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# The 32 kDa tonoplast polypeptide $D_i$ associated with the V-type $H^+$-ATPase of *Mesembryanthemum crystallinum* L. in the CAM state: A proteolytically processed subunit B?

An Zhigang[b], Rainer Löw[b], Thomas Rausch[b], Ulrich Lüttge[a], Rafael Ratajczak[a,*]

[a]*Institut für Botanik, Technische Hoschschule Darmstadt, Schnittspahnstraße 3–5, D-64287 Darmstadt, Germany*
[b]*Botanisches Institut, Ruprecht-Karls-Universität, Im Neuenheimer Feld 360, D-69120 Heidelberg, Germany*

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**Abstract In the facultative halophyte *Mesembryanthemum crystallinum*, the salt- or age-induced transition to crassulacean acid metabolism (CAM) leads to the occurrence of a tonoplast-bound 32 kDa polypeptide ($D_i$). The alignment of its N-terminal protein sequence with protein sequences of recently cloned higher plant V-ATPase B-subunits indicates that $D_i$ may be derived from subunit B by proteolytic removal of a protein fragment of about 20 kDa from its N-terminus. Furthermore, an antiserum directed against $D_i$ cross-reacts with subunit B from *Nicotiana tabacum*. It inhibits both proton pumping and ATP hydrolysis of the holoenzyme in *M. crystallinum*. As $D_i$ remains firmly attached to the holoenzyme the proteolytic processing may have functional implications.**

*Key words:* V-type $H^+$-ATPase; Tonoplast; Crassulacean acid metabolism; Protein turnover; Subunit B; *Mesembryanthemum crystallinum* L.

## 1. Introduction

Correlated with salt-induced and/or age-dependent shift from $C_3$-photosynthesis to crassulacean-acid metabolism (CAM) in the $C_3$/CAM-intermediate halophyte *Mesembryanthemum crystallinum*, the appearance of two polypeptides with apparent molecular masses of 31–32 ($D_i$) and 27–28 kDa ($E_i$) was observed [1–3] and their N-terminal amino-acid sequences were determined [4]. These polypeptides cross-react with a polyclonal antiserum against the tonoplast V-type $H^+$-ATPase (V-ATPase) holoenzyme of *Kalanchoë daigremontiana* [5] and co-purify with the subunits A (67 kDa), B, (56 kDa), C (41 kDa), D (34 kDa), and c (16 kDa) of the V-ATPase of *M. crystallinum* during size-exclusion and ion-exchange chromatography [1]. Furthermore, there is evidence from immunoprecipitation experiments with a polyclonal antiserum against subunit A of the V-ATPase of *M. crystallinum* that these polypeptides are attached to the holoenzyme even under mild solubilization conditions [3], in contrast to a 35 kDa polypeptide occurring in leaves of *Citrus sinensis* under salt stress conditions which was assumed to be a proteolytic breakdown product of the catalytic subunit A [6].

Preliminary results raised the question whether polypeptides $D_i$ and $E_i$ are genuine subunits of the V-ATPase or proteolytic products of subunits A or B [7] which, however, remain attached to the V-ATPase holoenzyme and might affect holoenzyme stability [8]. Recently, sequence information has been obtained for subunit B of *Hordeum vulgare* [9] and *M. crystallinum* [10]. The alignment of the N-terminal sequence of the $D_i$ polypeptide with the subunit B protein sequences indicates that $D_i$ may be derived from subunit B by proteolytic processing or breakdown. In agreement with this assumption an antiserum prepared against $D_i$ cross-reacts with subunit B and interferes with V-ATPase function as shown in this report.

## 2. Materials and methods

### 2.1. Plant material and tonoplast vesicle preparation

Plants of *M. crystallinum* L. were grown from seeds of the collection of the Botanical Garden, Darmstadt, Germany [3]. One set of plants was irrigated with tap water for 6 weeks after sowing, while another set of plants was irrigated for 3 weeks with tap water and for 3 weeks with 0.4 M NaCl. At the end of the light phase 20 g of leaves of the third leaf pair were harvested. As an indicator for the expression of CAM the night and day difference of the cell sap malate concentration (Δmalate) was determined [11]. For control plants and salt-treated plants Δmalate was 0.2 and 62.8 mM, respectively. Tonoplast vesicles were isolated by sucrose density centrifugation after homogenization of leaf tissue in a blender [8]. Immediately before homogenization the osmotic pressure of the cell sap of one leaf was measured with an osmometer (Osmomat 030, Gonotec). To avoid osmotic rupture the osmotic pressure of the homogenization buffer was adjusted to the actual osmotic pressure of the cell sap by the addition of mannitol. Immediately after the preparation the tonoplast vesicle fractions were frozen in liquid nitrogen and stored at −70°C. Under these conditions the polypeptide pattern of the preparations and the tonoplast $H^+$-ATPase activity remained constant for several months. Tonoplast vesicles of *Nicotiana tabacum* L. were isolated according to [12].

### 2.2. Antisera

Polyclonal rabbit antisera against subunit A and the 32 kDa polypeptide $D_i$ of the V-ATPase of *M. crystallinum* were prepared as previously described [3] following the method of Knudsen [13].

### 2.3. Electrophoresis and Western blot

Tonoplast vesicles (30 μg protein) were solubilized for 5 min at 70°C in 4% (w/v) SDS, 2% (v/v) β-mercaptoethanol, 10% (v/v) glycerol, and 2 mM PMSF. After centrifugation for 15 min at 12000×*g* the supernatant was filtrated. Proteins were precipitated by incubation in 80% (v/v) acetone for 20 min at −20°C. The pellet was washed 4 times with acetone and dried at 37°C. Proteins were resolved in 9 M urea, 1 mM EDTA, 1 mM EGTA, 5 mM ascorbic acid, 0.1 M dithiothreitol, 10 mM PMSF, 4% (w/v) CHAPS, 1.6% (w/v) ampholytes pH 5–7,

*Corresponding author. Fax: (49) (6151) 164808.
E-mail: RATAJCZAK@BIO1.BIO.TH-DARMSTADT.DE

*Abbreviations:* CAM, crassulacean acid metabolism; CHAPS, (3-[3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate


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0.8% (w/v) ampholytes pH 4–6, and 0.4% (w/v) ampholytes pH 3–10, and separated by isoelectric focusing on capillary acrylamide gels (0.3% (w/v) acrylamide monomer concentration) containing a pH gradient from 3 to 10 for 20 min at 200 V, 15 min at 300 V, and 15 min at 400 V. After isoelectric focusing the capillary gels were incubated for 5 min in 62.5 mM Tris, adjusted to pH 6.8 with HCl, 2% (w/v) SDS, 5% (v/v) β-mercaptoethanol, 10% (v/v) glycerol and positioned on 10% SDS-polyacrylamide gels [14] for the second electrophoretical dimension.

Western blots were performed by electrophoretical transfer of proteins from acrylamide gels to nitrocellulose. Antigens were detected after incubation with primary antibodies with goat anti-rabbit antibodies coupled to horseradish peroxidase or the Western Light chemoluminescence assay (Serva Tropix) following the manufacturer's instructions.

*2.4. Phenolic extraction of proteins from leaf tissue*

Leaf tissue from plants of *M. crystallinum* performing $C_3$-photosynthesis or CAM was ground in liquid nitrogen. Tissue powder (200 mg) was dissolved in 300 μl 2.5 M Tris-HCl, pH 7.0, 10 μl 1 M β-mercaptoethanol and 250 μl water-saturated phenol. The suspension was mixed and centrifuged for 5 min at $12000 \times g$. An aliquot of 100 μl of the phenolic phase was added to 1 ml ethanol, 10 μl 1 M dithiothreitol and 100 μl 1.21 M ammonium acetate and incubated for 2 h at −20°C. After centrifugation for 5 min at $12000 \times g$ the supernatant was discarded and the pellet containing precipitated proteins was washed with ethanol. Proteins were resuspended in 200 μl Laemmli sample buffer [14], incubated for 15 min at 80°C and subjected to SDS-PAGE (40 μl protein solution per lane) and Western blot analysis.

*2.5. Various assays*

Measurements of ATP hydrolysis were performed according to Palmgren [15]. The reaction assay contained 50 mM KCl, 25 mM Tricine, adjusted to pH 8.0 with Tris, and 15 μg protein. To inhibit ATP hydrolysis activity of the plasma membrane $H^+$-ATPase and the mitochondrial $H^+$-ATPase 0.1 mM vanadate and 1 mM sodium azide were added, respectively.

$H^+$-transport activity of the tonoplast $H^+$-ATPase was measured by determination of fluorescence quenching of the dye quinacrine [16] in the presence of 0.1 mM vanadate and 1 mM sodium azide.

Protein concentration was determined with Amidoblack $10^B$ (Merck) [17] using bovine serum albumin as a standard.

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B-H.v.      ERTYPEEMIQ TGISTIDVMN SIARGQKIPL FSAAGLPHNE -170
B-M.c.      ...YPEEMIQ TRISTIDVMN SIARGQKIPI FSAAGLPHNE
Di-M.c.

B-H.v.      IAAQICRQAG LVKRLEQSKH AAEGGEEDNF AIVFAAMGVN -210
B-M.c.      IAAQICRQAG LVKRLEKTEN LMEGGEEDNF AIVFAAMGVN
Di-M.c.                               XGGEEDNF AIVFA.....

B-H.v.      METAQFFKRD FEENGSMERV TLFLNLANDP TIERIITPRI -250
B-M.c.      METAQFFKRD FEENGSMERV TLFLNLANDP TIERIITPRI
Di-M.c.     .......... .......... .......... ..........

B-H.v.      ALTTAEYLAY ECGKHVLVIL TDMSSYADAL REVSAAREEV -290
B-M.c.      ALTTAEYLAY ECGKHVLVIL TDMSSYADAL REVSAAREEV
Di-M.c.     .......... .......... .......... ..........

B-H.v.      PGRRGYPGYM YTDLATIYER -310
B-M.c.      PGRRGYPGYM YTD.......
Di-M.c.     .......... ..........
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Fig. 1. Amino acid sequence alignment of V-ATPase subunit B of *H. vulgare* [9], subunit B of *M. crystallinum* deduced from a partial cDNA clone [10] and the N-terminal amino acid sequence of the 32 kDa tonoplast polypeptide $D_i$; X, unknown amino acid.

## 3. Results and discussion

*3.1. The N-terminal sequence of $D_i$ aligns with an internal protein sequence of subunit B*

The two CAM correlated polypeptides $D_i$ and $E_i$ of *M. crystallinum* were considered to be new subunits of the tonoplast $H^+$-ATPase [1]. Originally, the N-terminal amino acid sequence of both polypeptides did not show homologies to the known amino acid sequences of subunits of the V-type $H^+$-ATPase [4]. Nevertheless, doubts remained whether these two CAM correlated polypeptides really are genuine subunits of the V-ATPase [3] or proteolytic fragments of high molecular mass subunits of the tonoplast $H^+$-ATPase. Comparison of the N-terminal amino acid sequence of the 32 kDa tonoplast polypeptide $D_i$ [4] with the recently published sequence of V-ATPase subunit B (isoform 2) of *H. vulgare* [9] revealed that the known 12 amino acids of $D_i$ perfectly fit amino acids 194–205 of the *H. vulgare* B-subunit (Fig. 1). On the other hand, comparison of the N-terminal sequence of $D_i$ and known subunit A protein sequences revealed only minor homology. This was the first hint of the assumption that $D_i$ is a proteolytic breakdown product of subunit B. This assumption was confirmed after sequence information was available for subunit B of *M. crystallinum* deduced from a partial cDNA clone [10] and it turned out by sequence alignment that the B-subunit sequences of *H. vulgare* (isoform 2) and *M. crystallinum* were identical in the respective region (Fig. 1). Proteolysis of subunit B seems to be rather specific. The apparent molecular mass of the $D_i$ polypeptide of 31–32 kDa [1–3] indicates that $D_i$ represents the complete C-terminus of subunit B – including the putative 'regulatory' ATP binding site AIGEGMT (subunit B amino acids 387–393 and 380–386 of *Arabidopsis thaliana* and *Neurospora crassa*, respectively) [18] – after removal of a 20 kDa N-terminal fragment. Thus, the $D_i$ polypeptide might be a proteolytically processed B subunit.

*3.2. An antiserum directed against $D_i$ cross-reacts with subunits B and A and inhibits the function of the V-ATPase holoenzyme*

To obtain more detailed information about $D_i$ a polyclonal antiserum was raised. The antibodies cross-reacted with the $D_i$ polypeptide and subunits A (67 kDa), B (55 kDa) and D (34 kDa) of tonoplast vesicle preparations from *M. crystallinum* performing CAM (lane 4 in Fig. 2). In preparations from plants performing $C_3$-photosynthesis $D_i$ was not detectable (lane 3 in Fig. 2) indicating that the occurrence of $D_i$ in tonoplast vesicle preparations is CAM-specific. This is in agreement with previous experiments using an antiserum against the V-ATPase holoenzyme of *Kalanchoë daigremontiana* for detection of V-ATPase subunits [3,5]. To resolve the question of whether $D_i$ is present in vivo or if it is produced during the time-consuming preceedure of tonoplast vesicle preparation, we prepared crude phenolized protein extracts from leaf tissue of *M. crystallinum* plants performing $C_3$-photosynthesis or CAM. As shown in Fig. 2 (lane 2) $D_i$ was present in phenolic extracts from plants in the CAM state while it was absent in extracts from plants in the $C_3$ state (Fig. 2, lane 1). Since during phenolic extraction proteases present in the leaf tissue are rapidly inactivated, the $D_i$ polypeptide appears to occur in vivo in *M. crystallinum* performing CAM. Interestingly, in phenolic extracts a 40 kDa poly-

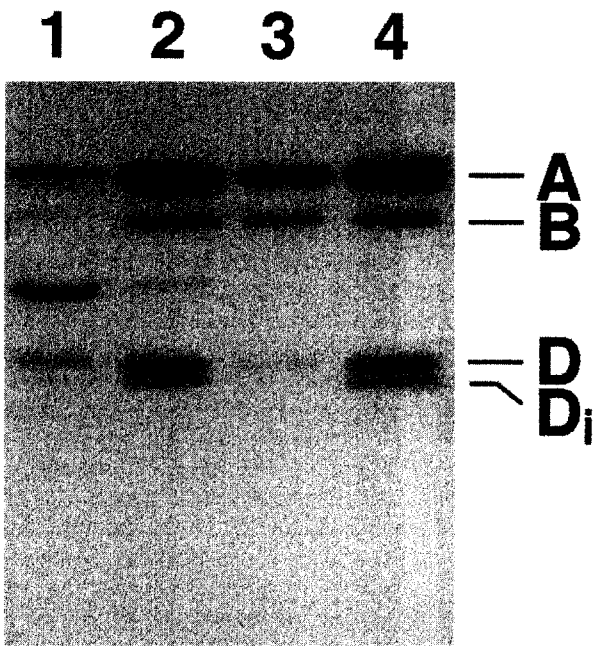


Fig. 2. Western blot of crude phenolized protein extracts of leaf tissue (lanes 1,2) and tonoplast vesicle preparations (lanes 3,4) from *M. crystallinum* plants performing $C_3$-photosynthesis (lanes 1,3) or CAM (lanes 2,4). Immunostaining was performed using an antiserum against the 32 kDa polypeptide $D_i$. Characters on the right-hand margin indicate subunits of the V-ATPase.

peptide was labelled by the antiserum against $D_i$ which was not present in tonoplast vesicle preparations.

In addition, we checked immunological cross-reactivity of the antiserum against $D_i$ in a heterologous system, i.e. tonoplast vesicle preparations of the $C_3$ plant *N. tabacum* (Fig. 3). Subunits of the *N. tabacum* V-ATPase were identified by Western blot analysis after two-dimensional gel electrophoresis using an antiserum against the V-ATPase holoenzyme of *K. daigremontiana* (Fig. 3A). Six polypeptides cross-reacted with the serum: a 70 kDa polypeptide (subunit A), a 60 kDa polypeptide (subunit B), a 40 kDa polypeptide (subunit C), and 3 polypeptides exhibiting molecular masses around 30 kDa. An antiserum directed against V-ATPase subunit A of *M. crystallinum* exclusively cross-reacted with subunit A of *N. tabacum* (Fig. 3B). The antiserum against $D_i$ strongly cross-reacted with subunits B and A of the V-ATPase of *N. tabacum* (Fig. 3C). Cross-reaction with subunit B corroborates the assumption that $D_i$ is a processed subunit B. The observed cross-reaction of the antiserum with subunit A is as yet unexplained but could be due to immunogenic splitting products of subunit A contaminating the $D_i$ antigen. Conversely, cross-reaction due to the partial homologous structure of subunits A and B seems to be less likely since the antiserum directed against subunit A did not cross-react with subunit B (Fig. 3B).

In addition to immunological cross-reactions with subunits B and A, azide- and vanadate-resistant ATP hydrolysis activity of tonoplast vesicles from *M. crystallinum* plants performing $C_3$-photosynthesis (Fig. 4A) or CAM (Fig. 4B) was inhibited by the $D_i$ antiserum (squares in Fig. 4). For comparison inhibition of enzyme activity by an antiserum against the catalytic subunit A is also presented. Incubation of tonoplast vesicles with preimmuneserum obtained from rabbits prior to the immunization with $D_i$ did not result in inhibition of ATP hydrolysis activity (triangles in Fig. 4). Moreover, the antiserum against $D_i$ inhibited proton transport activity of the V-ATPase as shown by measurements of fluorescence quenching of the dye quinacrine (Fig. 5) in preparations from plants in the $C_3$ state and the CAM state. Whether inhibition of both, ATP hydrolysis and proton transport activity of the V-ATPase of *M. crystallinum* in the CAM state and in the $C_3$ state – where $D_i$ is not detectable – by the antiserum against $D_i$ results from binding to subunit A, B, or both is not yet known.

### 3.3. Functional implications of the processing of subunit B

The polypeptides $D_i$ and $E_i$ were assumed to be necessary for regulation of the V-ATPase due to the special conditions of CAM, i.e. the diurnal accumulation of malic acid in the vacuole by a secondary active transport process which requires a strict control of vacuolar proton pump activity. From sequence comparisons and immunological investigations presented here it turned out that at least the polypeptide $D_i$ seems to be a proteolytically processed subunit B. Several observations indicate that the mechanism of proteolysis is very specific and might have physiological importance: (a) $D_i$ seems to represent the complete C-terminus of subunit B

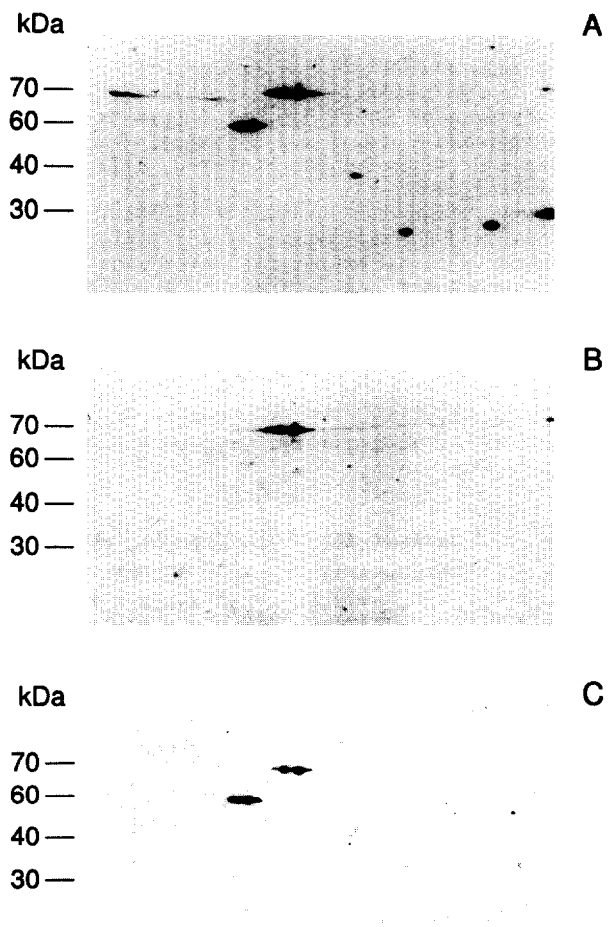


Fig. 3. Western blots of two-dimensional gel electropherograms used for separation of tonoplast proteins from *N. tabacum*. Blots were incubated with antisera against the holoenzyme of the V-ATPase of *K. daigremontiana* (A), against subunit A of *M. crystallinum* (B), or against $D_i$ (C). Numbers on the left-hand margins indicate the molecular masses of protein standards.

including its putative 'regulatory' ATP binding site; (b), $D_i$ remains coupled to the holoenzyme complex even under mild solubilization conditions [3]; (c) the occurrence of $D_i$ is strictly correlated to the expression of CAM [3]. Moreover, the specific ATP-hydrolysis activity of tonoplast vesicles from *M. crystallinum* in the $C_3$ and the CAM state related to the amount of V-ATPase is identical [3].

A possible function of the CAM correlated polypeptides was discussed to be the stabilization of the V-ATPase holoenzyme complex [3] since in the presence of $D_i$ and $E_i$ the V-ATPase is less sensitive against detergent treatment and incubation at high concentrations of malate. The molecular mass of these polypeptides is similar to that of the stalk forming subunits of the V-ATPase, which have been shown to be required for the appropriate attachment of the V-ATPase head to the membrane integral $V_o$-domain [19]. By cross-linking studies with 3,3'-dithiobis(sulfosuccinimidylpropionate) $D_i$ and $E_i$ were shown to be positioned in the stalk region of the V-ATPase of *M. crystallinum* in the CAM state (G. König and R. Ratajczak, unpublished).

Taken together, the processing of subunit B of *M. crystallinum* in the CAM state might be important for the physiological function of the V-ATPase and mediated by a tonoplast membrane protease cleaving the peptide bond between a methionine and a glutamine. Recently, a tonoplast-bound protease activity exclusively present in preparations of *M. crystallinum* in the CAM state had been demonstrated (An Zhigang, unpublished). The mechanism of processing bears resemblance to C-terminal processing of the plasma membrane $H^+$-ATPase which is activated by cleavage of a C-terminal auto-inhibitory domain by specific proteases [20].

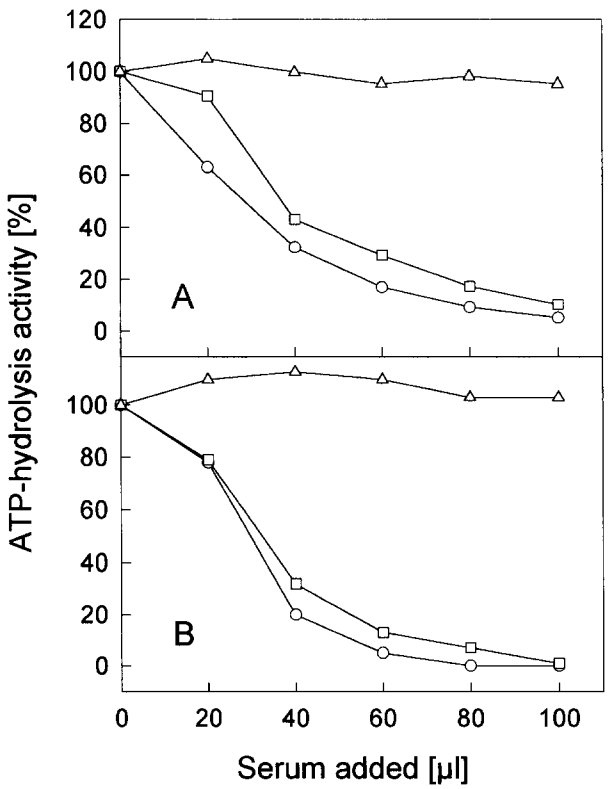


Fig. 4. Effects of antisera against subunit A (67 kDa; ○) of the tonoplast $H^+$-ATPase of *M. crystallinum*, the CAM correlated 32 kDa tonoplast polypeptide $D_i$ (□), or preimmuneserum (△) on ATP-hydrolysis activity of native tonoplast vesicles from *M. crystallinum* in the $C_3$ (A) or CAM state (B). ATP-hydrolysis activity in the absence of serum was 41.7 ± 5.2 ($n$=4) and 56.6 ± 3.6 ($n$=4) µmol $P_i$ $mg_{tonoplastprotein}^{-1}$ $h^{-1}$ for preparations from plants in the $C_3$ and the CAM state, respectively.

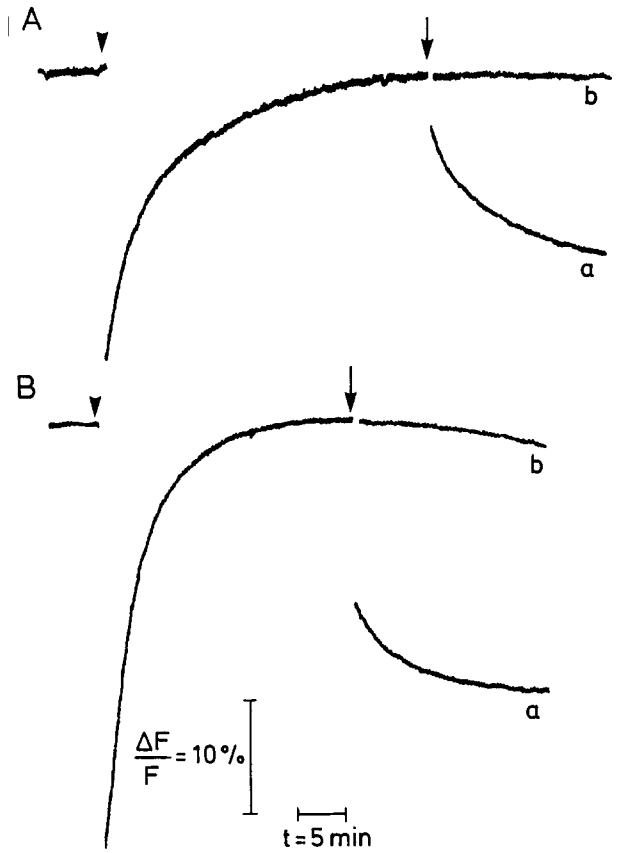


Fig. 5. Inhibition of Mg-ATP dependent proton-transport activity ($\Delta F/F$, quinacrine fluorescence quenching) of tonoplast vesicles of *M. crystallinum* (50 µg protein) in the $C_3$ (A) and CAM state (B) by an antiserum against the CAM correlated 32 kDa polypeptide $D_i$ (a); (b) preimmune serum. The volume of added serum was 100 µl in each case. Arrowheads and arrows indicate the addition of Mg-ATP and sera, respectively.

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