthovanadate (Refs. 1, 42 and references therein). Of these inhibitors, orthovanadate has been of particular interest, since its inhibitory action upon the plant plasma membrane H^+-ATPase suggested the involvement of a phosphoenzyme intermediate in the mechanism of ATP hydrolysis [48,49]. This provided the first indication that this enzyme was representative of the E_1E_2 class of transport ATPases (Refs. 48, 49, 50 and references therein). Recent studies on the effects of the iodinated fluorescein derivative erythrosin B [51] and the macrolide antibiotic bafilomycin A_1 [52] have shown that these compounds can be useful in distinguishing the plasma membrane H^+-ATPase from other ATPases potentially present in membrane fractions. In the case of erythrosin B, the plasma membrane H^+-ATPase shows an intermediate sensitivity; being fully inhibited in presence of 10 to 50 μM [51]. In contrast, the plasma membrane Ca^{2+}-ATPase is much more sensitive to this compound (full inhibition at 10 nM, see Ref. 53), while the tonoplast ATPase is less sensitive, requiring erythrosin B concentrations exceeding 100 μM [51]. As with erythrosin B, the plasma membrane H^+-ATPase shows an intermediate sensitivity to bafilomycin A_1 while the tonoplast H^+-ATPase shows high sensitivity and the mitochondrial ATPase is fully insensitive [52].

TABLE I

Selected procedures for purification of the higher plant plasma membrane H^+-ATPase

Species	Purification summary	Specific activity [a]	Fold-purification [b]	Ref.
Oats	Extract with Triton X-100 + KCl. Solubilize with lysophosphatidylcholine. Resolve on linear glycerol gradient	6.0	5.0	21
Tomato	Extract with Triton X-100 + KBr, octyl glucoside + deoxycholate. Solubilize with lysophosphatidylcholine. Resolve on linear glycerol gradient	17.6	18.7	22
Maize	Extract with Triton X-100. Solubilize with lysophosphatidylcholine. Resolve on linear glycerol gradient	4.65	12.6	24
Mung bean	Extract with Deoxycholate. Solubilize with Deoxycholate + Zwittergent 3-14. Resolve on linear glycerol gradient	6.23	10.74	55

[a] Specific activity expressed as μmol/min per mg protein

[b] Fold-purification is expressed relative to the activity associated with the initial membrane fraction prior to detergent treatment.

II-B. Purification of the plasma membrane H^+-ATPase

A prerequisite for any detailed biochemical studies on the plant plasma membrane H^+-ATPase is the availability of purified preparations of the enzyme. The plasma membrane H^+-ATPase has been purified from a limited number of plant tissues using procedures that have generally involved detergent extraction of the plasma membrane to remove proteins other than the ATPase, detergent solubilization of the ATPase and then resolution of the solubilized protein. A survey of procedures used in the purification of the higher plant plasma membrane H^+-ATPase is presented in Table I. Enrichment of ATPase activity over that present in the initial membrane fraction has ranged from about 5- to 18-fold. However, some care must be taken in assuming a direct relation between fold-enrichment of enzyme activity and actual quantitative purification of the en-

zyme, since all procedures require detergent treatment and there is evidence that detergents may directly activate the enzyme [23,54]. Procedures yielding the highest degree of purification have involved several extraction steps prior to ATPase solubilization, leading to a highly enriched insoluble preparation for the enzyme prior to solubilization. In terms of detergents that have been used for H^+-ATPase solubilization, the zwitterionic detergents zwittergent 3-14 [19,23,55] and lysophosphatidylcholine [22,24,56] have proven quite useful. In addition, the non-ionic detergent octyl glucoside [57,58] and the ionic detergent sodium deoxycholate [17,18] have been reported to solubilize the plant plasma membrane H^+-ATPase. Although the best resolution of the H^+-ATPase from other proteins has been obtained following centrifugation of the solubilized enzyme on linear glycerol gradients [22,24,55], this procedure suffers from the small amounts of enzyme preparation which can be produced and the long time required for the procedure to be conducted (about 18 h). Clearly, there is still a great need to develop a rapid and convenient procedure for the preparation of purified enzyme in bulk quantities.

II-C. Structure of the plant plasma membrane H^+-ATPase

II-C.1. Catalytic subunit structure

A consistent observation during purification of plasma membrane H^+-ATPase is enrichment of a 100 kDa peptide on dodecyl sulfate polyacrylamide gels [19,20,22,24,59,60]. That this peptide represents the catalytic subunit of the plant plasma membrane H^+-ATPase was shown by its catalytic phosphorylation by [γ-^{32}P]ATP during the course of ATP hydrolysis [60–63]. This formation of a phosphoenzyme intermediate on a 100 kDa peptide during ATP hydrolysis is again characteristic of E_1E_2-ATPases [50] and similar to what is observed for the Na^+/K^+-ATPase of animal cells [64–66], the Ca^{2+}-ATPase of the sarcoplasmic reticulum [66], the gastric mucosal H^+/K^+-ATPase [67] and the fungal plasma membrane H^+-ATPase [68,69]. Confirmation of a 100 kDa peptide as the catalytic subunit has also come from covalent labeling of the enzyme with [^{3}H]DCCD, where inhibition of the ATPase by this compound correlated with the degree of radiolabeling [70].

Since the gene encoding the plant plasma membrane H^+-ATPase [25–27] as well as those for other well-characterized E_1E_2 transport ATPases (Ref. 71 and references therein) have been cloned and sequenced and the amino acid sequences have been deduced, it has become possible to make comparisons between the primary sequences of these enzymes. As discussed by Serrano [28,29], although overall homology between these proteins is not high (about 25%) a strong homology is

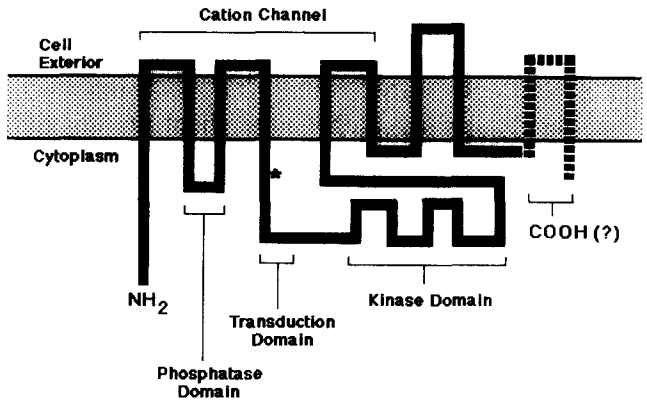

Fig. 1. Model for the proposed transmembrane arrangement of the plant plasma membrane H^+-ATPase catalytic subunit peptide. Functional domains conserved between different E_1E_2-type transport ATPases are indicated. The asterisk indicates the approximate location of the aspartic acid residue phosphorylated during the catalytic cycle of the ATPase. Uncertainty regarding a cytoplasmic or extracellular location for the carboxy terminus is indicated by the dashed segment of the peptide.

observed within specific conserved regions of these proteins thought to be commonly involved in ATP binding and phosphoenzyme intermediate formation and breakdown [71]. This was also indicated from studies of Schaller and Sussman [72] where several peptides produced by tryptic digestion of the oat root plasma membrane H^+-ATPase were found to be homologous to known sequences from other E_1E_2-type transport ATPases.

From what has commonly been observed for these enzymes in terms of conserved protein domains [64,71] and predictions with regard to transmembrane polypeptide regions for the higher plant and fungal H^+-ATPases [25–31], it has become possible to develop a generalized model for the transmembrane arrangement of the 100 kDa catalytic subunit. A model such as that proposed by Serrano [29] for plasma membrane H^+-ATPase is presented in Fig. 1.

As with other E_1E_2-type transport ATPases, the peptide of the plant plasma membrane H^+-ATPase catalytic subunit is thought to traverse the membrane several times. Hydropathy analyses of the amino acid sequences deduced from gene sequences have suggested at least eight transmembrane peptide segments [25–28]. However, there is some uncertainty about the transmembrane arrangement for the C-terminus of the protein (see Ref. 31 for discussion), with some workers suggesting either a single additional transmembrane segment leading to an extracellular C-terminal (i.e., Ref. 29) or two additional transmembrane segments forming a loop with a cytoplasmic C-terminal (i.e., Ref. 27). For the *Neurospora* plasma membrane H^+-ATPase, a cytoplasmic location for both the N- and C-terminal ends of the catalytic peptide has been supported by studies involving the binding of specific antibodies to N- and

C-terminal peptides [73] and limited proteolysis [74,75].

This 'lacing' of the catalytic peptide across the membrane delineates several common domains thought to have a central role in the mechanism of E_1E_2-type ATPases. These include a 'transduction' domain which contains the active site aspartic acid residue phosphorylated during the catalytic cycle, together with a few common adjacent amino acid moieties. There is also a separate 'phosphatase' domain which appears to be involved in the reactions associated with dephosphorylation. A 'kinase' domain represents the region of the catalytic subunit thought to be involved with ATP binding and formation of the phosphoenzyme. Finally, a series of transmembrane peptides may form a 'cation' channel possibly involved in H^+ translocation across the membrane (see Ref. 29 for discussion).

II-C.2. Oligomeric structure in the native membrane

In attempting to understand the native structure of the plant plasma membrane H^+-ATPase, radiation inactivation analysis has proven to be a particularly useful approach. This method involves irradiating enzyme samples with high energy radiation such as accelerated electrons, X-rays or γ-rays and then analyzing the decrease in enzyme activity with increasing radiation dose in terms of target theory (Refs. 76, 77 for reviews). From this analysis, it has been shown that the decline of enzyme activity with increasing radiation dose occurs exponentially in a manner related to the 'target molecular size' of the enzyme [76,77]. For a number of enzymes which have been investigated, this target molecular size has been shown to be a close estimate of the enzyme native molecular weight (Refs. 77 and references therein). With membrane-associated enzymes, this target molecular size has corresponded to the protein component regardless of the quantity of phospholipids tightly bound to the enzyme (Refs. 76 for discussion).

When plant plasma membrane preparations from red beet storage tissue [78] and radish seedlings [79] have been examined with this approach, target analysis has indicated a molecular size of 228 and 220 kDa, respectively. As the catalytic subunit is known to have a molecular weight of about 100 kDa, these results would suggest that the enzyme exists as a dimer in the native membrane. Similar results for a dimeric arrangement of catalytic subunits in the native membrane have also been obtained using radiation analysis for the *Neurospora* plasma membrane H^+-ATPase [80].

An interesting recent observation from radiation inactivation analysis is that the quaternary structure of the H^+-ATPase might change during detergent solubilization of the enzyme from the membrane. When the red beet plasma membrane H^+-ATPase was solubilized from the native membrane using the zwitterionic detergent, Zwittergent 3-14, radiation inactivation analysis revealed a decrease in the target molecular size from 225 to 129 kDa [81]. Since this latter target size is close to the molecular weight of an individual catalytic subunit and the solubilized H^+-ATPase displays full catalytic competency [23], this would suggest that a single catalytic subunit represents the minimal unit of ATP hydrolysis. This result is also significant because it implies that solubilization and purification of the plasma membrane H^+-ATPase may result in an enzyme preparation that exists in a much different overall structural state than that associated with the native membrane. It is also possible that detergent treatments could result in a greater aggregation of catalytic subunits than that associated with the native plasma membrane. This could explain the trimeric arrangement of catalytic subunits found for purified plasma membrane H^+-ATPase of tomato roots by glutaraldehyde cross-linking analysis [22] and the heximeric arrangement for a purified preparation of the *Neurospora* plasma membrane H^+-ATPase [82].

Although the single 100 kDa catalytic subunit may represent the minimal unit of ATP hydrolysis, it remains uncertain as to whether an oligomeric arrangement might be required for coupling of ATP hydrolysis to H^+ transport or any regulatory properties of the enzyme.

II-C.3. Essential amino acids required for activity

With the amino acid sequence of the plant plasma membrane H^+-ATPase now available from molecular studies, it becomes important to understand the relationship of moieties within this sequence to the overall process of ATP hydrolysis and its coupling to H^+ transport. From early studies involving [^{3}H]borohydride reduction and acid digestion of the plant ATPase phosphorylated intermediate it was shown that the acyl phosphate bond involved an essential aspartic acid moiety in the enzyme active site [83]. When limit peptides of phosphorylated intermediates of corn root plasma membrane ATPase, gastric H^+/K^+-ATPase and hog kidney Na^+/K^+-ATPase were produced and compared, the common amino acid sequence: -Cys-(Ser/Thr)-Asp(^{32}P)-Lys- was observed for all three preparations [84]. To date, this sequence around the site of phosphorylation is absolutely conserved in all E_1E_2-ATPases examined [28,29,71].

An alternative approach to this problem has been the use of methodology involving chemical modification of ATPase preparations with amino acid specific reagents (see Ref. 85 for review of this approach). Using the arginine modifying reagent 2,3-butanedione, Kasamo [86] demonstrated that an essential arginine moiety was involved in ATP hydrolysis carried out by the mung bean H^+-ATPase and an active site location for this moiety was proposed based upon protection against inhibition by treatment with the enzyme substrate. These results were also found for the plasma membrane H^+-

ATPase of red beet using both 2,3-butanedione and phenylglyoxal [87]. Kinetic studies on the mechanism of the red beet plasma membrane H^+-ATPase suggested the involvement of a histidine moiety in the mechanism of the enzyme [88] and this was confirmed using the histidine-specific reagent diethylpyrocarbonate [89]. Recent studies on the *Neurospora* plasma membrane H^+-ATPase using diethylpyrocarbonate have also shown the presence of an essential histidine moiety [90].

Preliminary studies have also suggested the involvement of an essential lysine residue, since ATPase activity was sensitive to fluorescein isothiocyanate [91] similar to what has been observed for the animal Na^+/K^+-ATPase [65,66], the Ca^{2+}-ATPase of the sarcoplasmic reticulum (Ref. 66 and references therein) and the gastric mucosal, H^+/K^+-ATPase [67]. Furthermore, the observation of inhibition of activity by sulfhydryl reagents (Ref. 42 and references therein) would suggest the involvement of essential cysteine groups in activity. This was further supported in studies by Katz and Sussman [92] where *N*-ethylmaleimide inhibition of ATPase activity was observed that was partially protectable by either ADP or Mg : ADP. When used in radioactive form, this reagent specifically labeled the 100 kDa catalytic subunit of the enzyme in a manner consistent with its effect upon catalytic activity.

II-D. Demonstration of H^+ transport coupled to ATP hydrolysis

II-D.1. Studies with reconstituted enzyme preparations

Although biochemical studies with plasma membrane fractions had suggested that plasma membrane associated ATP hydrolytic activity most likely represented an enzyme involved in mediating H^+ transport, this could not be definitively shown until ATP-driven transport could be demonstrated in vitro with this enzyme. As the plasma membrane fractions initially used for biochemical characterization of the enzyme contained predominately leaky vesicles (Refs. 3, 39, 93 and references therein), this was first shown by reconstitution of the solubilized enzyme into artificial liposomes. Reconstitution of the plasma membrane H^+-ATPase with transport capacity has been achieved with solubilized enzyme preparations from red beet [17,18,19], oats [20], tomato [22], radish [94] and mung bean [55]. For each of these preparations, the enzyme was solubilized from the membrane using detergents such as lysolecithin or Zwittergent 3-14, resolved to varying degrees from other membrane components by centrifugation and then reconstituted using either a freeze/thaw sonication technique [20,22,55] cholate dialysis [94] or column chromatography [17–19]. A recent alternative reconstitution approach to the problem of leaky plasma membrane preparations is that used by Brauer et al. [95] for maize root plasma membrane fractions. In their approach, detergent and lipids were added to plasma membrane fractions and then the detergent was subsequently removed. This resulted in the production of a sealed proteoliposome vesicle without prior resolution of the ATPase from other membrane associated components. Such a system could prove useful for studies on the plasma membrane H^+-ATPase in the presence of other native membrane protein components in maize root vesicles, since transport stability has tended to pose a problem for the use of native vesicles from this species (see Ref. 95 for discussion).

With reconstituted preparations produced as described above, ATP-dependent acidification of the interior of the liposome can be demonstrated using probes responding to an acid-interior ΔpH [17–22,55,94]. This proton transport was inhibited by orthovanadate [17–22], consistent with the involvement of an E_1E_2-type transport enzyme and demonstrated a number of properties similar to those shown for plasma membrane-associated ATP hydrolytic activity [17,18,22,95,96]. Although the plasma membrane H^+-ATPase is directly stimulated by cations, anions such as nitrate can also have a stimulatory effect upon proton transport activity through a reduction in the membrane potential [17–19,96].

II-D.2. Studies with transport-competent plasma membrane vesicles

Although reconstitution of the enzyme proved important for demonstrating the role of plasma membrane ATPase activity in driving H^+ transport, the ability to have functional transport capability in isolated membrane vesicles becomes important for studying the function of the enzyme in its native lipid and protein environment. This could prove particularly important for studies related to regulation of the enzyme or the coupling of its H^+ transport action to the movement of other solutes. Although plasma membrane vesicles isolated from many tissues according to methods developed by Hodges and co-workers were leaky, so that transport gradients could not be observed [33,93], minor modifications to these procedures for cell fractionation yielded transport-competent membrane vesicles (see Ref. 39 for review). These modifications included addition of additional 'protectant' chemicals to the medium used for homogenizing plant tissue and treatment with elevated levels of KI either during homogenization [13] or during the washing of the microsomal fraction [15]. In the former type of treatment with KI, this salt appeared to promote a selective sealing of the plasma membrane relative to other membrane vesicle types [13] while in the latter use of this salt, enrichment of transport-competent plasma membrane vesicles may reflect a selective inhibition of the tonoplast ATPase and hence a reduction in ATP-driven transport from this contaminating vesicle type present in the microsomal mem-

brane fraction used in transport studies [15]. In terms of protectant compounds found to enhance the recovery of transport-competent plasma membrane vesicles, proteinase inhibitors have often been found to be important [13,15,39]. This would suggest that earlier difficulties associated with obtaining transport-competent vesicles might have resulted from proteolysis of membrane protein causing a higher H^+ conductance of the vesicles. However, lipid degradation has also been suggested as a problem for membrane vesicles isolated from maize roots [95]. It has also been found that the choice of plant tissue may have a strong bearing on the ability to isolate sealed plasma membrane vesicles. For example, transport-competent plasma membrane vesicles can be readily isolated from young radish seedlings using a relatively conventional approach to cell fractionation [16].

As with the enzyme present in reconstituted liposomes, plasma membrane H^+-ATPase associated with native membrane vesicles demonstrates ATP-dependent H^+ translocation which can be measured using probes sensitive to an acid-interior ΔpH (Ref. 39 and references therein). Transport is inhibited by low concentrations of orthovanadate [13–15] and demonstrates properties similar to those observed for ATP hydrolytic activity with respect to pH optimum, substrate specificity for ATP, dependence on Mg:ATP concentration and sensitivity to phosphohydrolase inhibitors [13–16]. Proton transport driven by the plasma membrane H^+-ATPase in vesicles is electrogenic where the membrane potential can be measured using optical probes such as oxonol V [14]. Modulation of the membrane potential by inclusion of various anions during the measurement of ATP-driven proton transport results in corresponding compensatory changes in the measured ΔpH [14].

II-D.3. H^+/ATP stoichiometry

The ability to measure ATP-dependent H^+ transport mediated by the plasma membrane H^+-ATPase in reconstituted and native vesicle systems has allowed measurements of the stoichiometry of H^+ transport relative to ATP hydrolysis (H^+/ATP stoichiometry). Using reconstituted maize membrane vesicles and a mathematical analysis of proton transport, Brauer et al. [97] estimated a H^+/ATP stoichiometry of about 0.8. Based upon the use of three independent ways to estimate H^+ flux, Briskin and Niesman-Reynolds have found an H^+/ATP stoichiometry of about 1 for ATP-driven H^+ transport in native plasma membrane vesicles from red beet storage tissue (unpublished data). Taken together, these results would suggest that the plant plasma membrane H^+-ATPase pumps 1 H^+ per ATP hydrolyzed during its catalytic/transport cycle, similar to what has been observed for the *Neurospora* H^+-ATPase associated with a reconstituted vesicle system [99]. Assuming a membrane electrical potential of about -120 mV [100], it can be shown from thermodynamic analysis that a stoichiometry of either 1 or 2 H^+ transported per ATP hydrolyzed could account for the approx. 2 unit pH gradient often observed across the plasma membrane in vivo. However, a H^+/ATP stoichiometry of 1 would be more consistent with the steep pH gradients (ΔpH > 3 units) produced in maize roots in response to treatment with fusicoccin (see Ref. 100 and subsection II-G).

II-E. Role in K^+ transport?

In early studies on the plasma membrane H^+-ATPase in higher plants, it was proposed that the enzyme might be directly involved in mediating K^+ uptake as well as H^+ efflux [1,12,41,42]. Thus, it was envisioned that the plant ATPase could be similar to the animal cell Na^+/K^+-ATPase except that H^+ would be substituted for Na^+ in the operation of the pump. Several observations were often cited as supporting a direct role in K^+ ion uptake. In a comparative study involving barley, oats, maize and wheat, the relative level of K^+ stimulation of ATPase activity in membrane fractions correlated with the relative rates of K^+ ($^{86}Rb^+$) uptake into roots [101]. For both oats [45] and maize [35], the complex kinetics of K^+ stimulation of ATPase activity in plasma membrane fractions correlated with the complex kinetics of K^+($^{86}Rb^+$) uptake into root tissue. In addition, the relative order for monovalent cation stimulation of ATPase activity ($K^+ > NH_4^+ > Rb^+ > Na^+ > Cs^+ > Li^+$) often correlated with the relative level of uptake for these cations (Refs. 44, 41 and references therein). By analogy to other ATPases where a K^+ transport function is reflected in a stimulation of enzyme activity [65–67], these results were taken to suggest some relationship between K^+ stimulation of ATPase activity and K^+ transport for the higher plant enzyme.

However, an immediate criticism has been that the overall stimulatory effect of K^+ is relatively minor when compared to other ATPases that transport this cation directly [28,102]. Potassium stimulation is generally about 1-fold [23,40,47] as compared to the several-fold stimulation of ATPase activity as observed for enzymes such as the Na^+/K^+-ATPase (Refs. 65, 66 and references therein). In some cases K^+ stimulation may be absent [103] or much less than 1-fold in partially purified enzyme preparations (Refs. 56, 102 and references therein). Furthermore, in reconstituted oat ATPase preparations, Vara and Serrano [20] demonstrated that H^+ transport can occur in the absence of K^+, and K^+ stimulation of the enzyme occurred immediately, representing an effect occurring on the outer surface of the proteoliposome. As this would correspond to the cytoplasmic side of the enzyme in vivo, this has raised further doubt as to a transport relation associated with K^+ stimulation of ATPase.

An additional argument against a role of the plasma membrane H^+-ATPase in mediating K^+ influx has come from patch clamp studies with *Vicia faba* guard cell protoplasts [104]. When illuminated with red light or treated with fusicoccin in the dark, stimulation of an outward current could be detected under voltage-clamped conditions. That this outward current was blocked in the presence of a protonophore (CCCP) or orthovanadate would be consistent with it representing electrogenic H^+ extrusion mediated by the plasma membrane H^+-ATPase. However if this is assumed, the observation of no current flow in the presence of (CCCP) would strongly argue against the involvement of the H^+-ATPase in directly mediating K^+ influx. If the ATPase were to act as an obligate H^+/K^+ exchange pump, a net inward current should result in the presence of a protonophore (Ref. 104 for discussion).

At present, most workers now consider the plasma membrane ATPase to act as a primary H^+ transporting enzyme and attention has been focused towards $\Delta\mu_{H^+}$-linked secondary transport mechanisms such as H^+/K^+ symports or K^+ uniports as being involved in mediating K^+ uptake into plant cells [102,105,106]. Recent studies by Kochian and Lucas [106] using H^+- and K^+-sensitive micro-electrodes have suggested that a high-affinity system for K^+ uptake could be mediated by an H^+/K^+ symport or a separate K^+-ATPase similar to the *E. coli* K^+-ATPase [50]. Isolated plasma membrane vesicles could prove useful as a means to distinguish these two possibilities as a direct ATP-fueled K^+ transport system should mediate K^+ flux even in the presence of a protonophore which would collapse the proton electrochemical gradient. On the other hand, the notion of direct involvement of the plasma membrane H^+-ATPase (as opposed to a separate K^+-ATPase) in mediating K^+ uptake would require examining K^+ transport in a similar manner but using a reconstituted and highly purified preparation of this enzyme.

II-F. Lipid requirements for ATPase activity and transport

Use of detergents to delipidate the plasma membrane H^+-ATPase and the ability to reactivate the enzyme by lipid addition have allowed preliminary investigation into possible lipid requirements for enzyme activity. Serrano et al. [107] delipidated the oat root plasma membrane H^+-ATPase by treatment with cholate and then tested the effect of adding back known phospholipids and lysophospholipids. Lysophosphatidylcholine provided the greatest degree of stimulation of activity from the delipidated enzyme, while lysophosphatidylethanolamine and lysophosphatides with acidic polar heads were less effective. In terms of phospholipids, phosphatidylcholine and phosphatidylethanolamine were more effective than phosphatidylinositol, phosphatidylserine, phosphatidylglycerol or phosphatidic acid in providing reactivation of the delipidated enzyme. The stimulatory effect of lysophosphatidylcholine was also shown for the oat enzyme associated with native lipids. Palmgren and Sommarin [108] found that addition of low levels of lysophosphatidylcholine to oat root plasma membrane vesicles resulted in an increase in ATPase activity and ATP-driven H^+ transport. Taken together, these results would suggest a stimulatory effect of the zwitterionic head-group region of lipids. From selective delipidation of the oat plasma membrane using Triton X-100, Sandstrom and Cleland [109] further demonstrated an important role for sterols in the activity of the plasma membrane H^+-ATPase.

In contrast, Brauer and Tu [110] found that phosphatidylcholine had little capacity to stimulate activity for deoxycholate-treated plasma membrane H^+-ATPase from maize. Instead, maximal stimulation of ATPase activity was observed with phosphatidylserine or phosphatidylglycerol. Stimulation by phosphatidylglycerol was found to be roughly equal to that observed for the addition of a complex extract of soybean phospholipids (asolectin). When the approach of reconstitution into proteoliposomes was used by these authors, only small effects of lipid composition on ATPase activity and H^+ transport were observed. These differences in results between these two approaches tested for the maize enzyme were interpreted in terms of possible effects other than head-group composition influencing the activity of the enzyme. These effects could include the overall geometric shape of the lipids as well as their critical micellar concentration (see Ref. 110 for discussion).

II-G. Effects of fusicoccin on H^+ transport and the plasma membrane H^+-ATPase

Over the past 15 years there has been significant interest in the effects of the fungal toxin, fusicoccin, on transport and the plasma membrane H^+-ATPase in higher plants [79,111]. This compound, isolated from the fungus *Fusicoccum amygdali*, has proven to be a useful tool in demonstrating the role of the H^+-ATPase in driving in vivo H^+ extrusion (Ref. 79 and references therein). Furthermore, it has been of particular interest since its effects either mimic or antagonize those of natural plant hormones [111,112].

When added to a number of plant tissues, fusicoccin causes a strong stimulation of H^+ efflux and hyperpolarization of the electrical potential across the plasma membrane [79,100,111,112]. That the fusicoccin effect on H^+ extrusion in vivo is inhibited by orthovanadate would support a role for the H^+-ATPase in mediating this enhanced H^+ flux [113]. Maximal stimulation of H^+ extrusion by fusicoccin occurs in the presence of high extracellular concentrations of K^+ [100] where the

rate of K^+ influx was stimulated in parallel with H^+ efflux [100,111]. In contrast, the fusicoccin-induced hyperpolarization of the membrane potential does not display a requirement for K^+ (or other cations) and can occur even without electrolytes in the extracellular solution [114]. Since other cations such as Ni^{2+}, Co^{2+}, Mn^{2+}, Zn^{2+} [115] or a lipophilic cation such as tributylbenzylammonium [116] can allow fusicoccin stimulation of H^+ extrusion, this effect is not specific for K^+. Additional studies have shown that the requirement of a cation to observe maximal enhancement of H^+ efflux is based upon the ability of these extracellular solutes to reduce the membrane electrical potential [117].

Taken together, these results suggest that the parallel enhancement of H^+ efflux and K^+ influx does not represent a direct obligatory coupling of these two fluxes (i.e., an H^+/K^+-ATPase). Rather, enhancement of K^+ influx most likely depends upon the hyperpolarization of the membrane electrical potential associated with fusicoccin-enhanced H^+ extrusion (Ref. 100 and references therein). Therefore, work with fusicoccin has further supported a model where K^+ uptake occurs as a secondary transport process (K^+ uniport or H^+/K^+ symport) driven by the H^+-ATPase.

Studies with isolated plasma membrane vesicles and reconstituted liposomes have shown that at least some fusicoccin effects upon the H^+-ATPase can be explained at the level of the plasma membrane. When added to plasma membrane vesicles isolated from radish seedlings, fusicoccin causes a stimulation of ATP hydrolytic activity and ATP-dependent H^+ transport [118,119]. Recent work by De Michelis et al. [120] has shown that native plasma membrane vesicles contain a fusicoccin receptor with a dissociation constant of about 1.7 nM. From the observation that the kinetics of fusicoccin binding to this receptor were similar to the kinetics of fusicoccin stimulation of the H^+-ATPase, it was concluded that fusicoccin activation of the ATPase was related to binding of the toxin to this plasma membrane-associated receptor [120].

This plasma membrane fusicoccin receptor has been identified by photoaffinity labeling with a [^{3}H]azidofusicoccin analog [121] and purified by detergent solubilization followed by affinity chromatography [122]. These studies have suggested that the fusicoccin receptor is a plasma membrane-associated peptide with subunit molecular mass (or masses - see Ref. 122) in the 29 to 34 kDa range. The most conclusive evidence for this receptor in mediating fusicoccin effects upon the plasma membrane H^+-ATPase has come from studies with reconstituted liposomes. Aducci et al. [123] were able to co-reconstitute the solubilized fusicoccin receptor and plasma membrane H^+-ATPase into liposomes and demonstrate fusicoccin stimulation of ATP-dependent H^+ transport.

While most work has been focused on the effects of fusicoccin on the H^+-ATPase, it is also apparent that this compound can exert effects upon other transport systems associated with the plasma membrane. In *Vicia faba* guard cells, Blatt [124] demonstrated an additional effect of this compound upon secondary transport systems based upon current-voltage (*I-V*) analysis. In this approach the plasma membrane was considered in terms of the ensemble of outward H^+-pumps and coupled secondary transport processes where the latter would contribute to the return flow of current. Addition of fusicoccin reduced the return flow of current and it was proposed that this reduction in the electrical leak was based on the selective elimination of some components of the pathway for current return (secondary transport processes). The resultant redistribution of charge flux across the plasma membrane could also contribute to enhanced secondary transport of solutes, hyperpolarization of the membrane potential and enhanced net H^+ extrusion.

III. Mechanism of the plant plasma membrane H^+-ATPase

III-A. Demonstration of a phosphoenzyme intermediate in the catalytic cycle associated with ATP hydrolysis

Similar to other E_1E_2-type ATPases (Ref. 50 and references therein), the higher plant plasma membrane H^+-ATPase has been shown to form a phosphoenzyme intermediate during the course of its catalytic cycle. This finding has proven quite useful for studies on the reaction mechanism of the enzyme and its initial identification on electrophoretic gels [60–63]. The formation of a phosphoenzyme was shown by the presence of a rapidly turning over ^{32}P labeled enzyme intermediate when reactions were conducted using a (γ-^{32}P)-labeled ATP substrate [60–63]. Upon initiation of the phosphorylation reaction, the intermediate was rapidly formed and reached steady-state within seconds even when measured at ice temperature [60,63]. At 12°C, a steady-state level of phosphoenzyme was reached within about 150 ms [88]. If an excess of unlabeled ATP was added to phosphorylation reactions at steady-state, the labeled phosphoenzyme intermediate was rapidly discharged, consistent with its representing the reaction intermediate of an enzyme [60–63] rather than the product of a protein kinase reaction [125]. The phosphoenzyme intermediate demonstrated acid pH stability and sensitivity to hydroxylamine, suggesting the presence of an acyl-phosphate bond [60,61,63]. This was later shown to be associated with an aspartic acid residue associated with the active site of the enzyme [83,84].

When a predicted rate of ATP hydrolysis based upon the level of phosphoenzyme and its rate of breakdown was compared to the rate of ATP hydrolysis measured

under identical conditions, it was apparent that the phosphoenzyme was kinetically competent to represent an intermediate in the ATPase reaction mechanism [63]. When the effect of assay pH was examined on the level of steady-state phosphoenzyme and its turnover, the phosphoenzyme level was maximal at low pH (pH 5.5) while phosphoenzyme turnover was accelerated by high pH [126]. Hence, the measured pH optimum of 6.5 for ATP hydrolysis may represent a compromise balance between these two opposing effects upon the enzyme mechanism

Subsequent studies on catalytic phosphorylation of the plant H^+-ATPase revealed a further similarity to other E_1E_2-ATPases in that the total phosphoenzyme intermediate was found to consist of at least two forms (see Refs. 50, 66 for review). These two forms, designated with analogy to other transport ATPases as E_1P and E_2P could be distinguished by differing sensitivity to ADP and reaction ligands [88,126]. One intermediate form, presumably E_1P, was rapidly discharged by exogenous ADP, while the other form, presumably E_2P, was essentially ADP-insensitive. The presence of these intermediates was revealed during either transient formation of the phosphoenzyme in the presence of exogenous ADP [126], or from effects of exogenous ADP upon the kinetics of phosphoenzyme turnover measured following the formation of steady-state phosphoenzyme [88]. The ADP-insensitive E_2P form also appeared to interact with K^+, as this cation promoted its formation from E_1P and the release of inorganic phosphate from the E_2P form [126]. The concept of an E_2P form that interacts with K^+ is also supported by the observation that K^+ increases sensitivity of the plant plasma membrane ATPase to orthovanadate [48,49] as it is this E_2 form of the enzyme that binds this inhibitor (Ref. 66 and references therein).

With analogy to other E_1E_2-type ATPases (Ref. 66 and references therein), these two phosphoenzyme forms can be regarded as high- (E_1P) and low- (E_2P) energy forms in terms of the types of exchange reaction presumably conducted by each. The discharge of the E_1P form by ADP would be consistent with this form of phosphoenzyme being involving in an ATP/ADP exchange reaction [88,126]. Although not measured as yet, it is presumed that the E_2P form could participate in an H_2O/P_i exchange reaction. Some preliminary support for this latter reaction has come from recent studies where inorganic phosphate effects upon ATP hydrolytic activity associated with the red beet plasma membrane ATPase were investigated in the presence of low amounts of DMSO [127]. Although recent studies on the Na^+/K^+-ATPase have suggested the presence of a third phosphoenzyme intermediate in the mechanism of ATP hydrolysis denoted as E_xP [128], it is uncertain as to whether a similar intermediate exists within the mechanism of the higher plant enzyme.

III-B. Non-catalytic phosphorylation of the ATPase by protein kinase activity

In addition to the transient phosphorylation of the plasma membrane H^+-ATPase associated with the catalytic mechanism of the enzyme, evidence has also been presented for non-catalytic phosphorylation of the ATPase by protein kinase activity in isolated membrane fractions [129,130]. Observing this protein kinase related phosphorylation of the enzyme requires incubation with [γ-^{32}P]ATP over a much longer time (5–15 min) than that generally used to observe the phosphoenzyme reaction intermediates (150 ms to 10 s). In oat root membrane fractions, Schaller and Sussman [129] observed protein kinase-mediated phosphorylation of the plasma membrane H^+-ATPase that occurred at threonine and serine moieties of the protein. Phosphorylation was stimulated by Ca^{2+} and although these authors postulated a regulatory role of this phosphorylation, no effects upon ATPase activity or ATP-driven H^+ transport were shown. Bidwai and Takemoto [130] also demonstrated a protein kinase-mediated phosphorylation of the red beet plasma membrane H^+-ATPase that was promoted by the bacterial phytotoxin syringomycin. This kinase phosphorylation was distinct from the catalytic phosphorylation at an aspartic acid residue as shown by insensitivity of the protein phosphate bond to hydroxylamine. Since these authors had previously shown that syringomycin stimulated ATP hydrolytic activity associated with the red beet plasma membrane H^+-ATPase [131], they proposed that this protein kinase-mediated phosphorylation might be associated with this stimulatory effect. As it is likely that the plant plasma membrane H^+-ATPase may be regulated in vivo by protein kinases in response to hormonal or environmental stimuli (Refs. 28, 29, 59 and references therein), these results may reflect the action of parts of such regulatory systems associated with the isolated membrane fractions used in these in vitro studies

III-C. Role of reaction ligands in the mechanism of the enzyme

As mentioned earlier, a characteristic feature of the plant H^+-ATPase is that ATP hydrolysis is magnesium-dependent and further stimulated by potassium [1,12,41,42,47]. The ability to monitor phosphorylated reaction intermediates during the catalytic cycle of the plant plasma membrane H^+-ATPase has facilitated studies on the reaction mechanism for ATP hydrolysis to determine the roles that these reaction ligands might have. When the phosphorylated intermediate was allowed to reach its steady-state level under normal reaction conditions, and an excess of a magnesium chelator such as EDTA was added, the phosphoenzyme level declined exponentially at a rate comparable to that

observed when the [γ-^{32}P]ATP substrate was diluted with a 100-fold excess of unlabeled ATP [63,132]. This would suggest that magnesium has an important role in phosphoenzyme formation but not phosphoenzyme breakdown. That the phosphoenzyme can be transiently labeled when the enzyme is incubated with free [γ-^{32}P]ATP followed by magnesium addition with a 100-fold excess of unlabeled ATP would suggest that magnesium is involved in the reaction where the terminal phosphate group of ATP is transferred to the enzyme and that it is not needed for nucleotide binding [126,132]. This role for magnesium is also suggested from transient kinetic studies where phosphoenzyme formation was accelerated when reactions were initiated by magnesium addition to enzyme pre-incubated with [γ-^{32}P]ATP as compared to rapid mixing of enzyme with Mg:[γ-^{32}P]ATP [88]. The proposal that free ATP can bind to the plant ATPase without magnesium is also supported by chemical modification studies with arginine modification reagents where ATP or ADP protection against phenylglyoxal or butanedione inhibition occurred without a requirement for magnesium [87]. A similar independence of nucleotide binding with respect to magnesium has also been shown for the *Neurospora* plasma membrane H^+-ATPase [133]. It should be pointed out that under physiological conditions the true substrate for the enzyme is most likely the Mg:ATP complex [1,43] so that these reaction steps of free nucleotide binding and magnesium-dependent phosphoenzyme formation would presumably not be separable as they are in an in vitro kinetic experiment.

Studies to determine the mechanistic basis for K^+ stimulation of the ATPase have proven more complicated, since this cation appears to have multiple effects within the mechanism of the enzyme. Although initial studies suggested that K^+-stimulation of ATPase activity might be accounted for through a stimulation of phosphoenzyme turnover [60,62,63], subsequent work indicated an additional effect in accelerating the transition between the E_1P and E_2P phosphoenzyme forms [88]. While the stimulation of phosphoenzyme turnover by K^+ is similar to what has been observed for ATPases directly involved in K^+ transport (Ref. 66 and references therein), it is this latter effect on the E_1P to E_2P transition that predominates [88]. This effect on the E_1P to E_2P transition may represent a scalar effector role for this cation occurring on the cytoplasmic side of the plasma membrane. Alternatively, K^+ stimulation may simply reflect a more optimal condition for the functioning of the enzyme, since under in vivo conditions the cytoplasmic side of the enzyme would be in the presence of about 50 to 150 mM K^+ [5]. Again, this question of direct involvement in K^+ transport needs to be examined using a highly purified and reconstituted preparation of the H^+-ATPase.

III-D. Suggestions of different conformational states

Current concepts of the mechanism of energy coupling to ion transport by E_1E_2-type ATPases have emphasized the importance of different enzyme conformations within the reaction cycle of ATP hydrolysis and transport [50,134,135]. These proposed mechanisms involve two basic forms of the enzyme, E_1 and E_2, with their corresponding phosphorylated versions, E_1P and E_2P [50,65,66]. In terms of ion binding with a plasma membrane-associated enzyme involved in efflux, the E_1 form would have access to cytoplasm while the E_2 form would have access to the cell exterior. Thus, within the cycle of conformational changes driven by ATP hydrolysis, alternating access of ion binding sites to the cytoplasm or cell exterior would occur and effect vectorial ion transport (see Refs. 134, 135 for discussion).

At present there are only preliminary indications of different conformational states associated with the higher plant plasma membrane H^+-ATPase. The two phosphoenzyme forms appear to be present based upon kinetic studies involving ADP sensitivity [88,126] and it has been suggested that these forms differ by a conformational change rather than a transfer of the phosphate group from one amino acid moiety to another [136]. Further evidence of different conformational forms of the ATPase comes from studies involving chemical modification where it was found that Mg^{2+} or K^+ alone could have protective effects against inhibition by arginine-modifying reagents [87]. As it is difficult to envision these ions as being large enough to physically block an amino acid for derivatization by these reagents, a more reasonable suggestion would be that they promote a conformational change whereby the amino acid is made less accessible to the modification reagent.

III-E. Reaction mechanism for ATP hydrolysis and coupling to H^+ transport

From the information currently available and what is known regarding the mechanism of other E_1E_2-type ATPases it is possible to develop a preliminary reaction scheme for the mechanism of ATP hydrolysis as mediated by the plant plasma membrane H^+-ATPase. This is presented in Fig. 2. This mechanism includes only those steps which would be allowed for coupling of ATP hydrolysis to H^+ efflux and steps leading to uncoupling (i.e., direct hydrolysis of E_1P; $E_1 \cdot H^+ \rightarrow E_2 \cdot H^+$) are not included. In addition, the possible direct transport of K^+ by the enzyme is not considered, as there are insufficient data at present to support this concept.

Based upon the observation that phosphoenzyme formation is accelerated at low pH, it is proposed that H^+ binding might be a prerequisite for formation of the E_1 phosphorylated enzyme form, E_1P. Although it has been suggested that proton translocation mediated by trans-

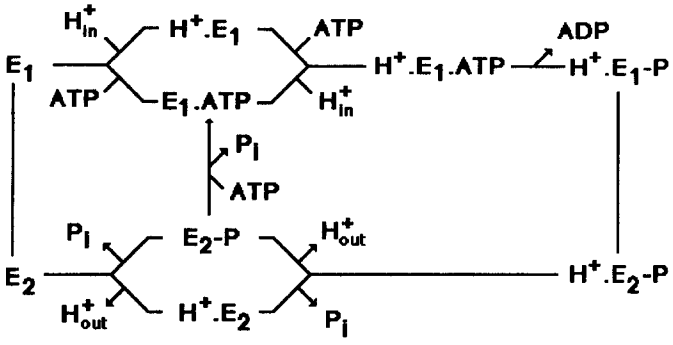

Fig. 2. Proposed reaction scheme for the plant plasma membrane H^+-ATPase. See text for details.

port proteins might occur via 'proton-wire' structures [137], the observations by Blostein and co-workers [138–140] that H^+ can substitute for Na^+ with the Na^+/K^+-ATPase at low pH and that Na^+ can substitute for H^+ with the gastric H^+/K^+-ATPase at high pH would suggest binding of H^+ as possibly H_3O^+ at a discrete ion binding site. As it is uncertain as to whether H_3O^+ binding is also prerequisite for ATP binding or vice versa, these steps are indicated in terms of a random mechanism. Hence, following a random binding of H^+ and ATP to the enzyme, the ternary complex, $H^+ \cdot E_1 \cdot ATP$, is formed which can undergo phosphorylation to produce E_1P with the release of ADP. Although the E_1P phosphoenzyme form can interact with ADP in an exchange reaction, it cannot be hydrolyzed by H_2O, as this can occur only following transition to the E_2P form.

Transition to the E_2P form of the phosphoenzyme would change access of H^+ bound from the cytoplasm to the cell exterior and the production of this form of the enzyme would be followed by steps of H^+ release to the cell exterior and dephosphorylation of the enzyme. Again, since it is uncertain as to whether E_2P hydrolysis leads to H^+ release or if H^+ release is required for E_2P hydrolysis, these steps are indicated in terms of a random mechanism. It is assumed that the E_2 form would also have a lower affinity for H_3O^+ and thus release H^+ to the cell exterior. These changes in affinity for H_3O^+ between the E_1 and E_2 forms would be required to effect net H^+ translocation from the cytoplasm with pH near neutrality to an acid pH cell exterior. In addition, these differences in binding affinity between the E_1 and E_2 forms would be necessary for a catalytic cycle to function without strong unidirectional equilibria in one isolated portion of the mechanism leading to a build-up of one intermediate state or another [134]. As a final step, the E_2 form would revert back to the E_1 form to initiate another catalytic/transport cycle. Alternatively, it is also possible that this final transition might be accelerated by ATP binding to the E_2 form to drive the E_2 to E_1 transition. It should be noted that this mechanism is preliminary and speculative so that further biochemical studies will be required in order to confirm these proposed reaction steps. A deeper understanding of this reaction mechanism and its relation to H^+ translocation could also be found by an application of the biophysical and mathematical modelling approaches utilized by Slayman and co-workers [141,142]. When applied to the *Neurospora* plasma membrane H^+-ATPase, important information regarding rate-limiting steps of the reaction/transport cycle, H^+ binding/release steps and voltage-dependent reaction steps has been found (Ref. 143 for discussion)

IV. Summary and perspective

Substantial progress has been made towards understanding the structure and mechanism of the higher plant plasma membrane H^+-ATPase since early studies conducted with membrane fractions over 20 years ago. Biochemical approaches have provided much insight into the transport function of this enzyme and its reaction mechanism of ATP hydrolysis. In addition, it has become possible to begin to assess the role of lipid interaction with the enzyme, the biochemical basis for modulation by chemicals such as fusicoccin and essential amino acid moieties required for enzyme function. The use of molecular approaches has provided important information regarding the primary amino acid sequence of the catalytic subunit and its relationship to other cation transport ATPases. Through sequence comparisons between ATPases it has also become possible to see an evolutionary relationship between E_1E_2-type ATPases (Refs. 28, 29 and references therein) and other H^+-transporting enzymes, in general [144]. An interesting recent finding from molecular studies on the higher plant plasma membrane H^+-ATPase is that the enzyme may exist in several isoforms encoded by multiple genes [27,31,32]. Further studies will be required to determine whether these isoforms represent H^+-ATPases with unique characteristics and whether they are differentially expressed in different cells of the plant (Ref. 31 for discussion).

Clearly, biochemical and molecular approaches will complement each other in attempts to understand the functional relationship between protein domains of the enzyme and the coupling of ATP hydrolysis to H^+ translocation. In conjunction with physiological studies, these approaches may provide future insight into the regulation of this enzyme within the context of plant growth and development.

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