The H^+-ATPase purified from maize root plasma membranes retains fusicoccin in vivo activation

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Abstract The activity of 'P-type' ATPases is modulated through the C-terminal autoinhibitory domain. The molecular bases of this regulation are unknown. Their understanding demands functional and structural studies on the activated purified enzyme. In this paper the plasma membrane H^+-ATPase from maize roots activated in vivo by fusicoccin was solubilised and fractionated by anion-exchange HPLC. Results showed that the H^+-ATPase separated from fusicoccin receptors retained fusicoccin activation and that it was more evident after enzyme insertion into liposomes. These data suggest that fusicoccin stimulation does not depend on a direct action of the fusicoccin receptor on the H^+-ATPase, but rather, fusicoccin brings about a permanent modification of the H^+-ATPase which very likely represents a general regulatory mechanism for 'P-type' ATPases.

Key words: Signal transduction; H^+-ATPase; 14-3-3 protein; Fusicoccin receptor

1. Introduction

Plasma membrane proton pumps are characteristic of plants, yeast, fungi and related organisms. These vanadate sensitive or 'P-type' ATPases play a central role in cell growth, generating the electrical gradient across the plasma membrane that provides the driving force for nutrient and ion uptake, regulation of intracellular pH and several other essential functions. Hence, it is not surprising that in plants its activity is subjected to regulation by a number of factors such as plant hormones, phytotoxins and light [1,2]. In recent years advances have been made which have shed some light on the molecular mechanism of the regulation of this enzyme. In particular, it has been shown that the C-terminus of the native plasma membrane H^+-ATPase is an autoinhibitory domain whose displacement by different effectors leads to increased enzyme activity [3,4].

The fungal phytotoxin fusicoccin (FC) is one of the most powerful effectors of the plasma membrane H^+-ATPase [5]. Upon binding to plasma membrane receptors [6–8], it rapidly and persistently stimulates the enzyme, thus triggering cell growth and membrane transport [5]. It has been established that activation of the H^+-ATPase is more remarkable when FC is administered in vivo to plant tissues and that it is maintained after plasma membrane isolation [9]. The biochemical features of the membrane-bound, FC-activated H^+-ATPase have been determined and, on the basis of its striking similarity to trypsin activation, it has been concluded that FC stimulation is mediated by the displacement of the C-terminal autoinhibitory domain of the enzyme [10–12]. The molecular mechanism which leads to the FC-induced C-terminus displacement is nevertheless still undetermined and at least two different models have been proposed. One favours a regulation through a direct interaction between FC receptors and the H^+-ATPase [13], whereas the other envisages the occurrence of a transduction chain in which phosphorylation/dephosphorylation events may be involved [14]. Recently, clues about the FC signaling machinery have been obtained by the discovery that 14-3-3 proteins are present in purified FC receptor preparations [6–8]. Nevertheless, this finding does not help to favour one of the two above models, since these proteins are known to regulate protein kinase activities as well as to mediate protein-protein interactions [15].

This study has been undertaken in order to clarify this point, namely to ascertain whether the activated state of the H^+-ATPase is maintained after solubilization from plasma membrane and purification from other components of the FC-signaling machinery, such as 14-3-3 proteins.

Furthermore, reconstitution of the purified H^+-ATPase into liposomes has been performed in order to evaluate whether the lipid environment is involved in the expression of FC stimulation.

2. Materials and methods

2.1. Chemicals

FC was prepared as described by Ballio et al. [16]. [^{3}H]FC with specific activity 20.5 Bq mol^{-1} was obtained according to Ballio et al. [17]. 9-Amino-6-chloro-2-methoxyacridine (ACMA), phosphatidylcholine (PC) (from soybean, type II), phosphatidylethanolamine (PE) (from sheep brain, type II) were purchased from Sigma. Analytical grade chemicals were used throughout.

2.2. Plant material

Maize seeds (*Zea mays* L., var Logos) from Italian Dekalb (Mestre) were germinated and grown in the dark as already described [18].

2.3. In vivo FC treatment

150 g of maize roots were harvested after 7 days, cut into small pieces, infiltrated under vacuum in 300 ml of 10 mM Tris-Mes buffer (pH 6), in the presence of 10 mM FC [10] and incubated for 60 min. An equal amount of roots was incubated under the same conditions

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Abbreviations: FC, fusicoccin; [^{3}H]FC, tritiated dihydrofusicoccin; ACMA, 9-amino-6-chloro-2-methoxyacridine; BTP, 1,3-bis[tris(hydroxymethyl)methylamino]propane; DTT, dithiothreitol; MOPS, 3-(*N*-morpholino)propanesulfonic acid; PMSF, phenylmethylsulfonyl fluoride; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PVDF, polyvinylidene difluoride

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but in the absence of FC and processed in the same way to obtain non-activated membranes as control.

2.4. H^+-ATPase purification

Two-phase partitioned plasma membranes from maize roots were obtained as described previously [19]. The H^+-ATPase was solubilised from two-phase partitioned plasma membranes and purified by anion-exchange HPLC, according to the method reported by Johansson et al. [20] with minor modifications. The two-phase partitioned plasma membrane fraction was incubated 30 min with 0.01 μM [^{3}H]FC, then diluted to 2 mg ml^{-1}protein in 10 mM Tris-HCl buffer (pH 7.6), containing 20% (w/v) glycerol, 1 mM DTT, 2 mM EDTA 0.2 mM PMSF, treated with 3.7 mg ml^{-1} Triton X-100 and 0.5 M KCl for 10 min at 4°C, and centrifuged at 100 000 × *g* for 45 min. The pellet was resuspended at a concentration of 4 mg ml^{-1} protein, in 10 mM MOPS-BTP buffer (pH 7), containing 20% (w/v) glycerol, 5 mM EDTA, 0.1 mM DTT, 0.5 mM ATP, 0.1 mM PMSF and 0.01 μM [^{3}H]FC. After addition of an equal volume of the same buffer, containing 20 mg ml^{-1} dodecyl-β-D-maltoside and 30 min incubation, the mixture was centrifuged at 100 000 × *g* for 45 min. The supernatant was reincubated with 0.01 μM [^{3}H]FC and loaded onto a 5 PW DEAE HPLC column (Bio-Rad), fluxed at 0.5 ml min^{-1} with 20 mM histidine-HCl buffer (pH 7), containing 10% (w/v) glycerol, 0.5 mM ATP, 0.1 mM DTT, 0.05 mg ml^{-1} dodecyl-β-D-maltoside. Elution was performed in 40 min, by a linear gradient from 0 to 0.5 M NaCl, in the same buffer. Fractions of 1 ml were collected and assayed for enzymatic activity and for radioactivity.

2.5. Proton transport assay

The partially purified H^+-ATPase was incorporated into preformed PC/PE liposomes. Each sample contained 10 μg protein from the purified fractions, which were mixed with 1 mg of sonicated liposomes, 5 μl of 0.5 M β-D-octylglucoside and processed as described [19]. H^+ translocation into liposomes was followed by monitoring the fluorescence quenching of ACMA, by means of a Perkin-Elmer LS50 spectrofluorimeter, at excitation and emission wavelengths of 430 and 500 nm, respectively.

2.6. Analytical methods

Protein concentrations were estimated by the method of Bradford [21], using the Bio-Rad protein assay kit, with BSA as standard. ATP hydrolysis was assayed according to Serrano [22]. Radioactivity was measured in a LKB Wallac 1410 scintillation counter, using the Beckman Ultima Gold scintillation cocktail.

2.7. Western immunoblotting

SDS-PAGE were performed according to Laëmmli [23], in a Mini Protean apparatus (Bio-Rad). Proteins were transferred onto PVDF membranes using a semidry LKB apparatus (2 h, 0.8 mA cm^{-2}). Anti-C-terminus H^+-ATPase antibodies were a generous gift of Prof. J.Y. Takemoto, Utah State University, Logan, UT, USA; anti-14-3-3 policlonal antibodies were obtained as already described [6]. After blocking with a solution of 5% fat-free, dry milk and incubating overnight with primary antibodies, immunoreactive proteins were revealed using horseradish peroxidase-conjugated secondary antibodies (Bio Rad) and the enhanced chemi luminescence detection system (ECL) from Amersham, according to the manufacturer's instructions.

3. Results

The H^+-ATPase has been solubilised and purified from two-phase partitioned plasma membranes obtained from maize roots incubated in vivo with or without 10 mM FC. As expected, membranes from FC-treated roots showed a stimulation of the vanadate-sensitive, ATP-hydrolysing activity tested at pH 7.4. The stimulation over the control ranged from 50 to 100%, depending on the preparation and the ratio of the ATP-hydrolytic activity between pH 6.5 and 7.4 was 2 for the control and 1 for the FC-activated enzyme [10–12].

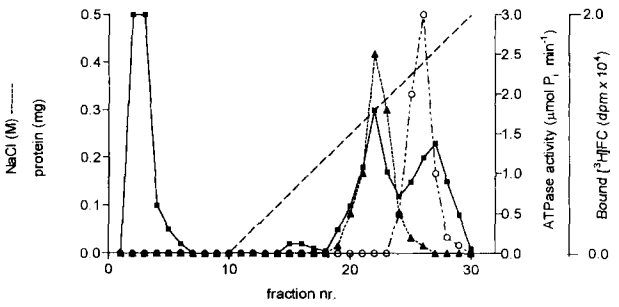

Fig. 1. Elution profile of solubilised plasma membrane proteins, ATPase activity and protein-bound [^{3}H]FC from the DEAE-HPLC anion-exchange column. (■) Protein, (○) bound [^{3}H]FC, (▲) ATPase activity. The salt gradient was applied at 20 min, after elution of unbound protein. H^+-ATPase and FC receptors were eluted at 0.28 and 0.4 M NaCl, respectively.

Interestingly, it was found that during two-phase partitioning the in vivo-bound FC was released (data not shown), so that purified membranes from FC-treated roots were still able to bind [^{3}H]FC. Hence, prior to solubilization, plasma membranes were incubated with 0.01 μM [^{3}H]FC, in order to follow the distribution of FC-binding activity during HPLC purification.

Plasma membranes were then washed with Triton X-100, which was more effective than the $C_{14}E_8$ (polyoxyethylene 8-myristyl ether) used by Johansson et al. [20]. This treatment removed about 50% protein, without significant loss of H^+-ATPase activity and only 20% of FC-binding activity.

Solubilisation of the H^+-ATPase from the Triton-washed pellet was achieved by dodecyl-β-D-maltoside treatment [20]. Purification of the H^+-ATPase from the soluble mixture was performed by anion-exchange HPLC. In Fig. 1 is shown the elution profile from a typical purification, performed from FC-activated plasma membranes. Maximal H^+-ATPase activity eluted in fractions 22 and 23, the specific activity in the purest fractions being 5–6 μmol min^{-1} mg^{-1}. The FC-binding activity eluted in fractions 26 and 27, thus neatly separated from the H^+-ATPase. Qualitatively the same elution profiles were obtained with control membranes, although a lower amount of bound [^{3}H]FC was usually recovered.

These results were confirmed by immunoblotting experi-

Table 1
Ratios of the ATP-hydrolytic activity and of the initial rate of H^+ pumping at pH 6.5 and 7.4 for membrane-bound and purified H^+-ATPase

H^+-ATPase treatment	Membrane-bound	Purified	
	Ratio of ATP hydrolysis	Ratio of ATP hydrolysis	Ratio of H^+ pumping
None	2.5	2.0	1.5
FC	1.0	1.7	1.0
Trypsin	1.0	1.7	n.d.

FC treatment was performed in vivo on maize roots. Trypsin treatment was performed on two-phase partitioned plasma membranes. Values refer to a typical purification. The experiment was repeated six times and ratios of activities were always lower for FC-activated purified H^+-ATPase than control, ranging between 10 and 20% for ATP hydrolysis and between 30 and 40% for proton pumping.

ments performed on purified fractions, probed with anti-H^+-ATPase and anti-14-3-3 antibodies [6]. As shown in Fig. 2, the immunodecoration of the 100 kDa polypeptide paralleled the distribution of the H^+-ATPase activity, whereas 14-3-3 immunoreactive proteins were detected in the same fractions in which the FC-binding activity was eluted. Immunoblotting also revealed that the amount of 14-3-3 proteins was considerably higher in fractions from FC-treated tissues. The presence of 14-3-3 proteins on the plasma membrane after Triton washing, namely under conditions removing 50% protein, suggests that they are very tightly associated to the plasma membrane.

The H^+-ATPases purified from control and FC-activated membranes were then used in comparative experiments, to test whether the activation by FC was still detectable on the soluble enzyme.

It has been demonstrated that FC activation is much more remarkable at neutral or alkaline pH [24]. As the direct comparison of ATP-hydrolysing activities at pH 7.4 was hampered by the uncertainty in the estimation of the amount of H^+-ATPase in the two samples, the ratio of the ATP-hydrolysing activities between 6.5 and 7.4, which is independent of protein concentration, was considered. Values of this ratio (Table 1) were slightly, but reproducibly lower (10–15%) for the H^+-ATPase purified from FC-treated tissues compared to control. This finding indicates that the soluble H^+-ATPase purified from FC-treated tissue still displays, although to a lesser extent, the higher efficiency for ATP hydrolysis at alkaline pH, typical of the membrane-bound enzyme.

Since it has been demonstrated that the trypsin-activated H^+-ATPase shows biochemical features very similar to those induced by FC activation [10–12], the same purification procedure was also used to solubilise and purify the membrane-bound, trypsin-activated H^+-ATPase. The ratio of its ATP-hydrolytic activity at pH 6.5 and 7.4 was compared to that of

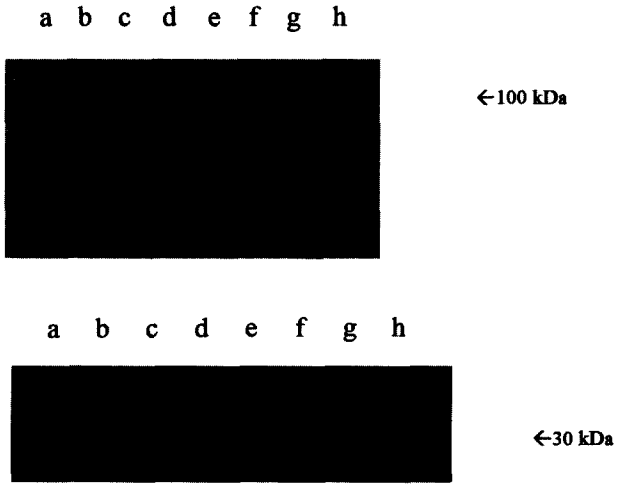

Fig. 2. Immunoblotting of fractions eluted from the DEAE-HPLC anion-exchange column. Immunodecoration was performed by the ECL system from Amersham. Upper: anti-H^+ATPase antibodies. Lower: anti-14-3-3 antibodies. Lanes a–d, correspond to fractions 26, 27, 22, 23 from FC-activated membranes. Lanes e–h, correspond to fractions 26, 27, 22, 23 from control membranes. Patterns of immunodecoration with the two antibodies demonstrated that H^+-ATPase is eluted in fractions 22 and 23, thus confirming the profile of activity, whereas 14-3-3 proteins are recovered in fractions 26 and 27.

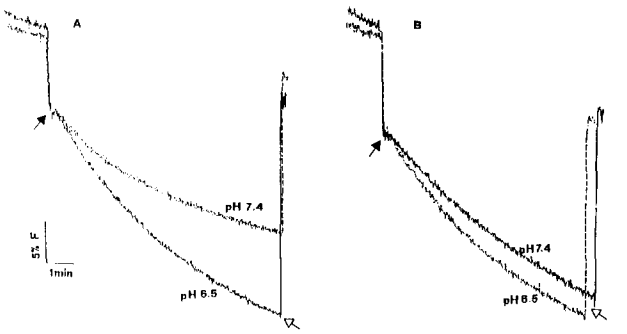

Fig. 3. H^+ pumping of the H^+-ATPases purified from control and FC-activated membranes, reconstituted into liposomes. (A) H^+ pumping at pH 6.5 and 7.4 of the control enzyme. (B) H^+ pumping at pH 6.5 and 7.4 of the in vivo FC-activated enzyme. The solid arrow indicates the addition of ATP, the open arrow the addition of ammonium sulfate. Traces refer to fluorescence of 1 μM ACMA.

the H^+-ATPase purified from control membranes. As shown in Table 1, the results exactly matched those obtained for the FC-activated enzyme.

As it has been observed that FC stimulation is more marked on proton pumping rather than on ATP hydrolysis [11,24], the purified H^+-ATPases were incorporated into liposomes and their proton-pumping activities at pH 6.5 and 7.4 were compared (Fig. 3). The ratio of the initial rate of ACMA fluorescence quenching at pH 6.5 and 7.4 was 50% lower for the FC-activated H^+-ATPase (Table 1).

4. Discussion

Recently, some light has been shed on the mechanism of activation of the H^+-ATPase by FC. Studies carried out by different groups on plasma membrane vesicles isolated from plant tissues incubated in vivo with FC have demonstrated that the increase of the ATP-hydrolysing and H^+-pumping activities of the enzyme is very likely mediated by the displacement of the C-terminal autoinhibitory domain [10–12]. Moreover, it has been shown that FC receptors can activate the H^+-ATPase from the FC-insensitive organism *Neurospora crassa*, thus suggesting that FC activation acts through a mechanism conserved among all the 'P-type' ATPases [25]. At the same time, during the last few years progress has been made concerning the identification and purification of FC receptors and/or components of the FC signalling pathway [26]. These investigations, however, did not help clarify the molecular mechanism of the C-terminus displacement triggered by FC.

Previous work has demonstrated that the H^+-ATPase can be phosphorylated on serine and threonine residues [27], but only indirect evidence has been obtained that phosphorylation/dephosphorylation events can play a regulatory role [28,29]. On the other hand, the finding that 14-3-3-like proteins are involved in FC perception or transduction allows one to envisage a transduction mechanism leading to C-terminus displacement by protein-protein interaction or by phosphorylation/dephosphorylation reactions [15]. Since up to now circumstantial evidence in favour of both hypotheses has been obtained, it appears that this question can be properly addressed only by the biochemical characterization of the purified FC-activated enzyme. In this paper, we report on the solubilisation and partial purification of the membrane-bound

FC-activated H^+-ATPase, as well as on its functional characterization. The results obtained have shown that the properties of purified H^+-ATPase are reminiscent of those of FC-activated enzyme at the plasma membrane. In fact, the ratio of the ATP hydrolysis between pH 6.5 and 7.4 for the purified enzyme was lower than that of the non-activated H^+-ATPase, although the values were markedly lower than those reported for the membrane-bound enzymes [11]. The reasons for this are unknown, but it has been shown that the solubilisation and purification of the H^+-ATPase reduces its sensitivity to pH [20].

A more remarkable effect was observed when the H^+-pumping activities were compared, after insertion of the enzymes into liposomes. In fact, values of the ratio of the initial rate of proton pumping of the FC-activated H^+-ATPase between pH 6.5 and 7.4 were 50% lower than the control.

This result seems to confirm previous indications that FC activation is expressed predominantly on proton pumping, possibly by determining a more efficient coupling of proton translocation to ATP hydrolysis [10]. This result is also in accordance with data recently reported on an heterologously expressed H^+-ATPase from *Arabidopsis*, lacking the 77-amino-acid, C-terminal peptide [30]. The truncated enzyme displays an enhanced coupling of proton transport to ATP hydrolysis. In conclusion, the finding that the soluble and purified H^+-ATPase retains the activation by FC in the absence of FC-binding activity and of 14-3-3-like, putative FC receptors [6–8], strongly indicates that a direct interaction of the enzyme with other components of the FC signalling machinery is not occurring. These data rather suggest that FC activation of the H^+-ATPase arises from a covalent modification (e.g. phosphorylation or dephosphorylation) which survives detergent treatment and chromatographic fractionation. The identification of the structural modification brought about by FC is currently in progress and may help to elucidate the general regulatory mechanism of 'P-type' H^+-ATPases.

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