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STUDIES ON YEAST MEMBRANE TRANSPORTERS – HOW CAN COMPUTATIONAL BIOLOGY HELP?

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ABSTRACT

With the availability of complete genome sequences, emphasis has shifted toward the understanding of protein function and this in turn has opened up a new "-omics"-field, i.e. functional proteomics. Structural studies of proteins are only one aspect of functional proteomics and are mostly carried out by computational means. However, these investigations must be completed by function studies resulting in structure/function relationships and this can only be accomplished at the lab bench.

Some examples of comprehensive investigations on transport proteins of yeast, *Saccharomyces cerevisiae*, can be used to illustrate these relationships. This research includes various methods and tools concerning visualisation, sequencing and annotation. In addition, the transport activity of a tonoplast-residing proton pump has been studied in detail by biophysical approaches. The result of these investigations on the structure/function relationships demonstrate a fruitful cooperation of so-called traditional "wet" biology and computational biology.

INTRODUCTION

In scientific discussions the question is often raised whether bioinformatics and cheminformatics are equivalent or at least overlapping disciplines since computational scientists from both disciplines often seem to work in the same field. This in turn leads to a further question, i.e. if this is not true then is it possible to bridge or at least narrow the gap between them (1)?

However, if we take a step back and examine the situation we see that although computer scientists and biologists are accustomed to working together, they still think of their respective disciplines as separate. The use of computers as a new tool for investigation and research has reached the apparent traditionally techniques-free biology which is often described as a change of paradigm in biology and begs the question whether the biologists themselves are ready for the integration of the "classic" biology, as an experimental and practical discipline, and the computer-aided area in which bioinformatics plays a central role as part of the computational biology.

However, science without computers is unimaginable, since they have inserted themselves into almost every aspect of laboratory life, for example, for collecting, analysing and plotting experimental data, for aiding research topics by retrieving corresponding databases such as PubMed to search for literature or genomic or proteomics databases to look for successful research candidates, for the modelling of enzyme reaction cycles, enzyme-substrate interactions, protein foldings and 3D structures, and last but not least, for writing papers and grant applications.

One important aspect of the "ditch" between traditional biology and bioinformatics might be that there are a number of various online databases available which allows researchers to carry out their investigations and discoveries without even setting a foot in a lab.

Thus, if one accesses PubMed or PDB database (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=PubMed; http://www.rcsb.org/pdb/) to query the actual literature or any protein, one will recognize that there are numerous reports on bioinformatic-handled proteins concerning their sequence, modelled structure or even perhaps their subcellular location. Consequently, the least reports deal in fact with proven functional properties or regulatory aspects of these proteins. Shortfalls in the ability of bioinformatics to predict both the existence and function of genes have also illustrated the need for protein analysis which has given rise to a new research field, called proteomics. The emergence of proteomics has been inspired by the realization that the final product of a gene is inherently more complex and closer to function than the gene itself. The most practical application of proteomics is the analysis of single proteins as opposed to entire proteomes. This type of proteomics, which is referred to as "functional proteomics", is always driven by a specific biological question and requires a huge arsenal of both experimental methods and techniques as well as computational approaches to model, predict and explain biological function at the molecular level. Thus, the combination of

protein identification by bioinformatics and characterization by "wet" biology has a meaningful outcome. Consequently, this is an unique chance to bridge the gap between the number of gene sequences in databases and the number of functionally characterized gene products which is currently a major challenge in biology.

As one example for the successful conquest of the scientific impediments, an important model organism for functional proteomics is presented, a short overview about both transport mechanisms across one biological membrane (the tonoplast) and some insight to a powerful method – the patch clamp technique – for the functional characterization of one representative of ion transporting proteins, which is an ATP-driven proton pump, is given. At the same time, the utilization of computers as an equally powerful tool for the collection, analysis and presentation of experimental data is demonstrated.

THE TEST ORGANISM IS AN IMPORTANT MODEL ORGANISM

Rather than isolate the pump for studies in an artificial environment, the enzyme was investigated in its "quasi" native environment. In keeping with tradition, the fungus baker's yeast *Saccharomyces cerevisiae* was chosen for this purpose. Since the mid-1980s, an ever-increasing number of molecular biologists and physiologists have used yeast as their primary research system and consequently, this has resulted in a virtually autocatalytic stimulus for continuing investigations of all aspects of molecular and cell biology. The "awesome power of yeast genetics" has become legendary and is the envy of those who work with higher eukaroytes. The complete sequence, published in 1996 and containing ca. 12 kb of DNA packed into 16 chromosomes with 6300 genes identified (2, 3), has proved to be extremely useful as reference for the sequencing of human and other higher eukaryotic genes. For example, of the 80 human disease genes so far identified, 12 yeast homologues have been found (4). Comparative studies have resulted in the suggestion that most basic biological functions of eukaryotic cells are carried out by a core set of orthologous "house-keeping" proteins. Thus, the assumption that analysis of yeast proteins will give insight into those of higher eukaryotes is valid.

Furthermore, this model organism provides a series of further advantages. Yeast is a free-living, unicellular eukaryote. It is the best characterized genetic system which makes it easily available for genetic manipulation and can be used for conveniently analysing and functionally dissecting gene products from itself and other eukaryotes. Yeast cells are highly versatile DNA

transformation systems and viable with numerous markers. They are very suitable for replica plating and mutant isolation and easy to handle in batch cultures in which cells reach stationary phase after 18 to 22h.

For researchers who are mostly interested in transport processes across the membrane, some additional features make yeast a very interesting organism. Yeast cells facilitate structure-function studies of any electrogenic or electrophoretic ion transporters which can be expressed in the plasma membrane or tonoplast. The membranes themselves act as an ample source of diverse membrane proteins, such as ion channels, pumps and cotransporters, which lend themselves to electrophysiological analysis and specifically to patch-clamping. There are currently about 258 recognized and putative transporters from bioinformatic studies within the genome of *S. cerevisiae* but only perhaps one dozen of these transport proteins have been so far functionally characterised.

SUBCELLULAR LOCATION OF THE PUMP

The subcellular location of the focused proton pump is the vacuolar membrane. Plant and fungal vacuoles are intracellular compartments, bordered by the tonoplast, the vacuolar membrane, and occupy up to 90% of the intracellular volume (Fig. 1). They are multifunctional organelles with specific properties which are central to the cellular strategies of development of plants and fungi and, furthermore, as an acidic compartment they share some of their fundamental properties with mammalian lysosomes. Many newly synthesized proteins are targeted to the vacuole through the secretory pathway, and there they undergo maturing, processing and sorting processes (5). They confer the ability to accumulate a wide variety of solutes to relatively high concentrations and separate these solutes from the mainstream metabolism. Vacuoles store diverse metabolites, such as carbohydrates, amino acids, organic acids as well as inorganic phosphate, sulphate, calcium, potassium, sodium and other ions (6, 7). Here, degradation of carbohydrates and peptides by hydrolytic enzymes also takes place. Consequently, the vacuole plays a key role in both cellular metabolism and homeostasis of the cytosolic pH and ion balance (8, 9).

TRANSPORT ACTIVITIES ACROSS BIOLOGICAL MEMBRANES

Storage, import and export of metabolites and ions, respectively, require corresponding transport systems across biological membranes. Balance between net cellular and vacuolar

accumulation and release for a given ion will be determined by the relative activities of three classes of transport systems. The simplest case is the transport of the given ion which passively follows thermodynamically downhill its concentration gradient via ion-selective channel proteins in membrane. Ion channels are water-filled pores usually with a discrete gating behaviour and are controlled by ligands, voltage or mechanical pressure. Carriers couple the uphill flux of ions to that of protons which follow their opposite directed downhill concentration out of the vacuole. The third class is that of pumps which couple the energy from ATP hydrolysis to the uphill transport of protons into the vacuole (10).

A small number of substrate transporting proteins are known in the vacuolar membrane (Fig. 1). In the yeast membrane transporter's community, a Ca^{2+} dependent and Ca^{2+} permeable cation channel, called YVC1, is probably the best known ion channel of the tonoplast. This channel has been extensive studied and described by patch clamp experiments (11, 12, 13, 14). The accumulation of amino acids and calcium in the vacuolar lumen has been demonstrated by biochemical means and corresponding transport systems are assumed to be proton-substrate antiporters for which the encoding gene for the H^+/Ca^{2+} exchanger (VCX1) has been found. There is to date are no genetic evidence for the amino acid antiporters (15, 16, 17, 18). An alternative Ca^{2+} uptake system is postulated from genetic studies which revealed an ATP dependent Ca^{2+} pump (PMC1) (19). Another ATP dependent transport system, a Glutathion-S-conjugate transporter YCF1, has been also postulated from genetic studies, and sequence alignments with known genomic sequences showed that this transporter belongs to the great family of ABC transporters (18). Besides NHX1, a H^+/Na^+ antiporter, which has only been postulated from biochemical transport studies without any genetic evidence, a further series of transporters for phosphate, sulphate and chloride have been postulated for the proper functioning of the entire vacuole but their existence has not been proven either from transport studies or from genetic studies.

The transport of substrates through the antiporters mentioned above is coupled to an electrochemical proton gradient across the tonoplast which is generated by a vacuolar proton-translocating adenosine triphosphate hydrolase (V-type H+-ATPase, TC 3.2.2) (18, 20). The H^+-ATPase couples the energy from ATP hydrolysis to the uphill transport of protons from the cytosol into the vacuole.

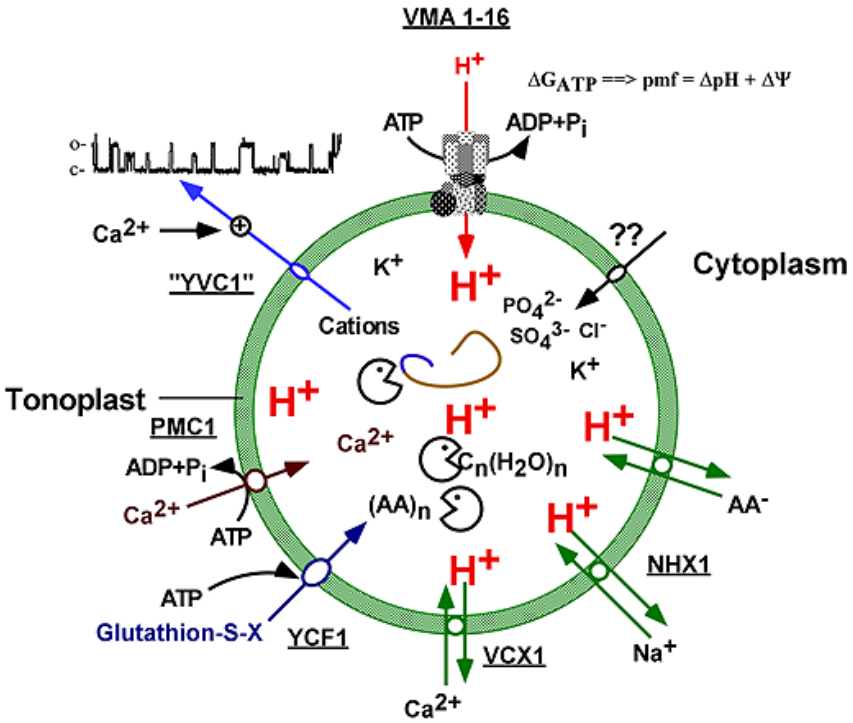

Figure 1. Schematic representation of the yeast vacuole with tonoplast-residing transport systems.

This proton translocation results in acidification of the vacuolar lumen and thus to the generation of a pH gradient across the membrane. Furthermore, the accumulation of positive charges within the vacuole creates an electrical potential difference across the membrane (which is defined as voltage) with positive voltage inside the vacuole. Voltage together with pH gradient build up a driving force, called proton motive force (*pmf*) (21), which in the case of the tonoplast results in low vacuolar pH creating a cytoplasm-directed proton gradient rather than in the generation of an electric voltage across the membrane due to parallel ion conducting transport systems with equilibrium voltages more positive than the maximum ATPase generated voltage (22).

MOLECULAR STRUCTURE OF THE V-ATPASE

Extensive studies on the structure and function of the pump have been carried out in detail by optical, biochemical and genetic methods on *S. cerevisiae*, *Neurospora crassa* (a fungus), as well as on plant and animal cells (for reviews, see: 6, 23, 24, 25, 26). Electron microscopic images of the ATPase holoenzyme show that the pump consists of at least two distinct sectors of which a peripheral domain is readily distinguishable from the integral membrane domain. The peripheral domain is connected by a stalk structure to the membrane-bound domain. Cross-sections of fungal vesicles showed that these ball-and-stalk structures seemed to be arranged

like a string of pearls on the surface of the membrane (25, 27). From optical investigations it could be demonstrated that, under certain conditions, the holoenzyme decays into its two main sectors (28).

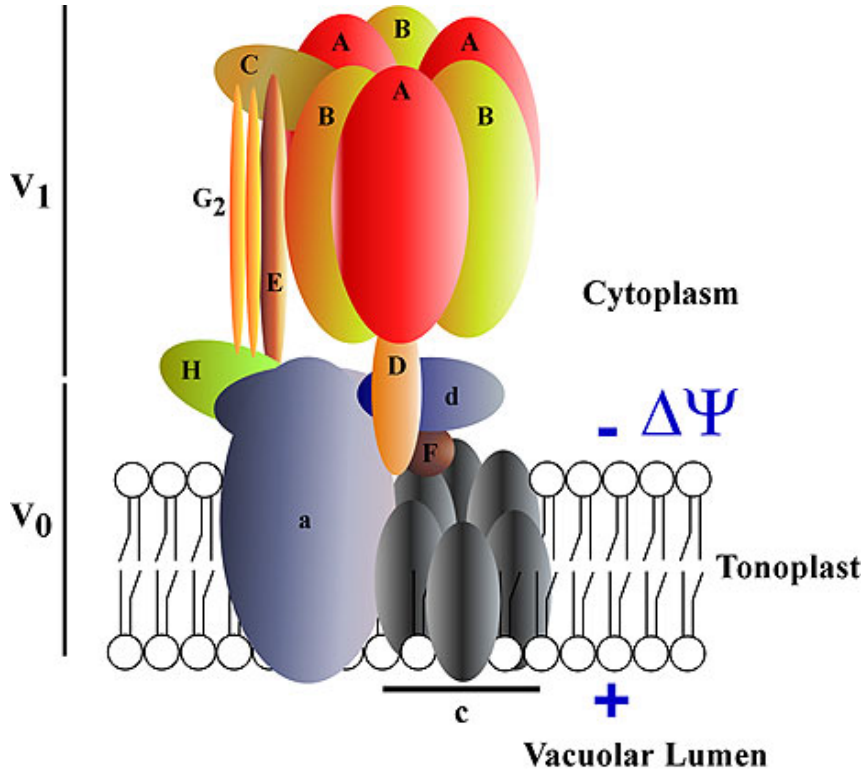

Figure 2. Topological model of the yeast V-ATPase (according to Arata *et al.* (53)). Subunits in the peripheral domain V_1 are indicated by capital letters, whereas subunits in the integral domain V_O are indicated by small letters.

Biochemical and genetic experiments revealed that the pump is a multisubunit enzyme complex composed of at least 7 different subunits (peripheral domain, called V_1) with stoichiometry A_3B_3CDEFG and at least 4 different subunits (integral part, called V_O), respectively (Fig. 2). The subunits A (69 kDa) and B (59 kDa) (encoded by the genes VMA1 and VMA2 (29, 30)) are the largest subunits of the V_1-domain which is the catalytic domain of the pump. Here, ATP binding and hydrolysis takes place (31). At the V_O domain a remarkable structural feature should be noted: the subunit c (VMA3, 17 kDa) forms – six-fold copied – a barrel shaped hexamer which forms the proton channel (32). The molecular weight of the pump is between 500 and 750 kDa, and the yeast V-ATPase is encoded by at least 16 different genes (33). ATP-dependent proton translocation by the pump was first observed by biochemical transport studies carried out with voltage- and pH-sensitive dyes (34).

However, the key determinant of the pump, the transport coupling ratio, is best estimated by electrophysiological methods. Detailed studies were first reported from plant V-ATPases because plant vacuoles with diameters up to 100 µm are more suited for patch clamp experiments than yeast vacuoles (10 to 15 µm in diameter), thus, little is known about the biophysical properties of the yeast pump.

In this overview, I shall expand on our preliminary report (34) and present some details of the yeast V-ATPase obtained from patch clamp experiments with special emphasis on the transport coupling ratio (Kettner & Bertl, submitted).

PATCH CLAMP EXPERIMENTS AND CELL PREPARATION

A common property of all the transport systems mentioned previously is that they transport charged substrates. Transport of these substrates, mostly ions, is recordable as an electrical current whose size is dependent on the basic electrical quantities such as membrane voltage and resistance of the conducting transport systems. The patch clamp technique allows recording of currents from a small membrane area (patch) in response to a defined command voltage (voltage clamp).

The investigation of the yeast ATP-driven proton transport by the V-ATPase has been carried out with the patch clamp technique in the whole-vacuole configuration (35).

Vacuoles were released from protoplasts in the recording chamber by subjecting 24-hour-old protoplasts to mild acid lysis in low-Ca^{2+} medium. Shortly after the first vacuoles have been released, releasing solution was replaced by standard recording buffer (150 mM KCl, 5 mM $MgCl_2$, 0.5 mM EGTA, pH 7 with Tris/MES) which removes residual cell fragments, lipids and dirt. A standard solution-filled glass microelectrode, the patch pipette, was connected via the pipette holder with a pre-amplifier and the movement of this recording system was controlled by micromanipulator (Fig. 3). The patch pipette was firmly attached to the tonoplast which resulted in a high mechanical stability and electrical resistance of up to 5 gigaohms (1 $G\Omega = 10^9\ \Omega$), called giga-seal. A tight seal is required for the current recording with high signal/ noise ratio to make sure that the small transport currents are masked neither by statistical noise nor by leakage currents between pipette and membrane.

This seal formation is called cell-attached configuration and determines the starting point for further configurations (36, 37). Establishment of the whole-vacuole configuration required that

the membrane underlying the pipette tip was ruptured by a brief voltage pulse (600 mV, 3 ms) whereas the seal resistance has to be kept significantly above 1 GΩ. The breakage was monitored by the change of the electrical properties of the glass-membrane system which are determined by the appearance of "slow" capacitative transients whose size is dominated by charge movement within the vacuolar membrane (35). The whole vacuole configuration allows current recording from the entire vacuolar membrane with defined voltages.

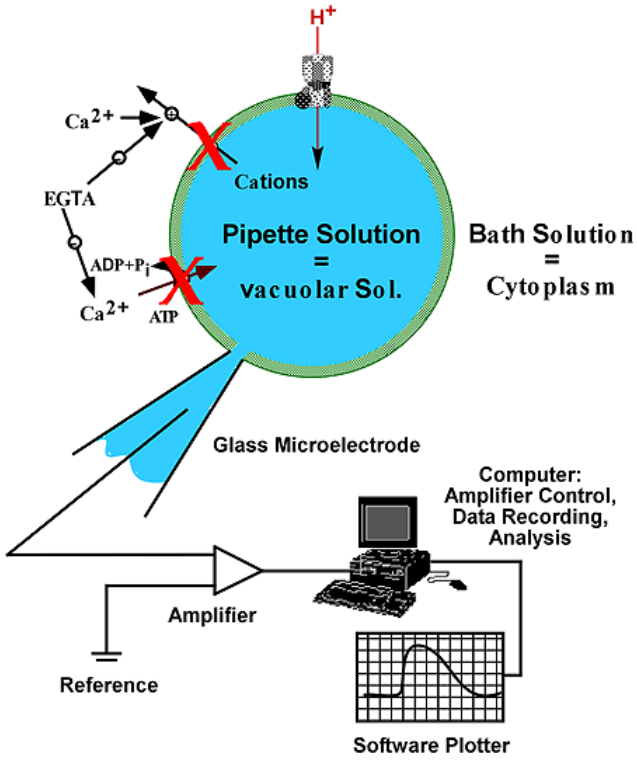

Figure 3. Representation of the experimental setup with patch clamp whole-vacuole configuration. EGTA in bath and pipette solution avoids activation by calcium of both the cation channel and the Ca^{2+} pump and thus masking pump currents.

Due to the larger volume of the patch pipette compared to that of the vacuole, the vacuolar solution was completely exchanged by the pipette solution within a few seconds so that the composition of the vacuolar solution can be assumed to be known (38). The cytosolic side of the tonoplast is exposed to the bath solution which simulates the cytosol and matches the vacuolar solution (standard recording solution, see above). This symmetric composition avoids the build up of any driving forces for ion transport across the tonoplast. Furthermore, the calcium concentration in pipette and bath solution was held at virtually zero by EGTA to avoid masking the smaller pump currents by the much larger channel currents from the cation channel YVC1. For continous data recording a software package for Macintosh (HEKA, Pulse/PulseFit

8.0) was used, supplemented with the chart recorder extension X-Chart, in combination with the EPC-9/ITC-16 amplifier/data acquisition system. Data were filtered at 100 Hz with a built-in 8-pole Bessel filter, sampled at 1 kHz and stored on the computer hard drive.

Determination of the transport coupling ratio was carried out by analysis of current-voltage (I/V) characteristics of the vacuolar membrane under different cytosolic and vacuolar pH conditions. Currents were recorded in response to a voltage ramp which clamped the membrane potential gradually from –80 to +80 mV within 2.5 s and were plotted against the applied voltage. The I/V characteristics were obtained both in the absence and presence of ATP/ADP and P_i when the ATP-dependent currents reached their maximum. Subtraction of both characteristics yields the I/V characteristics of the vacuolar H^+-ATPase. The sign convention for membrane voltage and current, as proposed by Bertl *et al.* (39) was used throughout. This convention defines a positive current (= outward current) as the flow of positive charges from the cytoplasmic side of a membrane to the extra-cytoplasmic side, which can be both the extra-cellular area and intra-organellar volume. This positive current is drawn upwards in all representations of current traces and I/V plots.

A tetraploid strain of *Saccharomyces cerevisiae* (YCC78, *MAT*a,a,α,α, *ura3-52 ade2-101*, (40)) was used throughout since it contains larger cells than haploid strains. It should be emphasized however, that patch clamp experiments are usually not limited by the size of the cells. Nevertheless, large cells are more convenient and easier to work with than small cells. General methods for growing, handling and protoplasting yeast and for isolating vacuoles have previously been described in detail (34).

RESULTS AND DISCUSSION

The whole vacuole configuration was obtained in symmetric standard solutions (150 mM KCl, 5 mM $MgCl_2$, 0.5 mM EGTA, pH 7 with Tris/MES). The membrane voltage was clamped at 0 mV and current recordings were commenced when the membrane current was stable at approximately 0 pA. Addition of 5 mM ATP to the bath solution induced a nearly instantanous current after a short lag time of about 10 to 15 s (Fig. 4). This *per definitionem* outward current reached a maximum of up to 15 pA ± 6 pA (n=10 vacuoles), corresponding to about 30 mAm^{-2} ± 7 mAm^{-2}. These current values are comparable to the plant V-ATPase current densities which are reported to be between 5 mAm^{-2} and 23 mAm^{-2} (41, 42).

It is remarkable that the current declined slowly and reached the baseline level within 15 to 20 min in the sustained presence of ATP. There is evidence that the inhibition of the pump results from tightly bound ADP at the catalytic side of the enzyme rather than by a chaotropic effect which has been reported for plant V-ATPases (54). For example, high salt concentrations – namely with chaotropically acting dissociated anions such as Cl^- and NO_3^- - in presence of ATP, have been shown to be responsible for the dissociation of the V_1 and V_O subunits of plant, fungal and animal V-ATPases (27, 43, 44). The loss of the activity of the V-ATPase and the regain, is reported to be a controlled mechanism in response to changed extracellular conditions.

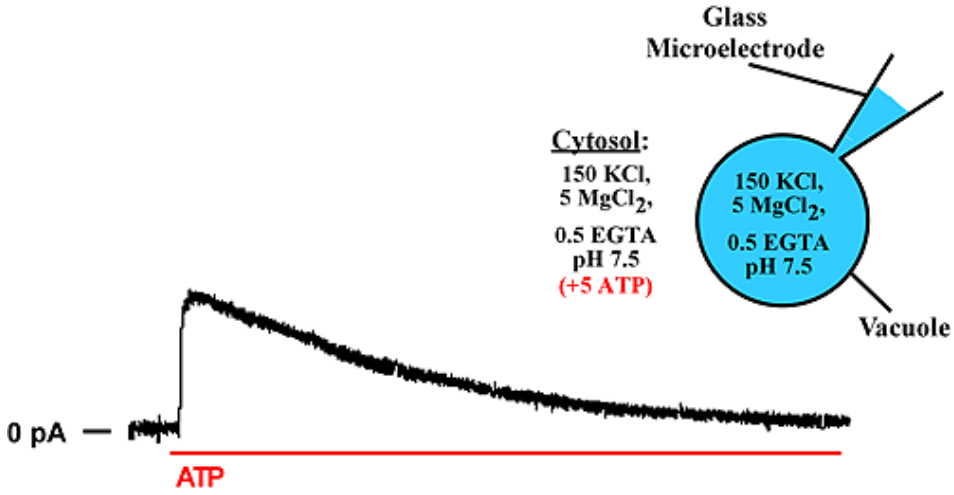

Figure 4. Whole vacuolar recording of ATP dependent current at 0 mV clamping voltage. The current reaches a maximum and decreases with ATP continuously present (bar of the bottom of the current trace).

In order to see whether this ATP-induced current was indeed generated by the V-type ATPase and not by other ATP-dependent transport systems inserted within the tonoplast, experiments were conducted in the presence of bafilomycin A_1. This substance and other bafilomycin-derived agents are known to be highly specific and potent inhibitors of V-type ATPases, whilst F-type and P-type ATPases are only slightly or not at all affected (45, 46). The addition of 100 nM bafilomycin A_1 to the bath solution containing ATP at the peak of the ATP-induced current resulted in a rapid decline of this current to the zero current level (Fig. 5). This complete inhibition of the ATP-induced current is evidence for the supposition that this current was indeed solely generated by the V-ATPase. Consequently, bafilomycin is a well-suited candidate to distinguish between V-ATPase activity and the activity of other ATP-dependent transport systems. Under these experimental conditions, the activity of other ATP-dependent transport system, such as Ca^{2+}-ATPase (PMC1) or ABC-transporter YCF1, was not detected.

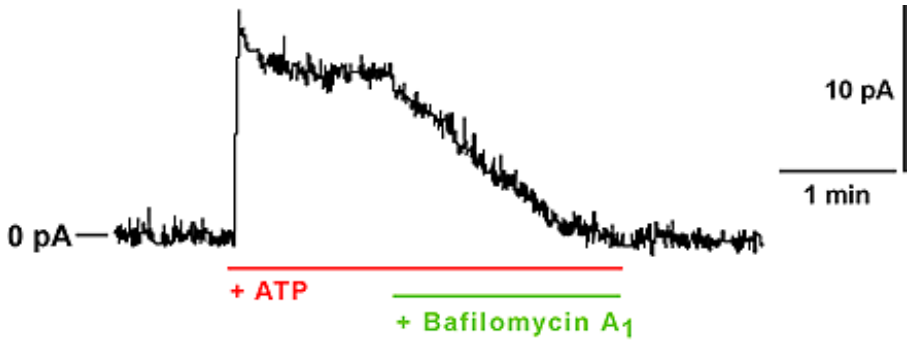

Figure 5. The ATP-induced whole vacuolar currents are bafilomycin-sensitive indicating that the ATP driven current is mediated by the vacuolar H^+-ATPase.

For definite identification of the ATP dependent current as the activity of the V-ATPase, it was necessary to study the key thermodynamic determinant of a pump which is the transport coupling ratio. The transport coupling ratio, which is often incorrectly described as stoichiometry of transports, is defined as the number of protons per ATP molecule hydrolysed.

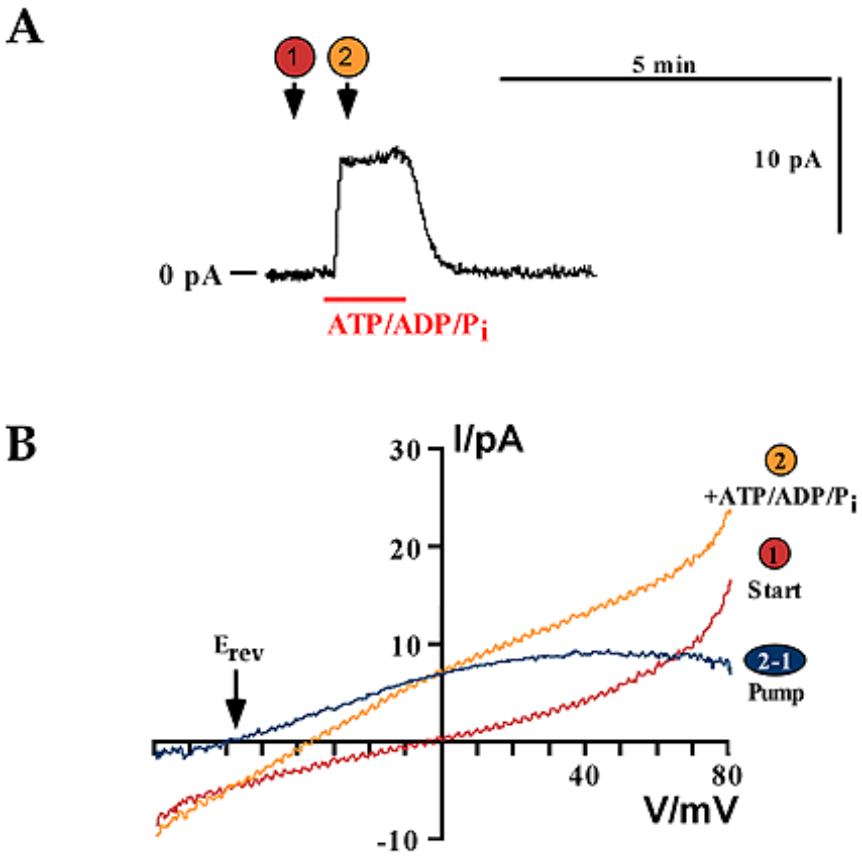

Figure 6. Determination of E_{rev} for the V-ATPase in presence of ATP, ADP and P_i (I). **A)** Whole vacuolar current trace at 0 mV in standard solution at pH_{cyt} 7.5 and pH_{vac} 5, and subsequent additon of 5 mM ATP, 5 mM ADP and 10 mM P_i. **B)** Current-voltage (I/V) characteristics of the vacuolar membrane in absence (*1*) and presence (*2*) of ATP/ADP and P_i. Subtraction (*2-1*) yields the pump characteristics. The intersection of this I/V curve with the voltage axis determines E_{rev}.

For example, H^+-translocating P-type ATPases in the plasma membrane of plants and fungi energize the transport of a single proton by the hydrolysis of one molecule ATP. They generate a membrane voltage up to –400 mV which is used to drive other transport systems, however, the pH gradient yields only about one pH unit. By contrast, plant V-ATPases transport 2 to 3 protons under hydrolysis of one molecule ATP and generate a steep pH gradient of up to 5 pH units (as for example in the vacuoles of lemon fruits (47)) but the voltage across the tonoplast yields only to –30 to –50 mV (see also Section 4).

According to previously published methods (48), the estimation of the coupling ratio was carried out by analysis of the I/V characteristics of the vacuolar membrane under different cytosolic and vacuolar pH conditions. The characteristics were obtained both in absence of ATP/ADP and P_i and in the presence of 5 mM ATP, 5 mM ADP and 10 mM P_i after the current reached its maximum (Fig. 6A).

As depicted in Fig. 6B, the first curve of the I/V plot shows the electrical properties of the entire vacuolar membrane which are determined by the ATP independent transport systems. The second trace consists of the sum of ATP dependent and independent transport systems. The result from the subtraction of both these current traces gives the characteristics of the pump which are marked by (i) sigmoidal shape with saturation of the current towards the limit of the applied voltage, (ii) the corresponding short circuit current at 0 mV in the current trace (Fig. 6A), and (iii) the intersection of the I/V curve with the voltage axis which represents the reversal potential of the pump and determines the thermodynamic equilibrium at which no ATP-dependent proton net-flux occurs. The value of the reversal potential was then used to calculate the coupling ratio according following equation:

$$\Delta\Psi = E_{rev} = \frac{59mV}{n} * \left(\log\left(K_{ATP} * \frac{[ATP]}{[ADP]*[P_i]} \right) + n * \log\left(\frac{[H^+]_c}{[H^+]_v} \right) \right)$$

where $\Delta\Psi$ is the membrane potential and corresponds to the reversal potential (E_{rev}), n is the coupling ratio of H^+ translocated per ATP hydrolysed, K_{ATP} is the equilibrium constant for ATP hydrolysis, the square brackets denote the activities of ATP, ADP and inorganic P (P_i) and the subscripts c and v refer to the cytosolic and vacuolar solution, respectively. The value for K_{ATP} depends strongly on pH and free pMg^{2+} of the ATP solution and was calculated using ΔG^0, the values for the free energy of ATP hydrolysis, given by the following equations:

$$\Delta G^0 = -RT * \ln K_{ATP}$$
$$\Rightarrow K_{ATP} = e^{-\Delta G^0 / RT}.$$

The values for ΔG^0 were obtained from Alberty (49) and, when pH and pMg^{2+} were taken into account, yielded the corresponding values for K_{ATP}.

Fig. 7 shows a set of several I/V characteristics of the pump at different cytosolic pH values and at a constant vacuolar pH of 5. The reversal potential of the pump is shifted toward negative voltages at higher pH values of the cytosol, i.e. from –20 mV (pa_{ct} 8.5) to –60 mV (pa_{ct} 6). At symmetrical pH 5, E_{rev} could not be determined because the intersection of the current curve with the voltage axis might be outside of the applied voltage. Table 1 shows that corresponding to the shift of the reversal potential, an increase of the calculated coupling ratios occurs from 2.5 H^+/ATP at pH 8.5 to 4.1 H^+/ATP at pH 6.

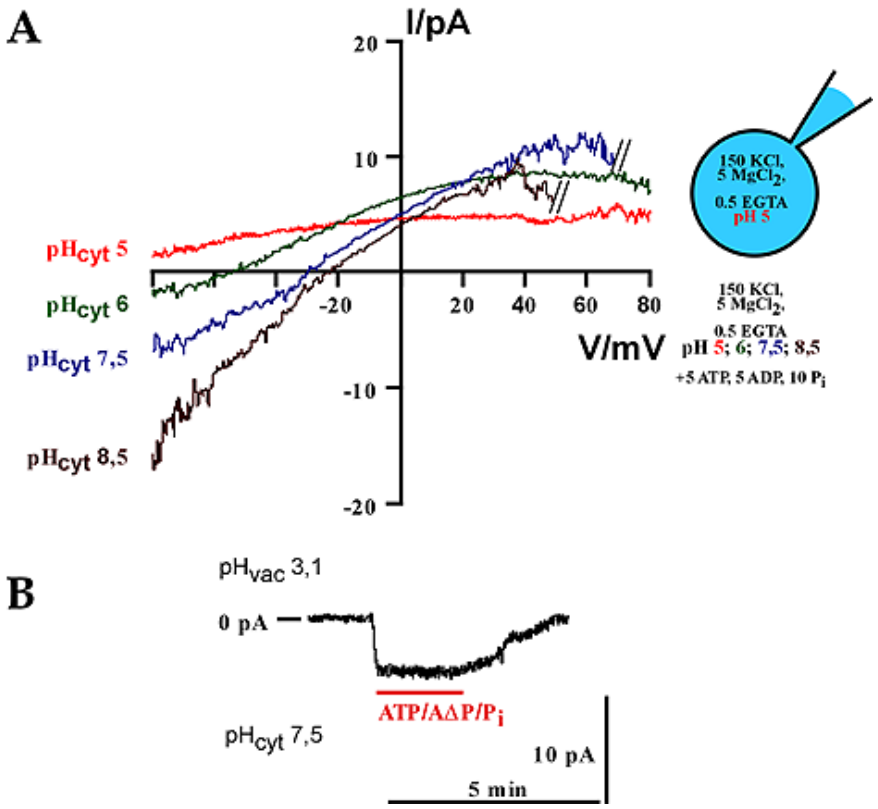

Figure 7. Determination of E_{rev} of the V-ATPase (II). **A)** Set of I/V characteristics of the pump showing dependence on cytosolic pH at constant vacuolar pH 5. E_{rev} shifts towards negative voltages with acidification of the cytosol. These E_{rev} values were used to calculate the transport coupling ratios. **B)** With the membrane voltage held at 0 mV and with a vacuolar pH of 3.1, the addition of ATP/ADP and P_i induced an inward current. The pump worked obviously in the reverse mode suggesting ATP synthesis.

At various vacuolar pH and at constant cytosolic pH of pH 7.5, the analysis of the pump characteristics shows a shift of the reversal potential towards positive voltages with acidification of the vacuolar lumen, i.e. from –40 mV (pH_{vac} 6) to –25 mV (pH_{vac} 4.1).

At symmetrical pH 7.5, the intersection of the current curve could also not be determined. Corresponding to the shift of the reversal potentials, the calculated coupling ratios decreased from 4.1 H^+/ATP at pH 6 to 2.3 H^+/ATP at pH 4.1 (Tab. 1).

It is remarkable that with the membrane voltage held at 0 mV and with a vacuolar pH of 3.1, the addition of ATP induced an inward current, out of the vacuole into the cytoplasm (Fig. 7). The pump worked obviously in the reverse mode under these conditions which suggests ATP synthesis coupled to the translocation of about 2.5 protons, however, we did not carry out any experiments to detect synthesized ATP molecules. This reversible behaviour of the pump has also been reported from plant V-ATPases (50).

Under physiological conditions (pH_{cyt} 7 to 8 and pH_{vac} 4 to 5), the coupling ratios were estimated to be between 2 and 3 H^+/ATP.

Table 1. Coupling ratios of the V-ATPase in dependence of ΔpH across the vacuolar membrane

pH_{vac} 5	**pH_{cyt}**	**E_{rev} ± SE (mV)**	**Coupling Ratios ± SE H^+/ATP**
	8.5	-20.2 ± 4	2.55 ± 0.05
	7.5	-27.7 ± 5.1	2.98 ± 0.1
	6	-58.3 ± 9.1	4.15 ± 0.3
	5	n.d.	n.d.
pH_{cyt} 7.5	**pH_{vac}**	**E_{rev} ± SE (mV)**	**Coupling Ratios ± SE H^+/ATP**
	7.5	n.d.	n.d.
	6	-37.3 ± 5.5	4.15 ± 0.2
	5	-31.8 ± 7.8	2.91 ± 0.12
	4.1	-25.7 ± 4.1	2.31 ± 0.05
	3.1	+50 ± 8	2.49 ± 0.09

The data show that the V-ATPase incompletely couples proton transport across the vacuolar membrane to the hydrolysis of ATP. The coupling ratios are non-integer, variable and dependent on ΔpH across the vacuolar membrane. These properties of the coupling ratios as well as their values are consistent with those found in plant vacuoles (41, 51). This phenomenon can be best described as slippage and tunnelling of the pump. The slippage effect was observed

for both plant and yeast V-ATPase: Due to the reduced acidification of the cytoplasm, the decreased coupling ratio demonstrates that the pump remains catalytically active even although occupancy of the H^+ binding sites was incomplete. By contrast, tunnelling, which is described by increasing coupling ratios when the cytoplasm was acidified, indicates that protons may have overcome the tonoplast without coupling to ATP hydrolysis (52).

The yeast V-ATPase shows high coupling ratios and low membrane voltages at pump equilibrium. This indicates that the capacity of the pump generates a steep proton gradient rather than high membrane voltages. Thus, the V-ATPase effectively maintains cytosolic pH homeostasis and generates a powerful *pmf* to drive the co transport of other substrates across the tonoplast.

CONCLUSION

The results presented here, demonstrate the feasibility of the electrophysiological recording techniques developed to investigate both the biophysical properties of ATP-driven active transport across the tonoplast of *S. cerevisiae* and other transport systems lacking channel properties such as cotransporters with a coupling ratio greater than 1.

Furthermore, this work also shows that computers are powerful tools for purposes other than doing *in-silico* biology, retrieving databases and modelling.

In the area of electrophysiology computers and appropriate software packages effectively help doing experiments by recording data in nearly real-time. Online analysis during the current experiments allows redesigning of the experimental setup if required. Collecting experimental data by computers allows storage and archiving of huge amounts of data on hard disks. Dependent of the sampling rate and filter setting as well as the extent of the experimental protocol, data amounts of up to 5 MB per experiment have to be stored. The great advantage of digitalisation of experimental data is the simple access to these data for analysis and preparation for publication.

Furthermore, the integration of hardware functions such as filter, oscilloscope, and voltage generator into software greatly reduces the equipment setup. Last but not least, this configuration makes it easy to combine electrophysiological approaches simultaneously with visualisation methods such as Ca^{2+}-imaging.

In conclusion, the predominant aim of this review was to show that only a combination of diverse methods and techniques as well tools – *in-silico* and "in-reality" – yield a comprehensive insight to the structure and function of complex molecules such as the vacuolar proton pump.

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REFERENCES

[1] Bradshaw, R. A. (2002). Proteomics – Boom or bust? *Molecular & Cellular Proteomics* **1**(3):177-178.

[2] Nelissen, B., Mordant, P., Jonniaux, J.-L., De Wachter, R., Goffeau, A. (1995). *FEBS Lett*. **377**:232-236.

[3] André, B. (1995). An overview of membrane transport proteins in *Saccharomyces cerevisiae*. *Yeast* **11**:1575 – 1611.

[4] Bassett, D. E., Jr. et al. (1997). Genome cross-referencing and XREFdb : Implications for the identification and analysis of genes mutated in human disease. *Nature Genetics* **15**:339-344.

[5] Klionsky, D. J., Nelson, H., Nelson, N. (1992). Compartment acidification is required for efficient sorting of proteins to the vacuole of *Saccharomyces cerevisiae*. *J. biol. Chem.* **267**:3416-3422.

[6] Klionsky, D. J. , Herman, P. K., Emr. S. D. (1990). The fungal vacuole: Composition, function and biogenesis. *Microbiol. Rev.* **54**:266-292.

[7] Bowman, B. J., Vazquez-Laslop, N., Bowman, E.J. (1992). The vacuolar ATPase of *Neurospora crassa. J. Bioenerg. Biomembr.* **24**:361-369.

[8] Davies, R. H. (1986). Compartmental and regulatory mechanisms in the arginine pathways of *N. crassa* and *S. cerevisiae. Microbiol. Rev.* **50**:280-313.

[9] Anraku, Y., Umemoto, N. Hirata, R., Wada, Y. (1989). Structure and function of the yeast vacuolar membrane proton ATPase. *J. Bioenerg. Biomembr.* **21**:589-603.

[10] Hille, B. (1992). Ion channels of excitable membranes. Sinauer Ass. Inc. Sunderland.

[11] Bertl, A. & Slayman, C. L. (1990). Cation-selective channels in the vacuolar membrane of *Saccharomyces*: Dependence on calcium, redox-state and voltage. *Proc. Natl. Acad. Sci. USA* **87**:7824-7828.

[12] Bertl, A., Gradmann, D., Slayman, C. L. (1992). Calcium- and voltage-dependent ion channels in *Saccharomyces cerevisiae*. *Phil. Trans. R. Soc. Lond.* **338** :63-72.

[13] Wada, Y., Ohsumi, Y., Tanifuji, M., Kasai, M., Anraku, Y. (1987). Vacuolar ion channel of the yeast, *Saccharomyces cerevisiae. J. biol. Chem.* **262**:17260-17263.

[14] Tanifuji, M., Sato, M., Wada, Y., Anraku, Y., Kasai, M. (1988). Gating behaviours of a voltage-dependent and Ca^{2+}-activated cation channel of yeast vacuolar membrane incorporated into planar lipid bilayer. *J. Membr. Biol.* **106**:47-55.

[15] Ohsumi, Y. & Anraku, Y. (1981). Active transport of basic amino acids driven by a proton motive force in vacuolar membrane vesicles of *S. cerevisiae. J. biol. Chem.* **258**:2079-2082.

[16] Ohsumi, Y. & Anraku, Y. (1983). Calcium transport driven by a proton motive force in vacuolar membrane vesicles of *Saccharomyces cerevisiae. J. biol. Chem.* **258**:5614-5617.

[17] Sato, T., Ohsumi, Y., Anraku, Y. (1984). An arginine/histidine exchange transport system in vacuolar membrane vesicles of *Saccharomyces cerevisiae. J. biol. Chem.* **259**:11509-11511.

[18] Sato, T., Ohsumi, Y., Anraku, Y. (1984). Substrate specificities of active transport systems for amino acids in vacuolar-membrane vesicles of *Saccharomyces cerevisiae. J. biol. Chem.* **259**:11505-11508.

[19] Paulsen, I. T., Sliwinski, M. K., Nelissen, B., Goffeau, A., Saier, M. H. Jr. (1998). Unified inventory of established and putative transporters encoded within the complete genome of *Saccharomyces cerevisiae. FEBS Lett.* **430**:116-125.

[20] Kakinuma, Y., Ohsumi, Y., Anraku, Y. (1981). Properties of H^+-translocating adenosine triphosphatase in vacuolar membrane of *Saccharomyces cerevisiae. J. biol. Chem.* **256**:10859-10863.

[21] Mitchell, P. & Moyle, J. (1965). Stoichiometry of proton translocation through the respiratory chain and adenosine triphosphate systems of rat liver mitochondria. *Nature* **208**:147-151.

[22] Nelson, N., Perzov, N., Cohen, A., Hagai, K., Padler, V., Nelson, H. (2000). The cellular biology of proton-motive force generation by V-ATPases. *J. exp. Biol.***203**:89-95.

[23] Nelson, N. & Harvey, W. R. (1999). Vacuolar and plasma membrane proton-adenosinetriphosphatases. *Physiological Reviews* **79**(2): 361-385.

[24] Sze, H., Ward, J. M., Lai, S. (1992). Vacuolar H^+-translocating ATPases from plants: Structure, function and isoforms. *J. Bioenerg. Biomembranes* **24**(4): 371-380.

[25] Stevens, T. H. & Forgac, M. (1997). Structure, function and regulation of the vacuolar H^+-ATPase. *Annu. Rev. Cell Dev. Biol.* **13**:779-808.

[26] Bowman, B. J., Dschida, W. J., Bowman, E. J. (1992). Vacuolar ATPase of *Neurospora crassa*: Electron microscopy, gene characterization and gene inactivation/mutation. *J. exp. Biol.* **172**:57-66.

[27] Lüttge, U., Fischer-Schliebs, E., Ratajczak, R., Kramer, D., Berndt, E., Kluge, M. (1995). Functioning of the tonoplast vacuolar C-storage and remobilization in crassulacean acid metabolism. *J. exp. Bot.* **46**:1377-1388.

[28] Nelson, N. (1992). The vacuolar H^+-ATPase – one of the most fundamental ion pumps in nature. *J. exp. Biol.* **172**:19-27.

[29] Hirata, R., Ohsumi, Y., Nakano, A., Kawasaki, H., Suzuki, K., Anraku, Y. (1990). Molecular structure of a gene, VMA1, encoding the catalytic subunit of H^+-translocating adenosine triphosphatase from vacuolar membranes of *Saccharomyces cerevisiae*. *J. biol. Chem.* **265**:6726-6733.

[30] Kane, P. M., Yamashiro, C. T., Wolczyk, D. R., Neff, N., Goebl, M., Stevens, T. H. (1990). Protein splicing converts the yeast TFP1 gene product to the 69-kDa subunit of the vacuolar H^+-adenosine triphosphatase. *Science* **250**:651-657.

[31] Webster, L. C. & Apps, D. K. (1996). Analysis of nucleotide binding by a vacuolar proton-translocating adenosine triphosphatase. *Eur. J. Biochem.* **240**:156-164.

[32] Nelson, N. (1989). Structure, molecular genetics and evolution of vacuolar H^+-ATPase. *J. Bioenerg. Biomembr.* **21**:553-571.

[33] Mewes, H. W., Frishman, D., Güldener, U., Mannhaupt, G., Mayer, K., Mokrejs, M., Morgenstern, B., Münsterkoetter, M., Rudd, S., Weil, B. (2002). MIPS: a database for genomes and protein sequences. *Nucleic Acids Research* **30**(1):31-4; http://mips.gsf.de/proj/yeast/catalogues/funcat/fc40_25.html.

[34] Uchida, E., Ohsumi, Y., Anraku, Y. (1985). Purification and properties of H^+-translocating, Mg^{2+}-adenosine triphosphatase from vacuolar membranes of *Saccharomyces cerevisiae*. *J. biol. Chem.* **260**:1090-1095.

[35] Bertl, A., Bihler, H., Kettner, C., Slayman, C. L. (1998). Electrophysiology in the eukaryotic model cell *Saccharomyces cerevisiae*. *Pflügers Arch.* **436**:999-1013.

[36] Hamill, O. P., Marty, A., Neher, E., Sakmann, B., Sigworth, F. J. (1981). Improved patch clamp techniques for high resolution current recording from cells and cell-free membrane patches. *Pflügers Arch.* **391**:85-100.

[37] Sakmann, B. & Neher, E. (1995). Single channel recording. 2nd Ed. Plenum, New York.

[38] Marty, A. & Neher, E. (1983). Tight seal whole-cell recording. Chapter 7 in: Single Channel Recording. 1st Ed. Editors Sakman, B. & Neher, E., Plenum Press New York.

[39] Bertl, A., Blumwald, E., Coronado, R., Eisenberg, R., Findlay, G., Gradmann, D., Hille, B., Köhler, K., Kolb, H.-A., MacRobbie, E., Meissner, G:, Miller, C., Neher, E., Palade, P., Pantoia, O., Sanders, D., Schroeder, J., Slayman, C. L., Spanswick, R., Walker, A., Williams, A. (1992). Electrical measurements on endomembranes. *Science* **258**:873-874.

[40] Mirzayan, C., Copeland, C. S., Snyder, M. (1992). The NUF1 gene encodes an essential coiled-coil related protein that is a potential component of the yeast nucleoskeleton. *J. Cell Biol.* **16**:1319-1332.

[41] Coyaud, L., Kurkdjian, A., Kado, R., Hedrich, R. (1987). Ion channels and ATP-driven pumps involved in ion transport across the tonoplast of sugarbeet vacuoles. *Biochem. Biophys. Acta* **902**:263-268.

[42] Davies, J. M., Hunt, I., Sanders, D. (1994). Vacuolar H^+-pumping ATPase variable transport coupling ratio controlled by pH. *Proc., Natl. Acad. Sci. USA* **91**:8547-8551.

[43] Puoplo, K. & Forgac, M. (1990). Functional reassembly of the coated vesicle proton pump. *J. Biol. Chem.* **265**:14836-14841.

[44] Kane, P. M. (2000). Regulation of V-ATPases by reversible disassembly. *FEBS Lett.* **469**:137-141.

[45] Bowman, E. J., Siebers, A., Altendorf, K. H. (1988). Bafilomycins: A class of inhibitors of membrane ATPases from microorganisms, animal cells and plant cell. *Proc. Natl. Acad. Sci. USA* **85**:7972-7975.

[46] Dröse, S., Bindseil, K. U., Bowman, E. J., Siebers, A., Zeeck, A., Altendorf, K. (1993). Inhibitory effect of modified bafilomycins and concanamycins on P- and V-type ATPases. *Biochemistry* **32**:3902-3906.

[47] Sinclair, W. B. (1984). The biochemistry and physiology of the lemon and other citrus fruits, pp. 109 – 156, University of California, Division of Agriculture and Natural Resources, Oakland, CA.

[48] Rea, P. A. & Sanders, D. (1987). Tonoplast energization: two H^+ pumps, one membrane. *Physiologia Plantarum* **71**:131-141.

[49] Alberty R. A. (1968). Effect of pH and metal ion concentration on the equilibrium hydolysis of adenosine triphosphate to adenosine diphosphate. *J. biol. Chem.* **243**:1337-1343.

[50] Hirata, T., Nakamura, N., Omote, H. Wada, Y., Futai, M. (2000). Regulation and reversibility of vacuolar H^+-ATPase. *J. biol. Chem.* **275**:386-389.

[51] Müller, M. L., Jensen, M., Taiz, L. (1999). The vacuolar H^+-ATPase of lemon fruits is regulated by variable H^+/ATP coupling and slip. *J. biol. Chem.* **274**:10706-10716.

[52] Läuger, P. (1991). Electrogenic ion pumps. Sinauer Ass. Inc. Sunderland.

[53] Arata, Y., Baleja, J. D., Forgac, M. (2002). Cysteine-directed cross-linking to subunit B suggests that subunit E forms part of the peripheral stalk of the vacuolar H^+-ATPase. *J. biol. Chem*. **277**:3357-3363.

[54] Kettner, C., Obermeyer, G., Bertl, A. (2003). Inhibition of the yeast V-type ATPase by cytosolic ADP. *FEBS Lett*. **535**:119 – 124.