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Modulation of the vacuolar H^+-ATPase by adenylates as basis for the transient CO_2-dependent acidification of the leaf vacuole upon illumination

Karl-Josef Dietz [a,*], Ulrich Heber [b], Tetsuro Mimura [c]

[a] *Lehrstuhl für Stoffwechselphysiologie und Biochemie der Pflanzen, Universität Bielefeld, 33615 Bielefeld, Germany*
[b] *Julius-von-Sachs-Institut für Biowissenschaften, Julius-Maximilians-Universität Würzburg, Julius-von-Sachs-Platz 2, 97082 Würzburg, Germany*
[c] *Biological Laboratory, Hitotsubashi University, Naka 2-1, Kunitachi, Tokyo 186, Japan*

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Abstract

Using tonoplast vesicles, we have investigated the activity of the vacuolar H^+-ATPase which is the dominant proton pump at the tonoplast of mesophyll cells. Bafilomycin-sensitive ATP hydrolysis or acidification of tonoplast vesicles in the presence of ATP were measured at varying ATP, ADP and P_i concentrations, and in the presence of oxidized or reduced glutathione. Increased ATP/ADP ratios as reported for the extrachloroplast cytoplasm during the induction phase of photosynthesis at high or low CO_2 (P. Gardeström, Biochim. Biophys. Acta 1183 (1993) 327–332) increased the activity of the V-ATPase in simulation experiments with vesicles. Depending on reported subsequent decreases in cytoplasmic ATP/ADP ratios in the presence of high or low CO_2, the ATPase activity of tonoplast vesicles changed in simulation experiments to lower values. More than 10 mM phosphate was required to decrease the ATPase activity in vesicles significantly at ATP/ADP ratios of 3 or higher, indicating that ATPase activity is controlled more by ratios of ATP to ADP than by phosphorylation potentials (ATP)/(ADP)(P_i). Oxidized glutathione was inhibitory. The results permit interpretation of the observation that on illumination of previously darkened leaves the pH of the vacuoles of mesophyll cells decreases indicating energized transport of protons across the tonoplast into acidic vacuoles, and that the extent of vacuolar acidification depends on the CO_2 concentration of the surrounding air (Z.-H. Yin, S. Neimanis, U. Heber, Planta 182 (1990) 253–261). We conclude that short term control of tonoplast ATPase activity in leaves during dark/light transients can essentially be understood on the basis of reported changes in cytoplasmic ATP/ADP ratios, with a possible participation of redox modulation.

Keywords: ATPase; pH homeostasis; Proton pumping; Redox control; Tonoplast

1. Introduction

A vacuolar-type H^+-ATPase occurs in all eukaryotic cells. It exports H^+ from the cytosol into the vacuole and other lysosomal organelles. Main physiological functions of the V-type ATPase are the maintenance of cytosolic pH homeostasis and crea-

Abbreviations: CDCF, 5-carboxy-2′,7′-dichlorofluorescein; GSH/GSSG, reduced/oxidized glutathione; P_i, inorganic phosphate

* Corresponding author. Fax: +49 (521) 1066039; E-mail: karl-josef.dietz@biologie.uni-bielefeld.de

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tion of a membrane potential and a proton motive force for energized transport of solutes into acidic vacuoles [3]. The enzyme has a complex subunit structure similar to that of the chloroplast ATPase, but in contrast to the latter enzyme little is known on its regulation. In intact leaves the activity of the H^+-ATPase can be monitored using pH-indicating dyes [2,4,5]. Following illumination of leaves, the cytosolic pH increases transiently and the vacuolar lumen acidifies. The kinetics and extent of the vacuolar acidification depend on the developmental state of the leaves, the photon fluence rate and the CO_2 concentration of the atmosphere which determines photosynthetic flux and the corresponding energy turnover.

Gardeström [1] has published a detailed analysis of metabolite levels in the chloroplast and extrachloroplast compartments during the initial phase of photosynthetic induction at low and high CO_2 concentrations. Using these data we have attempted to answer the question whether the light-induced and CO_2-dependent acidification of mesophyll vacuoles in leaves can be understood on the basis of cytosolic changes in the concentration or ratios of ATP, ADP, P_i, involving, possibly, also redox control of the vacuolar H^+-ATPase.

2. Methods

2.1. Monitoring of the vacuolar pH in intact leaves

The fluorescent dye 5-carboxy-2′,7′-dichlorofluorescein (CDCF, Molecular Probes, Eugene, OR, USA) was fed to excised leaves of *Fuchsia hybrida* for 4 h via the petiole. Gas flow, fluorescence excitation and emission were as described in [4]. For control purposes, a dextran conjugate of the fluorescein isothiocyanate-dextran conjugate (MW 4400, Sigma Chemicals, Deisenhofen, Germany) was infiltrated into the apoplast of leaves using the technique described by Jakob and Heber [6].

2.2. Measurement of ATPase activity

Tonoplast membranes were isolated from freshly harvested primary leaves of 9–11 day old barley seedlings. Leaves equivalent to 10 g fresh weight were ground into a buffer containing 250 mM sucrose, 50 mM Tris-Cl, pH 8.0, 8 mM EDTA, 0.12 mM phenylmethylsulfonylfluoride, 4 mM dithiothreitol (DTT). The homogenate was filtered through two layers of cotton cloth and one layer of nylon net with a mesh size of 10 μm. The suspension was spun at $8000\times g$ for 10 min; the supernatant was spun again at $8000\times g$ for 10 min, and then at $25\,000\times g$ for 30 min. The post-mitochondrial membrane pellet of the last centrifugation step was resuspended in 600 μl of a buffer containing 250 mM sucrose, 5 mM PIPES-KOH, pH 7.2, 0.5 mM DTT and layered on top of a two-step sucrose gradient of 600 μl 30% and 700 μl 35% sucrose solution supplemented with 5 mM PIPES-KOH, pH 7.2 and 0.5 mM DTT. Following centrifugation at $45\,000\times g$ for 2 h, the tonoplast enriched membrane fraction was taken from the 30%/35% interphase, sedimented and used for the experiments at a protein concentration of 10 μg in 150 μl reaction mix for tonoplast H^+-ATPase-dependent ATP hydrolysis. The assay contained 25 mM Tricine-Tris, pH 8.0, 50 mM KCl, 0.1 mM Na_2MoO_4, 0.002% (w/v) Brij 58, 2 mM $MgSO_4$, and, if not indicated otherwise, 1 mM ATP. Duplicate samples were incubated for each condition. Enzymic hydrolysis was terminated by transfer of 70 μl aliquots to 210 μl of Bencini test solution containing 100 mM Zn acetate, 15 mM $(NH_4)_2MoO_4$, 20 mM Na acetate, adjusted to pH 4.5 with HCl. In an alternative set of experiments, quinacrine quenching in tonoplast vesicle preparations was employed as direct measure of proton pumping activity. In a total volume of 500 μl, the assay contained 50 mM Tricine-Tris, pH 8.0, 2 μM quinacrine, 30 μg membrane protein, and ATP and other effectors or inhibitors at concentrations as indicated. Proton pumping was initiated by injection of 4 mM $MgSO_4$.

3. Results

Leaves of *F. hybrida* were used for in situ studies of vacuolar acidification rather than barley leaves which served as the source for tonoplast vesicles because homogenates of *Fuchsia* leaves have a pH close to 4.5 suggesting acidic vacuoles, whereas homogenates of barley leaves have a pH of about 6.2. With a

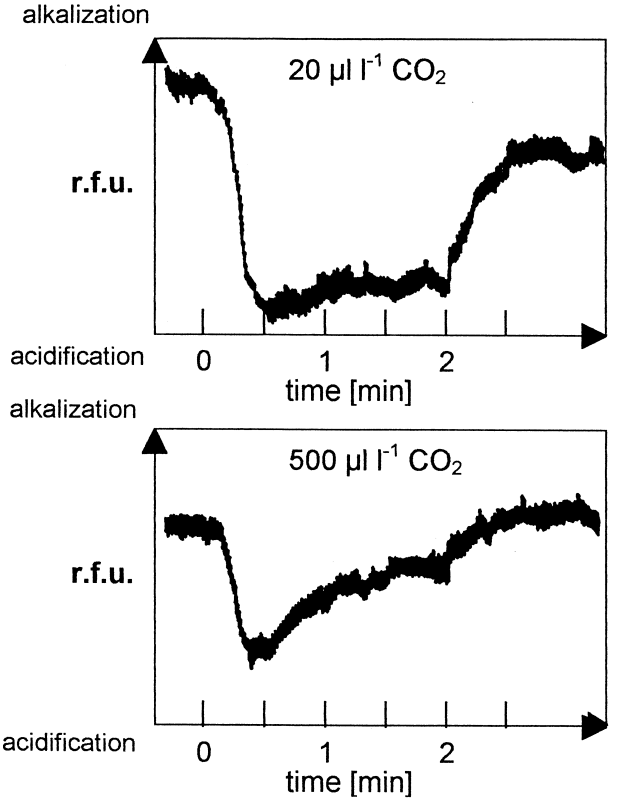

Fig. 1. Light-dependent change in CDCF fluorescence emission of a *F. hybrida* leaf upon illumination with 650 µmol quanta m^{-2} s^{-1}. Dark adapted leaves were illuminated at time 0. The leaves were darkened again after 2 min of illumination. The y-axes give relative fluorescence units. An increase in fluorescence corresponds to vacuolar alkalinization, a decrease in fluorescence yield indicates acidification of the vacuole. See Yin et al. [4] for further details. The leaves were flushed either with a low or a high concentration of CO_2 as indicated.

p*K* of 4.8, CDCF is sensitive to monitor pH changes in acidic compartments without appreciable interference by simultaneously occurring opposite pH changes in neutral or slightly alkaline compartments of the cytoplasm. For the measurement depicted in Fig. 1, CDCF (0.2 mM) was fed to an excised *Fuchsia* leaf. CDCF fluorescence emission from the leaf was monitored upon illumination and redarkening of the leaf in air containing either 20 or 500 µl l^{-1} CO_2. Illumination of the leaf with a photon flux density of 650 µmol quanta m^{-2} s^{-1} produced an acidification signal which is attributed to a pH decrease in the vacuoles [4] because it is kinetically different from a light-dependent apoplastic acidification signal which has been monitored after the introduction of another fluorescent pH indicator (C-NERF) into the apoplast of leaves (unpublished experiments). Upon darkening, the vacuolar lumen was alkalized again, i.e. the fluorescence emission increased to the initial value observed in the previous dark phase. The initial kinetics of light induced vacuolar acidification was similar in leaves gassed with the high CO_2 concentration of 500 µl l^{-1} or the low CO_2 concentration. However, following a transient minimum, the fluorescence increased and reached a steady state lower than before the illumination. The fluorescence change upon darkening revealed that the steady state acidification level was small in high CO_2 compared to exposure to low CO_2. A slight vacuolar acidification was usually maintained in the light at high CO_2 even during prolonged illumination times, but at CO_2 concentrations of 1000 µl l^{-1} or higher, or at reduced light intensities, some small alkalinization of the vacuoles (i.e. efflux of protons from the vacuoles) was often observed in the light (data not shown). A detailed analysis of the dependence of the light-induced acidification signal on irradiation and CO_2 concentration has been published before [2]. The kinetics and degree of fluorescence signals originating from the vacuole varied slightly between species [5].

In order to understand different light-dependent vacuolar acidification under low or high CO_2 concentrations in the gas stream, we investigated the effects of ADP and inorganic phosphate on the activity of the tonoplast ATPase. The portion of the hydrolysis reaction specific for the tonoplast ATPase

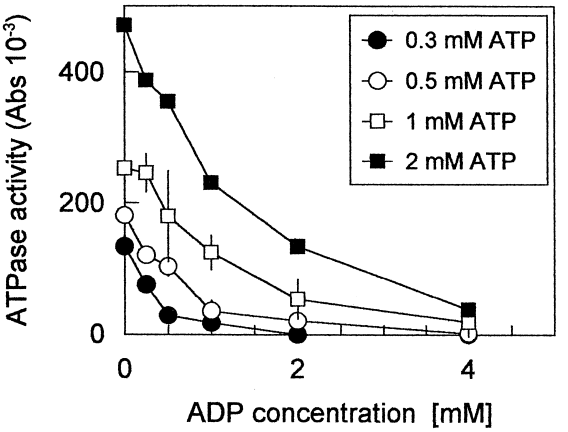

Fig. 2. Effect of increasing ADP concentration on the bafilomycin-sensitive ATP hydrolysis rate at varying ATP concentrations. Tonoplast enriched membrane vesicles of barley were tested at a protein concentration of 0.067 µg/µl. The data are means of four to eight measurements ± S.D.

was determined in each assay by control reactions performed in the presence of the specific V-type ATPase inhibitor bafilomycin at a concentration of 100 nM. All data refer to the bafilomycin-sensitive portion of ATP hydrolysis in the assay. Fig. 2 shows that ADP acted as inhibitor of ATP hydrolysis. At elevated ATP concentrations, increasing concentrations of ADP were required in order to achieve a similar degree of inhibition. The dissociation constant of the inhibitor/enzyme complex may be estimated to about 0.4 mM ADP.

The significance of the inhibition by ADP for the activity of the vacuolar ATPase in vivo was tested by simulating the changes in cytosolic ATP to ADP ratio which occur when previously darkened leaves are transferred into the light (Fig. 3). Gardeström [1] has reported a detailed analysis of the adenylate status during the first seconds and then during the following minutes after illumination of dark adapted barley leaves. In the simulation experiment, the total ATP plus ADP concentration was maintained constant at 1 mM in the assay. ATP hydrolysis rates increased when the ATP to ADP ratio increased from 2.9 in the dark to 9.4 within the first 15 s of illumination as was observed in vivo at high CO_2. The initial increase in the rate of ATP hydrolysis was similar when the low CO_2 condition was simulated. However, whereas ATPase activity remained high under the simulated low CO_2 condition, it decreased even below the dark level at the elevated CO_2 concentration.

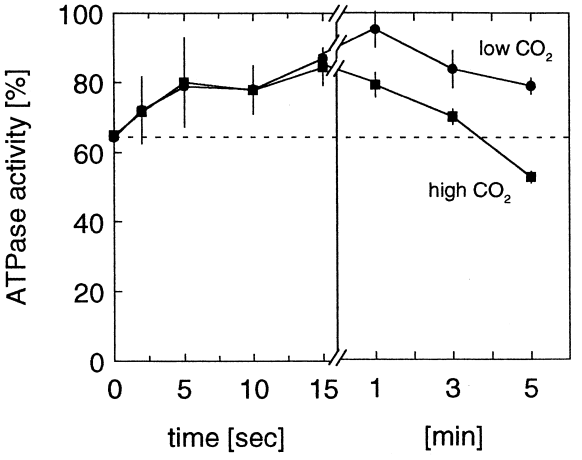

Fig. 3. ATP hydrolysis rates under conditions simulating the changes in ATP/ADP ratio during the first 5 min of illumination of previously dark adapted barley leaves. Using tonoplast enriched membrane preparations isolated from barley leaves, ATP hydrolysis was determined under the conditions reported by Gardeström [1] for the changing cytosolic ATP/ADP ratios during the first minutes of illumination ($n=4$, ±S.D.). The ATP to ADP ratios were in the low CO_2 simulation: dark, 2.9; 2 s, 5.1; 5 s, 6.0; 10 s, 6.5; 15 s, 10.5; 1 min, 13.2; 3 min, 9.1; 5 min, 7.2; in the high CO_2 simulation: dark, 2.9; 2 s, 4.3; 5 s, 6.2; 10 s, 6.6; 15 s, 9.4; 1 min, 6.0; 3 min, 4.3; 5 min, 2.2.

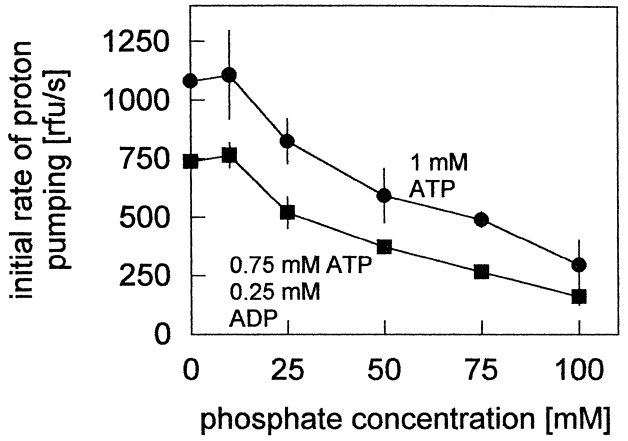

Fig. 4. Effect of increasing concentrations of inorganic phosphate on the initial H^+ pumping rate of tonoplast vesicles. Two substrate conditions were tested. In the first set of assays, ATP was present at a concentration of 1 mM. In the second set of experiments, ATP was added at a concentration of 0.75 mM, and the medium was further supplemented with ADP at a concentration of 0.25 mM. Mean values and standard deviations are indicated from four to five determinations.

This simulation experiment supports the view that light-induced changes in cytosolic adenylate status are mainly responsible for changes in the vacuolar ATPase activity which then account for the light- and CO_2-dependent changes in vacuolar acidity observed in leaves.

Other factors such as changes in the concentration of cytosolic inorganic phosphate or redox regulation of SH groups may also contribute to the regulation of the tonoplast ATPase. Therefore, pH-dependent quenching of quinacrine fluorescence in vacuolar vesicles was employed to test the effect of inorganic phosphate on the initial pumping activity of the ATPase. This experimental approach was necessary since in the ATP hydrolysis assay liberated P_i was determined as a direct measure of ATPase activity. It was not possible to reliably measure the rate of ATP hydrolysis by the liberation of P_i on top of a high level of P_i in the incubation assay. In the quinacrine assay, proton pumping was activated by addition of

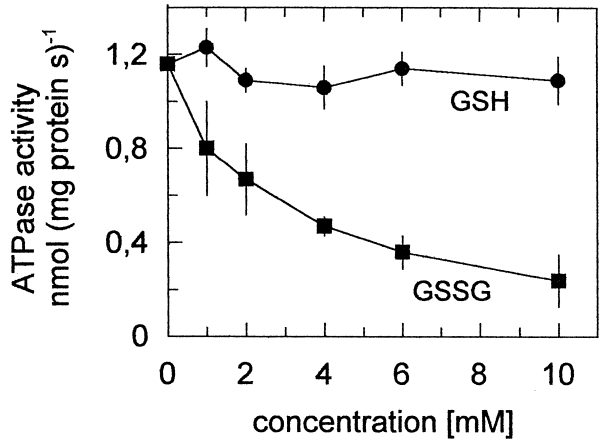

Fig. 5. Effect of glutathione in the reduced and oxidized form on ATP hydrolysis by the V-type H^+-ATPase. The data are means of four determinations. The ATP concentration was 1 mM.

Mg^{2+} to the tonoplast vesicles which were suspended in a buffer containing 1 mM Na_4ATP. The initial slope of fluorescence quenching was determined and plotted against the concentration of P_i (Fig. 4). Two conditions were tested. In the basal condition, ATP was present at a concentration of 1 mM. In the second set of experiments, 0.25 mM ADP was added to 0.75 mM ATP in order to simulate a cytosolic ATP/ADP ratio of 3. In both cases, the high concentration of 50 mM P_i was required to inhibit the rate of proton pumping by about 50%. Importantly, the ATPase activity responded to P_i by some inhibition only at P_i concentrations above 10 mM, i.e. below phosphorylation potentials (ATP)/(ADP)(P_i) of 300 M^{-1}.

Fig. 5 reveals that the ATPase is also sensitive to SH group oxidation. Its activity remained high in the presence of increasing concentrations of reduced glutathione but addition of oxidized glutathione inhibited the ATPase activity. An accurate redox dependence cannot be derived from the experiment of Fig. 5, since the ATPase had to be stabilized with dithiothreitol during the isolation of tonoplast vesicles. A small carry-over of dithiothreitol may have occurred.

4. Discussion

Developmental control of ATPase activity is known to proceed from the level of gene expression. It is realized within a time scale of days [7,8]. However, there is also strong evidence for regulation of the ATPase activity in the short term, i.e. by means of biochemical modulation of enzyme activity. This is particularly evident during the transition of leaves and algae from dark to light when proton transport across the tonoplast is accompanied by cytosolic alkalinization and vacuolar acidification [4,9], but also during cytoplasmic pH regulation when transport of excess protons from the cytoplasm into the vacuoles reestablishes original cytoplasmic pH values [10].

Usually, light induces an acidification of the vacuolar lumen which is reversed upon darkening. Little effort has been made to understand the biochemical basis of light-dependent changes in vacuolar pH values. Takeshige et al. [9] have proposed a dominant role of inorganic phosphate for the light-induced activation of the proton pumps located at the plasma membrane and the tonoplast of *Chara corallina*. Upon transfer of photosynthetic cells to the light, organic phosphate esters accumulate at the expense inorganic phosphate. The removal of inhibitory P_i was suggested to account for the activation of the pumps in the light. However, the accumulation of organic phosphate esters is slow after dark adaptation of photosynthetic systems [11,12] whereas vacuolar acidification is fast (Fig. 1). Moreover, cytosolic P_i concentrations are unlikely to be much higher than 10 mM. The data of Fig. 4 show that in this concentration range little effect on ATPase activity is to be expected.

Other possibilities for the light/dark regulation of the ATPase are changes in the level of ADP [13] or in redox modulation [14]. We show in this paper that changes in the cytosolic concentration of these effectors may indeed explain the transients observed in the vacuolar pH value upon transfer of leaves from the dark to the light and vice versa. Cytosolic ATP was reported to increase by about 20% upon illumination [1]. At a K_m value of the V-type H^+-ATPase for ATP of about 0.2–0.6 mM [9,15] and a cytosolic ATP concentration close to 1 mM, the ATP-dependent activation of the ATPase is by itself very small during a 20% increase in the ATP concentration. However, the concentration of ADP changes in the opposite direction when ATP changes. ADP acts as a competitive inhibitor of the ATPase. Thus, a combined action of ATP and ADP on the tonoplast

ATPase activity can explain vacuolar acidification during dark/light transients and effects of CO_2 on these transients as shown in Fig. 1. This is demonstrated in the simulation experiments of Fig. 3.

Since the observation by Hager and Biber [14] that chemical modification of SH groups inactivates the tonoplast ATPase, the possibility of redox control of the ATPase is also open. The inhibitory effect of GSSG (Fig. 5) may indicate that redox control can also contribute to the regulation of ATPase activity in vivo. It is well established that the redox systems of photosynthetic tissues are in a more reduced state in the light than in the dark. However, it is unlikely that glutathione is a physiological mediator since the cytosolic glutathione pool is usually highly reduced both in the light and in the dark. Thioredoxin may be a candidate for modulation of the tonoplast ATPase activity [16] as it is in the case of the chloroplast ATPase. It is noteworthy that oxidation of the yeast V-type H^+-ATPase results in a loss of vacuolar acidification of the glutathione-deficient mutant vma41-1 in vivo [17]. Nevertheless, at present it does not appear necessary to invoke redox control of the ATPase to explain vacuolar acidification in the dark/light transient as shown in Fig. 1. Reported changes in the cytosolic concentrations of ATP and ADP are sufficient for a consistent explanation. It remains to be seen whether changes in adenylates can also explain the activation of the tonoplast ATPase after cytoplasmic acidification which permits the fast pumping of excess protons from the cytoplasm into the vacuoles during pH regulation [10]. Since ATP hydrolysis consumes hydroxyl ions according to

$$ATP^{4-} + OH^- \rightarrow ADP^{3-} + P_i^{2-},$$

a decrease in pH by one unit may, at a constant phosphorylation potential, increase the ATP/ADP ratio by a factor of 10. This could produce substantial activation of the tonoplast ATPase.

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