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Different SH groups involved in H^+ translocation and PP_i hydrolysis of higher plant Mg^{2+}-PP_iase

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SH groups of the K^+-stimulated, H^+-translocating higher plant Mg^{2+} pyrophosphatase (Mg^{2+}-PP_iase) are functionally characterized. A pretreatment with 50 μM *N*-ethylmaleimide (NEM) in the absence of Mg^{2+} leads to about 50% inactivation of subsequent Mg^{2+}-PP_i-dependent H^+-pumping, whereas in the presence of Mg^{2+} no inactivation occurs. However, when PP_i and Mg^{2+} are present during the NEM pretreatment a 50% inhibition of the subsequent H^+-pumping rate is observed. In the presence of Mg^{2+} and the competitive inhibitor imidodiphosphate (IDP), the NEM pretreatment does not cause inactivation, indicating that this NEM-sensitive SH group only becomes exposed during the catalytic cycle. Conversely, PP_i hydrolysis is only slightly inhibited by the 50 μM NEM pretreatment, and Mg^{2+}-PP_i as well as Mg^{2+}-IDP protect PP_i hydrolyzing activity against NEM inactivation. The results demonstrate the presence of different SH groups involved in PP_i hydrolysis and H^+ translocation.

Mg^{2+} pyrophosphatase; Enzyme conformation; SH group; Mg^{2+}; Imidodiphosphate; (*Nicotiana tabacum*)

1. INTRODUCTION

The H^+-translocating Mg^{2+} pyrophosphatase (Mg^{2+}-PP_iase) of the tonoplast and Golgi membranes has been partially characterized for a number of plant species [1-10]. The enzyme (pH optimum at 8; K_m (PP_i) 15-60 μM in the presence of excess Mg^{2+} [3-5,10]) is dependent on K^+ and is inhibited by Na^+ [3-5,9,10]. *N*-Ethylmaleimide (NEM) and 7-chloro-4-nitrobenzo-2-oxa-1,3 diazole (NBD-Cl) inhibit the Mg^{2+}-PP_iase [1,3,8,10] indicating the presence of (an) essential SH group(s). The lower sensitivity towards dicyclohexylcarbodiimide (DCCD) suggests a different structure of the H^+-translocating portion of this enzyme as compared to other H^+ pumps [10]. Recently, Malsowski and Malsowska [11] have claimed the isolation of an H^+-translocating Mg^{2+}-PP_iase from corn by affinity chromatography; on SDS gels they found only one polypeptide of 64 kDa but an unequivocal identification was not presented.

The present study addresses the question whether the K^+-stimulated higher plant Mg^{2+}-PP_iase has different SH groups involved in H^+ translocation and PP_i hydrolysis. For this purpose the competitive inhibition of the enzyme by the substrate analogue imidodiphosphate (IDP) [1] is characterized to demonstrate its possible use as a reagent to protect the Mg^{2+}-PP_i-binding site from inactivation by NEM. The effect of an NEM pretreatment in the presence or absence of Mg^{2+}, PP_i, or IDP (or combinations of these) on subsequent H^+ translocation and PP_i hydrolysis shows that (i) different SH groups are involved in both functions, and (ii) under appropriate conditions a selective labelling of the enzyme with NEM may be possible.

2. MATERIALS AND METHODS

2.1. *Plant material*

The *Agrobacterium tumefaciens*-transformed *Nicotiana tabacum* cell clone (SR 1-C58) used in this study was a gift from

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Professor H. Van Onckelen, Antwerp, and is described elsewhere [12,13]. The transformed cell suspension culture was cultivated in the dark according to Rausch and Ranostaj [14].

2.2. *Isolation of membranes*

Cells were extracted at 4°C in a Moulinex blender with 2 ml buffer/g fresh weight: 250 mM sucrose, 100 mM Mops, 50 mM KCl, 10 mM EGTA, 20 mM ascorbic acid, 2.5 mM DTT, 0.1% (w/v) BSA, adjusted to pH 7.2 with KOH. A 20000–50000 × *g* microsomal membrane fraction was prepared as described earlier [15].

2.3. *H^+-pumping assay*

The assay medium contained 300 mM sucrose, 10 mM Hepes, 20 mM ascorbic acid, 50 mM KCl, 2.5 mM DTT, 1 mM $MgSO_4$, 0.1% (w/v) BSA, adjusted to pH 7.2 with KOH. Acridine orange (final concentration 5 μM) fluorescence (excitation, 495 nm; emission, 540 nm) was always adjusted to the same initial value by diluting with buffer. Membranes (final concentration 35 μg protein/ml) were allowed to equilibrate to room temperature. The reaction was started by adding PP_i and monitored in an FP 770 Jasco spectrofluorometer.

2.4. *Mg^{2+}-PP_iase assay*

K^+-dependent Mg^{2+}-PP_iase activity was determined as P_i release according to Cross et al. [16] with the exception that ascorbic acid (1%, w/v, final concentration) was used as reductant instead of $FeSO_4 \cdot 7H_2O$.

2.5. *Pretreatment of membranes*

Membranes were washed in extraction buffer without DTT. After resuspending in the same medium they were divided into aliquots (membrane concentration 1200 μg protein/ml) for the different treatments. Incubations with NEM and/or other additions were done at room temperature for 15 min. The pretreatment was stopped by ten-fold dilution in ice-cold extraction buffer (without DTT). The membranes were pelleted twice at 50000 × *g* for 45 min and then frozen in liquid N_2 and stored until use.

2.6. *Treatment of data*

Kinetic data for K_m or K_i determinations were obtained from 2 or 3 independent experiments by regression analysis using the Lineweaver-Burk plot. Results of Mg^{2+}-PP_iase and Mg^{2+}-PP_i-driven H^+ pumping after membrane pretreatments are means of 3 independent experiments. Standard error of the mean did not exceed 10%.

3. RESULTS

3.1. *Properties of the Mg^{2+}-PP_iase*

The microsomal membrane bound enzyme was K^+-stimulated ($K_m(K^+)$ 15 mM), had its pH optimum at 8, and a K_m for PP_i (in the presence of 1 mM Mg^{2+}) of 33 μM (not shown). The time course of H^+ translocation as monitored with acridine orange is shown in fig.1; the initial decrease of fluorescence was linear with time for about 6 min

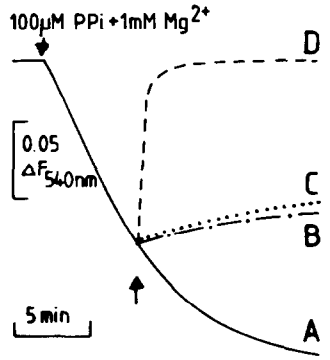

Fig.1. Time course of Mg^{2+}-PP_i-dependent H^+ pumping in microsomal vesicles from *A. tumefaciens*-transformed tobacco cells (SR 1-C58) as determined by acridine fluorescence quenching (membrane protein: 35 μg/ml). (A) Control; (B) inhibition of H^+ pumping by 5 mM EDTA; (C) inhibition of H^+ pumping by 100 μM imidodiphosphate (IDP); (D) reversal of acidification by 2.5 μM gramicidin. Time of addition of EDTA, IDP, or gramicidin is indicated by an arrow.

and proportional to membrane concentration. The V_{max} of initial H^+ pumping was dependent on culture age, light and hormone status, but the K_m for PP_i remained fairly constant (R.V. and T.R., in preparation).

3.2. *Inhibition by IDP*

The Mg^{2+}-PP_iase was drastically inhibited by IDP as determined by the effect on H^+ transloca-

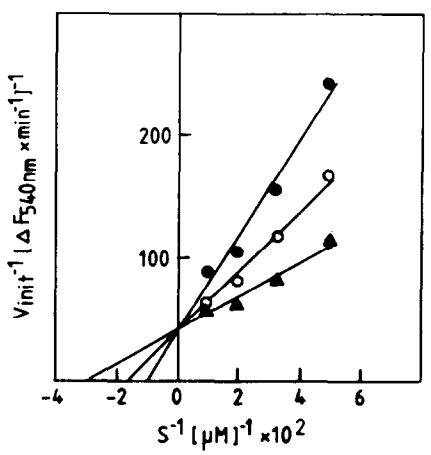

Fig.2. Kinetics of IDP inhibition of Mg^{2+}-PP_i-driven H^+ pumping (membrane protein: 35 μg/ml) presented in the Lineweaver-Burk plot. Total Mg^{2+} was always 1 mM, PP_i was varied between 20 and 100 μM. (▲) Control; (○) plus 2.5 μM IDP; (●) plus 5 μM IDP.

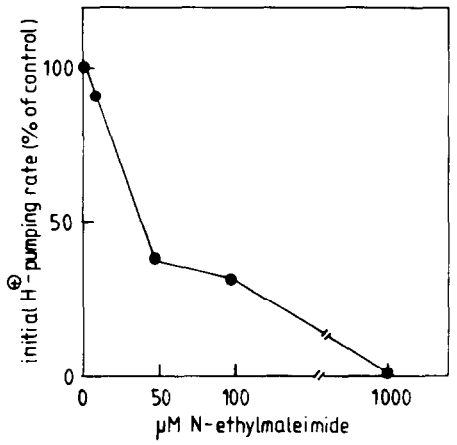

Fig.3. Concentration dependence of the effect of the *N*-ethylmaleimide (NEM) pretreatment (15 min at 25°C; membrane protein 1200 μg/ml) on subsequent H^+ pumping at 100 μM PP_i and 1 mM Mg^{2+} in the absence of NEM (membrane protein 30 μg/ml).

tion (fig.2). IDP did not affect passive H^+ conductivity as judged from a comparison with EDTA when being added to acidified vesicles (fig.1). Substrate kinetics of the IDP inhibition of Mg^{2+}-PP_iase demonstrated a competitive type with an apparent K_i value of 1.1 μM.

3.3. *Inhibition by NEM pretreatment plus or minus Mg^{2+}, IDP, and PP_i*

First the effect of different NEM concentrations (in the absence of DTT) during a 15 min pretreatment on subsequent H^+ pumping of washed membranes was determined (fig.3). Half-maximum inhibition was observed at about 50 μM NEM. This concentration was used for the following experiments. Table 1 shows the effect of NEM pretreatments plus or minus Mg^{2+} in the absence or presence of PP_i (1 mM) or IDP (100 μM). While the presence of Mg^{2+} protects the enzyme from inhibition by NEM, the simultaneous presence of the substrate PP_i leads to the same inhibition as found for PP_i alone; with 5 mM Mg^{2+} and 1 mM PP_i the same inhibition was observed (not shown). However, in the presence of Mg^{2+} plus IDP the H^+-translocation activity was even higher than in the control, whereas with IDP alone the inhibition by NEM was about the same as found for PP_i. The apparent stimulation by IDP plus Mg^{2+} over the control was of the same magnitude as the one observed for DTT.

In contrast to these results PP_i hydrolysis was much less inhibited by the NEM pretreatment, irrespective of the absence or presence of Mg^{2+},

Table 1

Effects of the *N*-ethylmaleimide pretreatment on subsequent Mg^{2+}-PP_iase-driven H^+ translocation and K^+-stimulated PP_i hydrolysis

Pretreatment	Initial H^+-pumping rate		K^+-stimulated PP_i hydrolysis	
	$\Delta F_{540\ nm}$/min	% of control	nmol PP_i min × mg protein	% of control
Control	0.021	100	55	100
2.5 mM DTT	0.027	133	n.d.	
50 μM NEM	0.010	47	50	90
50 μM NEM + 1 mM Mg^{2+}	0.023	106	51	93
50 μM NEM + 1 mM PP_i	0.011	52	n.d.	
50 μM NEM + 100 μM IDP	0.010	47	n.d.	
50 μM NEM + 1 mM Mg^{2+} + 1 mM PP_i	0.009	43	70	128
50 μM NEM + 1 mM Mg^{2+} + 100 μM IDP	0.027	130	69	126

After a 15 min pretreatment membranes were washed and PP_i hydrolysis and H^+-pumping activities were determined at 200 μM PP_i/1 mM Mg^{2+} in the presence of 2.5 mM DTT. For the H^+-pumping assays the membrane concentration was 30 μg protein/ml. n.d., not determined

whereas 1 mM Mg^{2+}-PP_i did not only protect against the inhibition by NEM but increased PP_i-hydrolyzing activity above the control (table 1), and the same effect was found for Mg^{2+} plus IDP.

4. DISCUSSION

The H^+-translocating Mg^{2+}-PP_iase in microsomal membranes from *A. tumefaciens*-transformed tobacco cells which was used as an experimental tool in our study has characteristics identical with those reported for other higher plant H^+-Mg^{2+}-PP_iase [1–10]. The kinetics analysis of IDP inhibition (fig.2) in the presence of excess Mg^{2+} together with the observation that IDP does not modify passive H^+ flux across the membrane (fig.1) shows that this competitive inhibitor may be used to specifically protect SH groups at the Mg^{2+}-PP_i-binding site without supporting H^+ translocation.

The differential effect of a 50 μM NEM pretreatment on subsequent PP_i hydrolysis and H^+ translocation (table 1) indicates that different SH groups are involved in Mg^{2+}-PP_i-driven H^+ transport. While NEM pretreatment inhibits H^+ pumping by about 50%, PP_i hydrolysis is reduced by not more than 15%. The substrate Mg^{2+}-PP_i does not protect the H^+-pumping activity from NEM inhibition, but it does so for PP_i hydrolysis; it even leads, like 100 μM IDP in the presence of 1 mM Mg^{2+}, to an increase of subsequent PP_i hydrolysis above the control incubation. This observation as well as the DTT effect on H^+ pumping suggest that in the control incubation some inactivation of the Mg^{2+}-PP_iase occurs at the substrate-binding site. Mg^{2+} alone protects against the NEM inhibition of H^+ pumping, but as it does not lead to a significant increase above the control treatment (as observed for the competitive inhibitor Mg^{2+}-IDP) it is obvious that Mg^{2+} exerts its effect not at the Mg^{2+}-PP_i-binding site.

The combined data may be rationalized by assuming at least two SH groups, one (A) localized at the Mg^{2+}-PP_i-binding site, the other (B) exposed during the H^+-translocation step. After an NEM plus Mg^{2+}-PP_i pretreatment PP_i hydrolysis would proceed uncoupled from the H^+ translocation. The fact that Mg^{2+} on its own protects against NEM inhibition indicates a change in enzyme conformation with SH group B not accessible to NEM.

The following equations summarize the assumed reaction sequence:

$$E_r + Mg^{2+} \rightleftharpoons E_t Mg^{2+} \quad (1)$$

$$E_t Mg^{2+} + Mg^{2+}\text{-}PP_i \rightleftharpoons E_{t'} Mg^{2+} \cdot Mg^{2+}\text{-}PP_i \quad (2)$$

$$E_{t'} Mg^{2+} \cdot Mg^{2+} \rightleftharpoons E_t Mg^{2+} + Mg^{2+} + 2\ P_i \quad (3)$$

where eqn 1 represents the formation of the Mg^{2+}-activated enzyme (E_r, enzyme relaxed; E_t, enzyme tense), eqn 2 the formation of the enzyme-substrate complex, and eqn 3 the splitting of the PP_i bond normally coupled to the H^+-translocation step. SH-group B would be exposed only in E_r and $E_{t'} Mg^{2+} \cdot Mg^{2+}$-$PP_i$.

Wang et al. [10] reported a rather significant inhibition of PP_i hydrolysis by NEM, which seems to contradict our results; however, the NEM inhibition is also dependent on the absolute NEM/membrane ratio and in our study membrane concentration during the NEM pretreatment was rather high (1200 μg protein/ml). The results indicate that SH-group A is less sensitive to NEM inhibition than SH-group B. In agreement with this Takeshige et al. [17] recently showed that in the tonoplast of *Chara corallina* the Mg^{2+}-PP_i-driven H^+ pumping is almost 10 times more sensitive to NEM as compared to PP_i hydrolysis. In NEM-inhibition assays at low membrane concentration we equally observed a stronger inhibition of PP_i hydrolysis (not shown).

Knight et al. [18] demonstrated that the inorganic PP_iase from yeast has two Mg^{2+}-binding sites one of which exhibits a very low K_i ($\leqq 1$ μM), while according to Barry and Dunaway-Mariano [19] the second Mg^{2+} would bind after the binding of the substrate (which was Cr^{3+} PP_i in their study). Recently, Helmich-de Jong et al. [20] reported that the gastric mucosa ($K^+ + H^+$)-ATPase showed a specific change in conformation after the addition of Mg^{2+} as judged from the tryptic digestion pattern. Thus an Mg^{2+}-induced change of conformation may be a more general feature of ion pumps.

We conclude that our data, besides demonstrating the presence of SH groups with different functions, will also provide the basis for the envisaged selective labelling of the plant

H^+-Mg^{2+}-PP_iase with radioactive NEM which may help to identify the H^+-Mg^{2+}-PP_iase polypeptide(s).

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