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The tonoplast proton–translocating ATPase of higher plants as a third class of proton–pumps

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Summary – Taken together, all the data reported recently in the literature suggest that tonoplast ATPase belongs to a new class of proton pumps.

To date, the most studied system is the proton–pumping ATPase from the tonoplast of *Hevea* latex. Its main characteristics are presented. It resembles the mitochondrial ATPase in its specificity, its substrate affinity, and its sensitivity to different inhibitors. However, for some aspects, it resembles the plasma membrane system in its response to other inhibitors tested (quercetin for example). It differs from both ATPases in its sensitivity to nitrate as well as by its molecular structure, *i.e.* a complex exhibiting a least 4 or 5 polypeptides.

These results favor the existence of a third class of proton pumps, intermediate between the F_1F_0-class and the E_1E_2-class.

tonoplast / lutoidal membrane / proton pump / subunits

Résumé – L'ATPase tonoplastique des plantes supérieures appartient à une troisième classe de pompe-à-proton. *Le système le mieux décrit actuellement est l'ATPase tonoplastique du latex* d'Hevea. *Son étude ne se heurte pas aux difficultés rencontrées chez les autres végétaux: leur faible quantité, leur contamination par d'autres structures membranaires et une dénaturation non contrôlée à l'origine de nombreux résultats difficiles à interpréter.*

Les propriétés de cette activité pompe-à-proton sont évoquées. Elle s'avère très proche de l'activité mitochondriale en considérant une partie de ses propriétés: son affinité pour le substrat, sa spécificité et sa sensibilité à certains inhibiteurs caractéristiques des ATPases du type F_0F_1 (trimethyltin, DCCD). De plus, elle est sensible aux protonophores. Mais, par d'autres aspects, elle se rapproche des ATPases du type E_1E_2.

Dès lors, tout contribue à penser que ce type d'ATPase appartiendrait à une troisième classe de pompes-à-protons, intermédiaire entre les deux classes actuellement décrites. Les rares études faites sur la structure moléculaire de cette activité solubilisée et purifiée le confirment. Par ailleurs, de par leurs propriétés comparables à celles décrites pour l'ATPase tonoplastique, toutes les ATPases situées sur les membranes des compartiments endocellulaires appartiendraient à cette nouvelle classe de pompe-à-protons.

tonoplaste / membrane lutoidique / pompe-à-protons / sous-unités

This review represents part of the seminar given on this topic during the XIth Yamada Conference: *Energy Transduction in ATPases* (27-31 May 1985, Kobe, Japan).

Introduction

The vacuoles of fungi and higher plants accumulate, apparently irreversibly, a large variety of compounds involved in secondary metabolism. These molecules are synthesized in the cytoplasm, they cross the tonoplast often against a transmembrane concentration gradient in an energy-requiring reaction, and they are stored in vacuoles where they are not metabolized. This has been very well described in the latex of *Hevea brasiliensis* (see [1]). This illustrates a major type of regulation in plant cells, involving the compartmentation of solutes and its bioenergetic aspect.

Under the physiological conditions generally reported for the plant cell, the energy needed for solute transport across the tonoplast is provided by an Mg^{2+}-dependent ATPase which is located in this membrane and which translocates H^+ electrogenically (see [1]).

The primary active transport process across the plasmalemma and vacuolar membranes in plant tissues is considered to be the ATP-dependent electrogenic translocation of protons. Recently, two types of electrogenic H^+-pumping ATPases in microsomal (non mitochondrial) membrane vesicles have been distinguished [2–6]. The plasmalemma H^+–pumping ATPase is vanadate-sensitive, whereas the tonoplast H^+–pumping ATPase is vanadate-insensitive (Table I). The tonoplast ATPase is also found to be anion-sensitive, *i.e.*, stimulated by Cl^- and inhibited by NO_3^- [1, 7–11].

By extension of the chemiosmotic hypothesis, the proton electrochemical potential difference generated by proton translocation is thought to provide the requisite motive force for the secondary transport of a wide range of solutes. Such a hypothesis has been clearly verified for the compartmentation of citrate in *Hevea* latex [12, 13]. Consequently, the role of the tonoplast H^+–translocating ATPase must be considered as fundamental under the physiological conditions observed and described in the literature (see [1]).

Recent data on the characterization of this activity and studies on its properties indicate that the tonoplast H^+–pumping ATPase could belong to the F_0F_1 family of ATPases. Nevertheless, it could also be regarded as a species representative of a third type of ATP-driven pumps. This point of view has been discussed recently in light of the developing knowledge concerning the evolution of ion pumps [14]. In this review, we will report all the data, published or not, which support such a hypothesis.

Table I. Some characteristic properties of the different ATPases from plant cells.

Parameter	Tonoplast ATPase	Plasma membrane ATPase (E_1E_2-type)	Mitochondrial ATPase (F_1F_0-type)
K_m (MgATP)	0.1–0.4 mM	0.3–0.4 mM	0.2–0.3 mM
Substrate specificity	ATP > GTP > PP_i > NTP	ATP ⋙ NTP	ATP ≫ NTP
Optimum pH	6.5–8.5	6.5–7.0	8.0–9.0
Anion sensitivity	stimulated $Cl^- > Br^- > I^- > HCO_3^-$	little or no effect	stimulated HCO_3^-
Cation sensitivity	little or no effect	stimulated $K^+ > Rb^+ > Cs^+ > Na^+$	little or no effect
Inhibitors			
vanadate	−	+	−
nitrate	+	−	+
DES	+	+	?
TMT	+	−	+
DCCD	+	+	+
oligomycin	−	−	+
azide	−	−	+
ouabain	−	−	+
M_r (kDa)	200 (or 400)	100–110	400 for F_1 part
Tentative identification of subunits (if any)			
M_r weight	13, 23, 54, 66	none	14, 20, 31, 50, 55
catalytic subunit	66 kDa	100–110	50
DCCD-binding subunit	13 kDa	−	10

shown to modulate stalk cell formation during developmental progression, decreases the intramitochondrial pH without altering cytosolic pH. The effect of weak acids on differentiation was perhaps linked to a quite specific perturbation of the transmitochondrial pH gradient and, in turn, of ion fluxes directly coupled to H^+ gradients. Metabolically produced weak acids (or bases) might be the natural regulators of developmental pathways in *D. discoideum*.

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The different classes of ATP-driven pumps

Among the different families of ATP-driven pumps found in all cell types, there are at least two different classes, as shown in Table II (see [14, 15]).

The F_0F_1–ATPase type

This class is exemplified by the H^+–pump known as the F_0F_1 type, an enzyme capable of synthesizing ATP during oxidative or photosynthetic phosphorylation. Bacteria, mitochondria, and chloro-

Table II. Characteristic features of F_1F_0– and E_1E_2–ATPases.

	F_0F_1	E_1E_2
Ions transported	H^+ only	H^+, Na^+, K^+ or Ca^{2+}
Number of polypeptides:	many	few
peripheral	5 (*E. coli*)	0
integral	3 (*E. coli*)	1, 2 or 3
Covalent intermediates	none	β-aspartyl-phosphate
Physiological poise (direction of the reaction)	ATP synthesis or ATP hydrolysis	ATP hydrolysis
Common cellular distribution:		
prokaryotes	plasma membrane	plasma membrane
eukaryotes	internal membranes	plasma membrane

Table III. Overall structure of F_1F_0–ATPase.

	Subunits	Function	M_r (kDa)			
			E. coli[a]	Thermophilic[b] bacterium PS 3	Beef heart[c] mitochondria	Chloroplast[d]
F_1 (ATPase) part	nucleotide binding subunits	α allosteric	55.4	54.0	53	55.4[++]
		β catalytic	50.2	51.0	50	53.9
	connecting subunits	γ (αβ-connecting)	31.4	30.2	33	37.0
		δ (F_0-αβγ-connecting)	19.6	21.0	17	20.8
		ε (F_1-inhibitory)	14.2	16.3	8	14.9
		[5]according the $\alpha_3\beta_3\gamma\delta\varepsilon$[e]	382	380	367	401
	DCCD-binding protein		8.4–9.5	7.3	6.5	6.0–9.0
F_0 (proton channel)[f]	F_1-binding protein		17.0–19.0	13.5	none	present but not determined exactly
	or OSCP and other proteins:		24.0	?	18.0–24.0	none
	or coupling factor (F_6)		none	?	8.0–9.0	none
	or factor B (F_2)		none	?	13.0	none

[a, c] from [17]; [b] from [18]; [d] from [17] for F_1 part: [++] for tobacco CF_1; other values for spinach CF_1; [e] the molecular weight assigned to F_1 subunits (α–ε) are those reported in [17] recent data consider $\alpha_3\beta_3\gamma\delta\varepsilon$ as the most reasonable estimate of stoichiometry; [f] compilation of data from [19] and [20].

plasts have very nearly the same molecular form of F_0F_1–ATPase. Recent information concerning their amino acid sequences is consistent with the enzyme being derived from a common ancestral species [16]. Tables I, II and III summarize the structure and the chemistry of the different ATPases belonging to this type.

A characteristic feature of the F_0F_1-type ATPase is its extraordinary complexity. The enzyme of *Escherichia coli* plasma membrane, for example, consists of eight different proteins and approximately 18 peptide chains (Table III). The mitochondrial enzyme is even more complex. In contrast, the ATPase of animal (or plant) plasma membranes contains only two proteins (Tables I and II).

The overall organization of this ATPase is as follows: the enzyme is built from two major functional units, distinguishable by their solubilities and their modes of binding to the membrane (Table III). The F_1 part possesses all the catalytic sites. Usually, this part can be extracted from the membrane by non-destructive treatments (dilution in a solution of a chelating agent at low ionic strength, for example). All known examples of the F_1 part, described in the literature, comprise five different subunits, named α through ε in order of decreasing size (Table III). The properties of each subunit are well established [19]. The isolation of the F_1 and F_0 components of H^+–ATPase from a thermophilic bacterium and *E. coli* has enabled us to determine their physicochemical and biological properties [18, 19]. Five subunits are found in F_1: α and β are the nucleotide binding sites, δ and ε are the connecting bridge between F_1 and F_0 and link the $\alpha\beta$ complex to the $\delta\varepsilon$ complex. In addition, it is often suggested that α is an allosteric site, β is a catalytic site, and $\gamma\delta\varepsilon$ is an H^+ gate. The binding of nucleotides to α and β changes the conformation of the latter. The protein components of F_0 of the ATPase are more integrated into the membrane than the F_1 part, and treatments with solvents or detergents (which destroy the membrane) are required to solubilize them. The complexity of F_0 polypeptides varies from one coupling membrane to another. It depends also upon the purity of the isolated H^+–ATPase. Thus, the F_0 part of *E. coli* contains three polypeptides of approximate molecular weights of 24, 17 and 9.5 kDa [17]. Chloroplast F_0 also appears to contain only three or four polypeptides, while mitochondrial F_0 contains two or three additional proteins [20]. In addition, the stoichiometry of F_0 polypeptides has not been established with certainty. Nevertheless, the smallest subunit which has the remarkable property of being soluble in chloroform–methanol is present in 6–10 copies per F_1 [17]. All H^+–ATPases of this F_0F_1 type contain this hydrophobic subunit defined as the DCCD-binding protein. Thus, in *E. coli*, among the three subunits of F_0, the DCCD-binding protein forms a highly hydrophobic part of the H^+ channel, and the F_0-binding protein specifically binds F_1 δ and ε [19].

F_0F_1-activity can be reconstituted from a highly purified F_1 part and the crude F_0 part. F_0 is hydrophobic and embedded in biomembranes and thus is difficult to purify without a loss of activity. F_0's H^+ channel activity is measured after its incorporation into liposomes. The flow of protons through F_0 could be coupled to ATP synthesis or hydrolysis by F_1, under appropriate conditions [21]. When an artificial proton–motive force is provided through imposed electrical and proton concentration gradients, the reconstituted vesicles catalyze ATP synthesis. The main function of this type of enzyme, the mitochondrial H^+–ATPase for example, is to utilize the transmembrane electrochemical gradient of protons for the synthesis of ATP.

F_0F_1 is most often considered as an ATP–synthase, but it operates in either a synthetic or hydrolytic mode (Table II). In anaerobic growth of *E. coli*, for example, F_0F_1 mediates ATP hydrolysis and H^+ extrusion to maintain the proton–motive force used by secondary porters [15]. Indeed, this is normal for anaerobic bacteria. Similarly, F_0F_1 (or a nearly related form, as suggested in Tables II and III) also functions as an ATP–synthase or hydrolase in eukaryotes, with its mode of operation depending upon location rather than circumstance. Thus, F_0F_1 in mitochondria or chloroplasts is an ATP–synthase driven by the proton–motive force from respiratory or photoredox reactions. In the other eukaryotic F_0F_1–ATPases, its function is to hydrolyze ATP under the physiological conditions observed and described.

Many other biological membranes have F_0F_1-like ATPases, orientated to yield a reversed proton–motive force, acid and positive inside. Nevertheless, as suggested elsewhere [14], assignment to the F_0F_1 family requires an ATP-dependent H^+ transport, sensitive to proton conductors but not affected by vanadate. A survey of the literature permits us to establish the list of candidates (Table IV). This table simply suggests that mechanistic homologies link these groups of H^+ pumps. We do not imply necessary structural resemblances. In fact, the structure of such newly studied F_0F_1-like H^+ pumps is unclear and the published data on chromaffin granules, for

Table IV. Distribution of F_1F_0–ATPases.

Group	Location	References
Prokaryotes	all forms studied	[17, 22–24]
Eukaryotes	all mitochondria	[17, 25]
	all chloroplasts	[17, 26–29]
Eukaryotes	other locations	
fungi	vacuoles:	
	N. crassa	[30, 31]
	S. cerevisiae	[32, 33]
	S. carlsbergensis	[34, 35]
plant cells	microsomes:	
	Avena sativa	[10, 11]
	Beta vulgaris	[36]
	Glycine max	[5, 37]
	Raphanus sativus	[4, 38, 39]
	Zea mays	[7–9, 40]
	vacuoles:	
	Beta vulgaris	[6, 41, 42]
	Hevea brasiliensis	[43–52]
	Kalanchoe daigremontiana	[53–55]
	Tulipa sp.	[56]
	Golgi vesicles	[57–59]
animal cells	lysosomes	[60–73]
	endoplasmic reticulum	[74]
	Golgi vesicles	[75, 76]
	chromaffin granules	[71, 72, 77–80]
	synaptic vesicles	[81]
	synaptosomes	[73, 82]
	mast cell granules pituitary storage granules	[83, 84]
	(renal) exocytic vesicles	[85]
	platelet granules	[86, 87]
	coated vesicles (endosomes)	[88]
	clathrin-coated vesicles	[89, 90]

Table V. Distribution of E_1E_2–ATPases.

Location	Ion substrate(s)	References
Prokaryotes		
S. faecalis	$Na^+/(H^+?)$	[91]
S. faecalis	$Ca^{2+}/?$	[92]
S. faecalis	$K^+/?$	[93]
E. coli	$K^+/?$	[94]
Acholeplasma laidalwii	$(H^+, Na^+?)/?$	[95]
Eukaryotes		
fungi		
plasma membrane	$1\ H^+/?$	[96–103]
plant cells		
plasma membrane	$H^+/(K^+?)$	[104–109]
animal cells		
plasma membrane	$3\ Na^+/2\ K^+$	[110–111]
plasma membrane	$1\ Ca^{2+}/2\ H^+$	[112]
gastric mucosa	$1\ H^+/1\ K^+$	[113–115]
sarcoplasmic reticulum	$Ca^{2+}/?$	[116]

example, indicate a very different subunit composition [71, 72].

The E_1E_2–ATPase type

The second class of ATP-driven pumps has been collectively termed the E_1E_2–ATPase type according the terminology introduced recently [14]. These ATP-driven pumps cycle between two stable states, with or without covalently bound phosphate (Table II). To classify an ATP-driven pump as E_1E_2-type, it must exhibit either of the two following criteria: ATP-driven transport sensitive to vanadate and/or identification of a phosphoprotein. In Table V, the direction of ion transport is shown to be away/toward the ATP hydrolytic site. If known, stoichiometry is indicated. References cited are some recent examples that give an available entry into the literature concerning this class of ATP-driven pumps.

E_1E_2-type pumps show a broad ionic specificity (H^+, Na^+, K^+, or Ca^{2+}) accompanied by a remarkably simple structure. All known pumps of this class use a 100 kDa polypeptide that becomes phosphorylated at aspartate. The plasma membrane ATPase of *Neurospora crassa* and *Beta vulgaris* involves a covalent intermediate, the β-aspartyl-phosphate [99, 106, 107]. Amino acids flanking this residue are nearly the same in examples of very different origins [117]. The presence of phosphoprotein provides a useful operational criterion for family membership, since its formation confers sensitivity to the transition state inhibitor, vanadate. As sequence data emerge, relatedness in the family may become even more apparent: J.E. Hesse *et al.* [94], for example, find a homology in the 100 kDa polypeptides of prokaryote and eukaryote E_1E_2–ATPase.

Present knowledge about the structure of tonoplast ATPase

Little information exists about the polypeptide composition of tonoplast ATPase. This is due in part to the difficulty encountered in the solubilization and purification of the entire enzyme (see [1, 39, 52, 118]).

The tonoplast ATPase of *Hevea* latex has been solubilized with dichloromethane and purified 100-fold [52]. The resulting ATPase enzyme (bearing a hydrolytic enzyme) has a molecular weight of approximately 200 kDa (Fig. 1). Its probable assembly consists of five subunits with molecular weights of 66, 23, 13 kDa and two 54 kDa subunits which normally remain together except for drastic SDS treatment. This composition indicates that this ATPase is probably not related to the plasma membrane system [97, 98, 101, 104–108]. The subunit pattern is also not strictly comparable to those reported for the F_1 part of the F_0F_1–ATPase (see [18] and Tables I and III). In addition, the lack

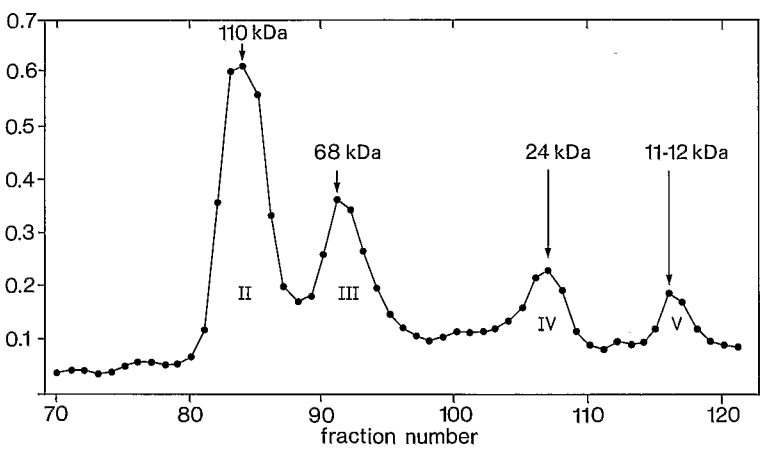

Fig. 1. Gel filtration on Sephacryl S–200 of *Hevea* tonoplast ATPase extracted by dichloromethane. The purified ATPase fraction (65 mg), prepared as described elsewhere [52], was subjected to mild SDS-treatment for a few minutes. It was layered on top of the column at a concentration of approximately 30 mg of protein/ml. The column (2.5 × 93 cm) was eluted with 100 mM Tris/sulfate, pH 7.5, at 8°C, at a flow rate of 10 ml/h maintained by a peristaltic pump. Proteins were determined by their absorbance in ultraviolet light. The native ATPase with an apparent molecular weight of approximately 200 kDa was decomposed into four subunits in equal ratio, probably in a 1:1:1:1 ratio. These subunits eluted according to molecular weights of approximately 110, 65, 25 and 12 kDa. The SDS–PAGE electrophoresis of these four protein peaks gave polypeptides of 54 (peak II), 66 (peak III), 23 (peak IV) and 13 (peak V) kDa. Consequently, two polypeptides of molecular weight 54 kDa seem to be closely associated and do not dissociate with mild SDS-treatment (from [52]).

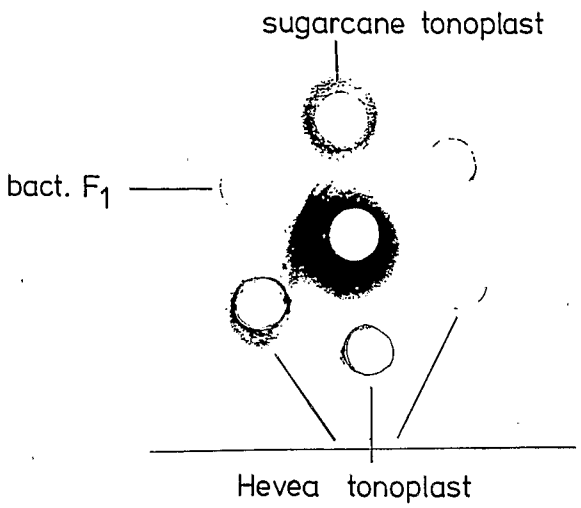

Fig. 2. Antibody diffusion test of *Hevea* tonoplast ATPase proteins with anti-F_1. Solubilized proteins from tonoplast membranes were allowed to diffuse against antiserum to *E. coli* F_1–ATPase. The reaction with bacterial F_1–ATPase is also shown for control. These data clearly show that a polyclonal antibody against F_1–ATPase of *E. coli* does not react with the vacuolar ATPase of *Hevea* latex (from [52]).

of cross-reaction between anti-F_1 and the ATPase does not suggest a close relationship with F_1-type ATPases (Fig. 2).

Another tonoplast ATPase has been solubilized from vacuolar membranes of *Saccharomyces cerevisiae* with the zwitterionic detergent *N*-tetradecyl-*N*,*N*-dimethyl-3-ammonio-1-propane sulfonate and purified by glycerol density gradient centrifugation [33]. It was found to be composed of two major polypeptides *a* and *b*, respectively, of $M_r = 89$ and 64 kDa. Such a subunit composition of the purified enzyme is entirely different from that of F_0F_1–ATPase and that of E_1E_2–ATPase. This enzyme was not inhibited by antiserum directed against mitochondrial F_1–ATPase or mitochondrial F_1–ATPase inhibitor protein. All these data clearly indicate that tonoplast ATPase is different from yeast plasma membrane H^+–ATPase and mitochondrial F_1–ATPase.

A NO_3^--sensitive ATPase activity, described as being of tonoplastic origin, has been solubilized from microsomal membranes of radish seedlings with octyl-β-D-glucoside [39]. The method led to an approximately 35-fold purification from the microsomes. The final preparation exhibited several bands. Eight components whose molecular masses were not indicated. It was strongly enriched in an 86 kDa polypeptide. This author reported that if this large polypeptide belonged to the tonoplast ATPase, it would not agree at all with a structural similarity with the F_0F_1–ATPase.

Actually, on the basis of the recent data concerning the purified tonoplast ATPase from *Hevea* latex, a function has been assigned to each component. The 66 kDa subunit seems to bind nucleotides and SH reagents. Different studies with various ATP analogs indicate that this subunit may contain the catalytic binding site. Significant results have been obtained with NBD–Cl (7-chloro-4-

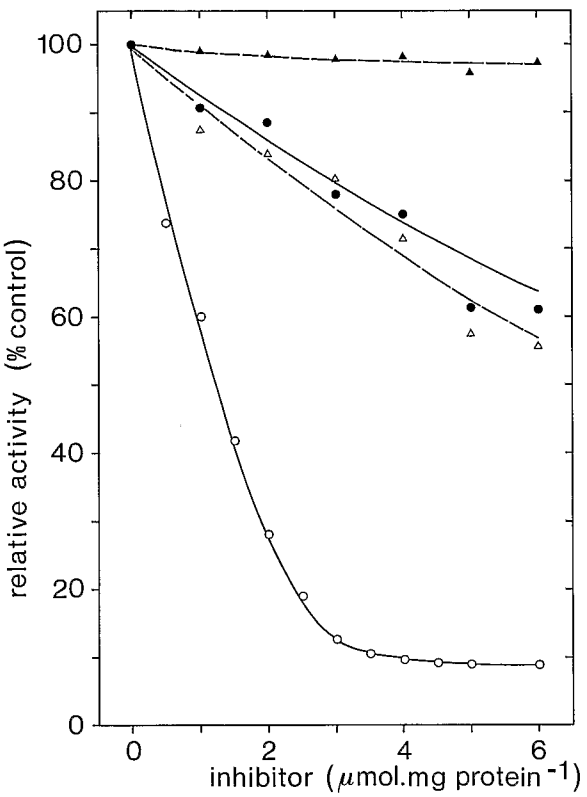

Fig. 3. Effect of the different carbodiimides on *Hevea* tonoplast ATPase. Experiments were performed at 30°C with the following incubation medium: 50 mM Mes, 50 mM Hepes and 5 mM β-mercaptoethanol adjusted to pH 7.0 with Tris-base. The medium also contained 0.1 mM ammonium molybdate and 5 mM Mg^{2+} (as SO_4^{2-} salt). Usually, the mixture was preincubated for 10 min before starting the reaction with the addition of ATP (5 mM final concentration). Tonoplast membranes were assayed for ATPase activity in the presence of increasing amounts of inhibitor: 1-cyclohexyl-3-(2-morpholinyl-4-ethyl)carbodiimide metho-*p*-toluenesulfonate (CMCD) (-▲-▲-), *N*,*N*′-dicyclohexylcarbodiimide (DCCD) (-○-○-), *N*,*N*′-diisopropylcarbodiimide (DIPCD) (-△-△-), or 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC) (-●-●-). Each assay contained 100 μg of protein/ml. Control activity was 0.21 μmol/min/mg of protein (from [47]).

Table VI. ATP-hydrolyzing activities present in plant homogenates.

Activity	Optimum pH	Substrate specificity	Inhibitors	Requirements for divalent cations
Plasma membrane ATPase	6–7	absolute for ATP	vanadate, DCCD, DES	Mg^{2+} (no Ca^{2+})
Mitochondrial ATPase	8–9	ATP, GTP, ITP, UTP	oligomycin, azide, DCCD	Mg^{2+} or Ca^{2+}
Tonoplast ATPase	7–8	ATP, GTP, ITP, UTP	DIDS, TMT, DCCD	Mg^{2+}, Ca^{2+}?
Pyrophosphatase	7.5–9	pyrophosphate and nucleotide triphosphate	fluoride	no effect
Acid phosphatase	5–6	many nucleotides and phosphoric esters	vanadate	none
Alkaline phosphatase	8–9	many nucleotides and phosphoric esters	vanadate	none
Apyrases (diphosphohydrolases)	6–7	nucleotide di- and triphosphate	unknown	Mg^{2+} and/or Ca^{2+}

Abbreviations: DCCD: *N,N'*-dicyclohexylcarbodiimide; DES: diethylstilbestrol; DIDS: 4,4'-diisothiocyano-2,2'-stilbene disulfonic acid; TMT: trimethyltin.

nitrobenzo-2-2-oxa-1,3-diazole). The 53 kDa subunit can bind nucleotides with different affinities. Perhaps, it also participates in ATPase reactions. Such an interpretation must be confirmed. In addition, the 13 kDa subunit binds DCCD and TMT. Consequently it may be tempting to consider it as a part of the proton channel. Such results will reveal some components common to all ATPases capable of catalyzing a transmembrane flux of protons.

The usefulness of inhibitors in classifying the tonoplast ATPase

As described elsewhere [12, 48, 119], the lutoids of *Hevea* latex must be considered as a vacuolar compartment. The lutoid preparations were uncontaminated with membranes from other organelles of the plant cell (chloroplasts, mitochondria, *etc.*). Usually, plant homogenates contained a lot of activities capable of hydrolyzing ATP (Table VI). A problem of purification exists and many difficulties are encountered when studying the tonoplast. Consequently, with *Hevea* latex, it has been possible to determine the precise sensitivity of the tonoplast ATPase to a great variety of agents, which selectively inhibit the different classes of H^+-translocating ATPases (Table I and Figs. 3, 4, 5 and 6).

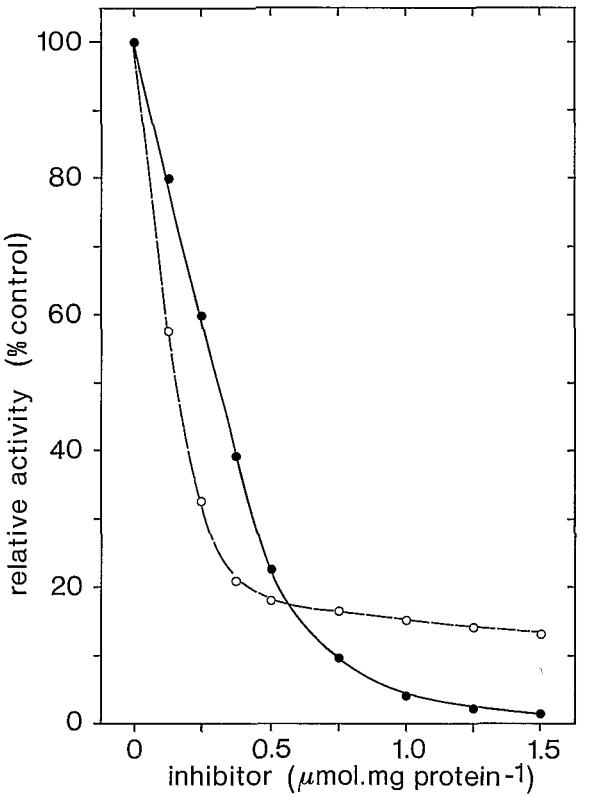

Fig. 4. Effect of trimethyltin chloride on *Hevea* tonoplast ATPase. Reaction conditions are the same as those described for Fig. 3. Control activity was 0.24 μmol/min/mg of protein (from [47]).

Effect of DCCD, TMT, NBD-Cl and ATP analogs on tonoplast ATPase. Relationship with F_0F_1–ATPases

DCCD has previously been described as an effective inhibitor of *Hevea* tonoplast ATPase [43, 44, 47]. 50% inhibition was observed at 1.26 μmol of inhibitor/mg of protein with purified enzyme. DCCD is the most effective of the carbodiimides tested [47] (Fig. 3).

Like DCCD, the organotins inhibit proton conductivity through the F_0 portion of the H^+–ATPase [120]. They act by binding to a proteolipid subunit, probably near but not identical with the site of DCCD binding. Among the different trialkyltin chlorides tested, trimethyltin and tributyltin were the most effective. *Hevea* tonoplast ATPase was inhibited 50% at 0.32 μmol of inhibitor/mg of protein and a nearly complete inhibition was obtained at 1 μmol of inhibitor protein (Fig. 4). Such inhibition has also been reported for the vacuolar ATPase of fungi [31, 33] and higher plants [11, 39, 53]. The organotins inhibit the ATPases of mitochondria [121, 122], chloroplasts [123] and chromaffin granules [77, 79, 80] in the same range of concentrations. These organotins also inhibit the plasma membrane ATPase but at significantly higher concentrations, as shown in *N. crassa* [31].

NBD–Cl constitutes a very powerful inhibitor of *Hevea* tonoplast ATPase. The half maximal effect was obtained at 1.4 μM and a value of 2.0 μM was calculated for K_i. Comparable values were obtained for the vacuolar membrane of *N. crassa* [31]. NBD–Cl was also shown to inhibit F_0F_1–ATPases from mitochondria [121, 124, 125] and chloroplasts [126, 127]. It also inhibits the H^+–ATPase of *E. coli* [128, 129] and the ATPase from chromaffin granules [77–80]. This molecule was effective on the soluble and membranous form of the F_0F_1–ATPase. It interacts specifically with the β-subunit of the F_1–ATPase [124, 125, 127, 129]. The mitochondrial ATPase was inhibited 50% at 3.0 μM for *N. crassa* [31], whereas the plasma membrane ATPase of *N. crassa* showed a 5–10-fold lower affinity, with 50% inhibition being obtained at 14 μM [31].

Different ATP analogs have been described as effective inhibitors of F_0F_1–ATPase [126, 130–132]. TNP–ATP and AMP–PNP were found to inhibit *Hevea* tonoplast ATPase with 50% inhibition at 10–15 μM and 3–5 μM, respectively, (Fig. 5). The K_i value calculated for the tonoplast enzyme was approximately 0.6–0.8 μM. The same results have been described for the mitochondrial and vacuolar membrane ATPases of *N. crassa* with somewhat similar effectiveness (50% inhibition at 6 and 10 μM, respectively, and a K_i value of 0.6 μM for both enzymes) [31]. The plasma membrane ATPase of *N. crassa* was found to be approximately 10-fold less sensitive (50% inhibition at 100 μM) to ATP analogs [31].

Effects of orthovanadate and quercetin on tonoplast ATPase. Relationship with E_1E_2–ATPases

Orthovanadate has been described as a specific inhibitor of plasma membrane ATPases in animal and plant cells [101, 133, 134]. This molecule produces no significant inhibition of *Hevea* tonoplast ATPase, even at a concentration as high as 1 mM (Fig. 6).

Hevea tonoplast ATPase was inhibited by quercetin with 50% inhibition at 20–25 μM (see [1, 118, 135]). This inhibition was competitive (Fig. 7). The *Hevea* enzyme was found to be nearly as sensitive to this inhibitor as the tonoplast membrane ATPase of *N. crassa* (50% inhibition at 22 and 24 μM, respectively [31]). This molecule was 10-fold less effective against the plasma membrane ATPase of *N. crassa* (50% inhibition at 170 μM [31]).

Phenolic compounds, especially quercetin, have been reported to inhibit a great variety of membrane ATPases [101, 127, 128, 136, 137]. Quercetin is not a specific inhibitor of this class of ATPase because it can also inhibit the hydrolytic activity of the mitochondrial F_0F_1 enzyme, without inhibiting the ATP–synthase [121].

Such studies confirm the originality of the tonoplast ATPase. This ATPase activity is clearly distinguished from plasmalemma ATPase by its insensitivity to vanadate. It can also be casily distinguished from mitochondrial ATPase by its insensitivity to azide and oligomycin.

The sensitivity of tonoplast ATPase to anions

The effect of Cl^- and other anions on the tonoplast ATPase from *Hevea* latex has been investigated [1, 48, 50].

Cl^- and other anions stimulated the ATPase activity of tightly-sealed vesicles prepared from *Hevea* tonoplast, with the following decreasing order of effectiveness: $Cl^- > Br^- > SO_4^{2-} > NO_3^-$. NO_3^- could be regarded as a potent inhibitor of this enzyme.

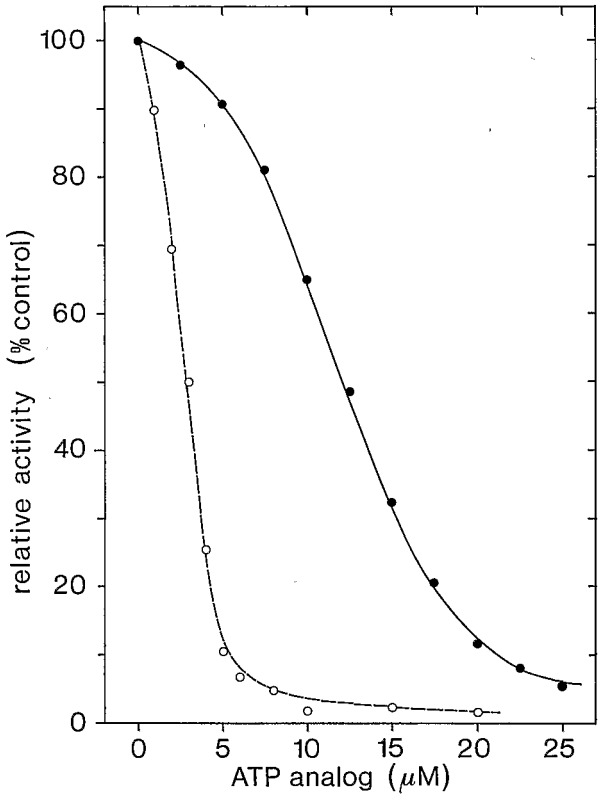

Fig. 5. Effect of ATP analogs on tonoplast ATPase activity of *Hevea* latex. Reaction conditions are the same as those described for Fig. 3. 2′,3′-*o*-(2,4,6-trinitro-phenyl)-adenosine/-5′-triphosphate (TNP-ATP) (-●-●-); 5′-adenylylimidodiphosphate (AMP-PNP) (-○-○-). Control activity was 0.30 μmol/min/mg of protein (measured according to [47] and [52]).

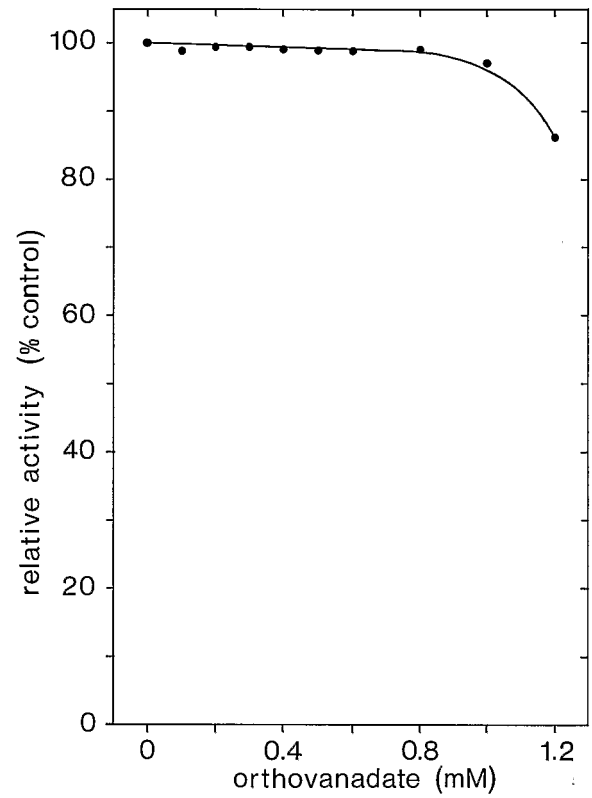

Fig. 6. Effect of orthovanadate on *Hevea* tonoplast ATPase. Reaction conditions are the same as those described for Fig. 3. Control activity was 0.25 μmol/min/mg of protein (from [47]).

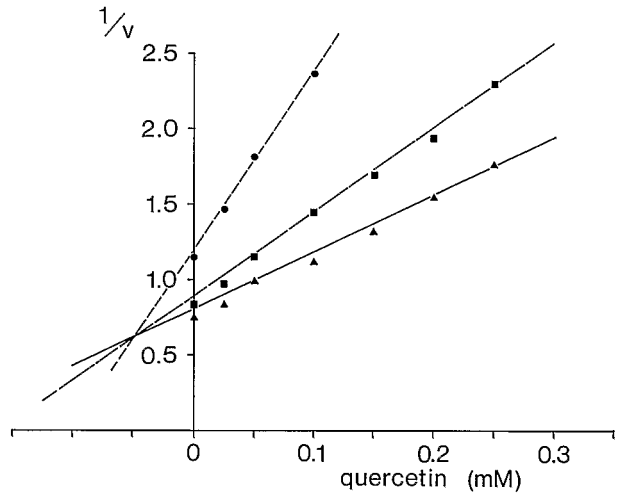

Fig. 7. Dixon plots for quercetin inhibition of *Hevea* tonoplast ATPase. The assay was started after 15 min of preincubation with either 2 mM ATP (-●-●-), 4 mM ATP (-■-■-) or 6 mM ATP (-▲-▲-) (see [1] and [118]).

As indicated by changes in the proton–motive potential difference, anion stimulation of tonoplast ATPase was caused in part by the ability of these anions to dissipate the electric potential. This interpretation assumes not a channeling of these anions against a membrane potential, negative inside, but a modification of the permeability of these anions through the tonoplast membranes [50]. Similar anion stimulation was found with solubilized ATPase from the tonoplast membrane. Consequently, the tonoplast H^+–pumping ATPase can be considered as an anion-stimulated enzyme. Cl^- has a direct effect on tonoplast ATPase as seen for the anion-sensitive H^+–pumping ATPase in membrane vesicles from corn roots [8, 9] and from oat roots [10].

A characteristic feature of tonoplast ATPase is its sensitivity to NO_3^-. The tonoplast ATPase differs from both mitochondrial (F_0F_1) and plasma membrane (E_1E_2) enzymes in its response to this anion. Thus, under conditions which completely inhibit the tonoplast ATPase, little effect is seen on the other ATPases. Its activity is lowered by up to 90% by 50 mM nitrate [1, 39, 52]. The mode of action of this anion on the tonoplast ATPase is actually unknown, in spite of a considerable amount of work.

The sensitivity of tonoplast ATPase to protonophores

Tonoplast ATPase was responsible for the electrochemical proton gradient across the tonoplast [1].

Table VII. Effect of ionophores on *Hevea* tonoplast ATPase activity.

Addition(s)	pH	$\Delta\Psi$ (mV)	Tonoplast ATPase activity (units/mg of protein)
None (control)	0.7	−140	1.20
50 mM $(NH_4)_2SO_4$	0.025	n.d.	2.51
2 μM SF-6847	n.d.	n.d.	1.91
100 μM S-13	n.d.	n.d.	1.96
0.5 μM FCCP	n.d.	n.d.	2.01
0.5 μM Nigericin	0.8	−136	1.14
0.5 μM Nigericin + 10 mM K_2SO_4	0.1	−140	2.30

From [1, 48, 49, 52, 139]; n.d.: not determined. Tonoplast vesicles were preincubated for 30 min at 30°C, with the above additions. The pH and mV were measured as described elsewhere [49, 139]. The ATPase activity was then initiated by the addition of 5 mM ATP and 5 mM $MgSO_4$ at an external pH of 7.0.

Such a demonstration has often been made difficult by the fact that heterogeneous populations of vacuoles and tonoplast vesicles contained a large proportion of leaky structures. As shown by Marin *et al.* [46, 51], the vacuo-lysosomes of *Hevea* latex could be lyophilized without loss of tonoplast ATPase. They could be gently homogenized to yield tonoplast vesicles that are useful for transport.

The tonoplast ATPase from *Hevea* latex was very sensitive to protonophores [49, 138]. When tightly-sealed vesicles were used, protonophores such as S13 (5-chloro-3-*t*-butyl-2′-chloro-4-nitrosalicylanilide), SF-6847 (3,5-di-*t*-butyl-4-hydroxybenzylidene malonitrile), FCCP (carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone) or 2,4-DNP (2,4-dinitrophenol), were very effective (Table VII). Their presence in the incubation medium resulted in a stimulation of the tonoplast ATPase between 53 and 83%. Other ionophores, such as nigericin in the presence of potassium, also have positive effects [139]. At a low concentration of nigericin and in presence of 10 mM K_2SO_4, tonoplast ATPase was stimulated 92%. These ionophores have been found to have no effect on the solubilized ATPase [1, 49, 52]. Consequently, ATP hydrolysis is stimulated by ionophores that decrease the constraint on the pump by the electrochemical gradient of protons [1].

Hevea tonoplast ATPase is modulated by changes in the transmembrane H^+ gradient across the tonoplast [49]. A similar suggestion has been made for the electrogenic H^+ translocase on vacuoles from *S. cerevisiae* [35]. A control by H^+ and/or the magnitude of the proton–motive force has been clearly established for the different types of F_0F_1–ATPases. Such a property is characteristic of all systems involved in chemiosmotic proton circuits in biological membranes [140].

Discussion and Conclusions

As shown in the different Tables, taken together, all the data reported recently in the literature suggest that tonoplast ATPase could be regarded as a species of the F_0F_1-family of ATPases. Nevertheless, in some cases, since it will be difficult to be sure of having no contamination by other cellular membranes, a mitochondrial-type proton–ATPase could be present in the vacuolar preparation. This case has been described with chromaffin granules [71, 72].

The literature offers two cases without ambiguity: the vacuolar membrane ATPase of *N. crassa* [31] and the lutoidal (=tonoplast) ATPase of

Hevea latex [1, 43–52, 118, 119]. In the first case, a careful analysis of the different mutants of the mitochondrial ATPase allowed rigorous testing to determine whether the vacuolar membrane ATPase activity could be due to mitochondrial contamination. In addition, it was possible to compare directly the vacuolar membrane ATPase with the mitochondrial and plasma membrane ATPases. This comparison is not possible with *Hevea* latex. Nevertheless, the principal advantage of this material is the ability to obtain a large fraction of uncontaminated vacuoles involving neither cell rupture treatments nor lytic attack by cell-wall-degrading enzymes. Consequently, the first evidence as to the nature of the tonoplast ATPase has come from the inhibitor studies on these two materials.

On thinking it over, it must be concluded that the tonoplast ATPase is different from the E_1E_2–ATPase type. It is insensitive to vanadate, even if it is sensitive to quercetin. The effect of vanadate on this ATPase class is to prevent the formation of a covalent phosphorylated intermediate (often a β-aspartyl-phosphate) during their reaction cycle [101, 104–107, 133, 134]. At least with *Hevea*, such an intermediate has not been evidenced. Vanadate inhibits phosphorylation of the plasma membrane ATPase because this pentavalent ion competes with phosphate for binding and adopts a stable trigonal bipyramidal structure resembling the transition state of phosphate during the reaction. Consequently, the insensitivity of tonoplast ATPase to vanadate suggests that such a phosphorylated intermediate is not involved in the mechanism of this enzyme. Certainly, quercetin is often regarded as a specific inhibitor of the E_1E_2–ATPase type, however, its effect is not conclusive because it also inhibits the hydrolysis of ATP catalyzed by the mitochondrial ATPase [121, 137].

The tonoplast ATPase also differs from the F_0F_1–ATPase type because it is insensitive to various chemicals such as azide and oligomycin. These molecules, described as typical inhibitors of the mitochondrial ATPase, have no effect on the tonoplast ATPase even at high concentrations. But, these differences are not sufficient to exclude the tonoplast ATPase from the F_0F_1–ATPase type. Its response to inhibitors which block proton translocation such as DCCD [138] and organotins [120, 122, 124, 125] resembles the mitochondrial ATPase. In addition, the sensitivity of the tonoplast ATPase to inhibitors which probably react at the active site of the enzyme (such as NBD-Cl or the different ATP analogs) is very similar to that of the mitochondrial ATPase. The substrate specificity of tonoplast ATPase is comparable with that described for the mitochondrial enzyme. The K_m values for MgATP are about 0.3 mM for both enzymes. In some cases, Ca^{2+} and Mg^{2+} are similarly effective for their ATPase activities. Consequently, the tonoplast ATPase must be considered as a good candidate for the F_0F_1–ATPase class. It complies with all the criteria which define this class of ATP-driven ion pump (see Table II). Nevertheless, the structure is somewhat different, not as complex (at least 4 or 5 polypeptides for tonoplast ATPase instead of 20–24 subunits for the ATP–synthase). Such a molecular composition will be able to assume only the hydrolysis of ATP.

The different proton pumps located inside the eukaryotic cell could belong to the F_0F_1 family. Table IV may be considered as speculative, linking all the ATPases by their mechanistic homologies. This does not necessarily imply structural resemblances [14]. The different electrophoresis patterns of the purified tonoplast ATPase prepared from *Hevea* latex, *Raphanus* seedlings and *Saccharomyces* confirm this point of view. The tonoplast ATPase is clearly distinguishable from the mitochondrial F_0F_1–ATPase and from the plasmalemma E_1E_2–ATPase. The same situation could be obtained with the ATPase of chromaffin granules: the purified enzyme consists of four major polypeptides with apparent molecular weights of about 125, 80, 40 and 20 kDa [72]. The ATPase of the cholinergic synaptic vesicle membrane corresponds to a 250 kDa ATP-hydrolyzing molecule, composed of five polypeptides of 50 kDa each [141]. Any conclusions as to phylogenetic relationships have to wait for comparison of antibody specificities and amino acid sequences.

If the differences observed in the structure of these new F_0F_1-like ATPases justify the definition of another type, it could suggest the existence of a third class of ATP-driven pumps, intermediate between F_0F_1–ATPases and E_1E_2–ATPases. A characteristic feature of this class should be its sensitivity to anions: inhibition by NO_3^- and stimulation by Cl^-.

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