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Cercospora beticola Toxins[1]

X. Inhibition of Plasma Membrane H^+-ATPase by Beticolin-1

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Beticolin-1 is a toxin produced by the fungus *Cercospora beticola*. The chemical structure of this toxin was previously elucidated. The effects of beticolin-1 on purified corn root plasma membrane H^+-ATPase were studied in a solubilized form or were reconstituted into liposome membranes. The ATP hydrolysis activity of the purified solubilized enzyme was inhibited by micromolar concentrations of beticolin-1, and this inhibition was noncompetitive with respect to ATP. When this purified enzyme was inserted into liposome membranes, a competitive inhibition of the H^+-ATPase hydrolysis activity by beticolin-1 was observed. The effect of beticolin-1 on the formation of H^+-ATPase-phosphorylated intermediate was also studied. With the purified enzyme in its solubilized form, the level of phosphorylated intermediate was not affected by the presence of beticolin-1, whereas micromolar concentrations of the toxin led to a marked inhibition of its formation when the enzyme was reconstituted into liposomes. These data suggest that (a) the plasma membrane H^+-ATPase is a direct target for beticolin-1, and (b) the kinetics of inhibition and the effect on the phosphorylated intermediate are linked and both depend on the lipid environment of the enzyme.

The plasma membrane H^+-ATPase (EC 3.6.1.35) plays a major role in the control of many cell processes. Using ATP as the energy source, it pumps protons from the cytoplasm to the cell exterior, thus creating an electrochemical gradient across the plasma membrane that constitutes the driving force for nutrient uptake. This enzyme is also involved in cell growth and division, and its activity is likely to be regulated by various factors, including plant hormones, light, and fungal toxins (for a review, see Serrano, 1990).

It was shown previously that CBT inhibits the proton extrusion in corn root segments (Macri et al., 1980) and the ATP-dependent proton pumping in pea stem microsomes (Macri et al., 1983) or corn root microsomes (Blein et al., 1988). Since only minimal data concerning the structure of CBT were known (Schlösser, 1971; Assante et al., 1986), we focused our previous research on isolation, purification, and structure elucidation of the yellow secondary metabolites of *Cercospora beticola*. We isolated 15 compounds and named them beticolins. The structures of some of these compounds have been determined (Milat et al., 1992, 1993; Ducrot et al., 1994a, 1994b; Prangé et al., 1995): they share the same octocyclic skeleton with a chlorine atom and partially hydrogenated anthraquinone and xanthone moieties (Fig. 1). One of them, beticolin-1, co-migrates with CBT on different TLC systems. These results led us to study further the effect of beticolin-1 on plasma membrane H^+-ATPase activity.

Previous results concerning effects of CBT on ATP-dependent proton transport of corn root microsomes suggested that this toxin could inhibit the plasma membrane H^+-ATPase (Blein et al., 1988). This observation led us to determine whether this enzyme was indeed a target for beticolin-1. Therefore, the corn root plasma membrane H^+-ATPase was purified according to the method of Grouzis et al. (1990), and the effects of beticolin-1 on both ATP hydrolysis and phosphorylated intermediate formation were measured. Since beticolin-1 is able to interact with artificial or biological membranes (Mikes et al., 1994b), we investigated the influence of the lipid environment of the enzyme using purified H^+-ATPase in a solubilized form or inserted into liposomes.

MATERIALS AND METHODS

Plant Material

Corn seeds (*Zea mays* L., var Mona) were surface-sterilized for 20 min with calcium hypochlorite (20 g/L), rinsed with distilled water, and germinated on stainless steel screens above distilled water for 7 d in the dark at 25°C.

Membrane Preparation

All steps of isolation were performed at 4°C. Corn roots (400 g) were homogenized with a blender in 800 mL of grinding medium (50 mM Tris-Mes, pH 8.0, 500 mM Suc, 20 mM EDTA, 10 mM DTT, 1 mM PMSF). After the samples were filtered through Miracloth (Calbiochem) and centrifuged at 25,000*g* for 20 min, supernatants were collected and centrifuged at 96,000*g* for 35 min. The pellets were suspended in 36 mL of buffer A (10 mM Tris-Mes, pH 7.3, 250 mM Suc, 1 mM ATP, 1 mM EDTA, 1 mM DTT, and 1 mM

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Abbreviations: CBT, *Cercospora beticola* toxin; lysoPC, L-α-lysophosphatidylcholine.

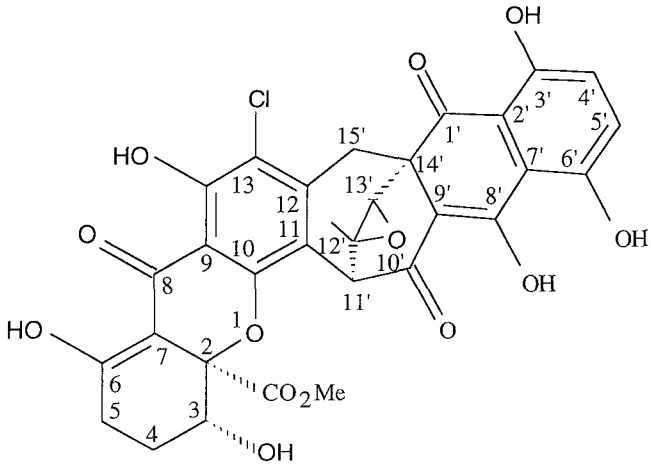

Figure 1. Structure of beticolin-1.

PMSF). Aliquots of 6 mL of this preparation were layered on cushions (12 mL each) of 30% (w/w) Suc and centrifuged at 100,000*g* for 75 min. The pellets were suspended in 2 mL of buffer B (5 mM phosphate buffer, pH 6.8, 250 mM Suc, and 1 mM DTT). This microsomal fraction was then added to a polymer phase mixture (Widell et al., 1982) to give a final concentration of 6.5% (w/w) Dextran T-500 and 6.5% (w/w) PEG 4000 in buffer B. The sample was mixed by 40 inversions of the tube and centrifuged in a swing-out rotor at 1,000*g* for 5 min. The upper phase was carefully removed and washed three times with an equal volume of fresh lower phase. The last upper phase was diluted with 5 volumes of buffer A and centrifuged at 96,000*g* for 35 min. The pellets were suspended in buffer A and centrifuged at 120,000*g* for 40 min. The resulting pellets of plasma membrane were suspended in the same buffer containing 20% glycerol and stored at −80°C.

This plasma membrane fraction was diluted with 20 mL of buffer A containing 500 mM potassium bromide and 0.25% Triton X-100, gently stirred for 20 min at 4°C, and centrifuged at 120,000*g* for 40 min. The pellet was washed with 20 mL of the same buffer without Triton X-100, and the final membrane fraction was suspended in buffer A containing 20% glycerol and stored at −80°C.

Solubilization and Purification of H^+-ATPase

The purified plasma membrane H^+-ATPase was obtained according to the method of Grouzis et al. (1990). Briefly, plasma membranes washed with Triton X-100 and potassium bromide were diluted with buffer A containing 20% glycerol, and lysoPC was added to a final concentration of 2 mg/mL. After 15 min of incubation at room temperature, the sample was centrifuged for 40 min at 120,000*g*. The supernatant fluid was layered on a linear glycerol gradient (25–50%, v/v) and centrifuged at 150,000*g* for 15 h in a vertical rotor. Fractions of 1.5 mL were collected from the bottom, and the fractions of highest phosphohydrolytic specific activity were pooled and stored at −80°C.

Liposomes Preparation and Reconstitution of Purified H^+-ATPase

Soybean phospholipids (25 mg/mL L-α-phosphatidylcholine, type II-S; Sigma) were sonicated to clarity under N_2 in 25 mM Tris-Mes, pH 6.5, and 50 mM KCl. Purified H^+-ATPase and liposomes were vortexed for 5 s in a lipid-to-protein ratio of 100 (w/w) according to the method of Simon-Plas et al. (1991).

Measurement of ATPase Activity

ATP hydrolysis activity was determined by measuring the release of Pi according to the method of Ames (1966). The standard assay medium (0.5 mL) contained, unless otherwise stated, 25 mM Tris-Mes, pH 6.5, 50 mM KCl, 3 mM $MgSO_4$, ATP (variable amounts indicated in legends of figures), 2 mM PEP, and 6 units of pyruvate kinase. Beticolin-1 was dissolved in ethanol, and the corresponding amount of solvent was added to the control. Assays were performed at 38°C. Controls were performed to verify that beticolin-1 had no effect on pyruvate kinase activity.

Phosphorylated Intermediate Formation

Phosphorylation

Plasma membrane proteins (40 μg) or purified ATPase (3 μg) were incubated with or without beticolin-1 for 30 min at room temperature in 50 mM Tris-Mes (pH 6.5), 50 mM KCl, and 1 mM $MgSO_4$. When indicated, 200 μM orthovanadate was included in the medium. After incubation, the reaction was started at 4°C with 10 μM (12 μCi) [γ-^{32}P]ATP and stopped after 15 s by the addition of ice-cold TCA at a final concentration of 10% (w/v). In pulse and chase experiments, 2 mM unlabeled ATP or ADP was added after the phosphorylation with [γ-^{32}P]ATP, 30 s before stopping the reaction with ice-cold TCA. After the sample was centrifuged at 12,000*g* for 15 min at 4°C, the pellet was washed once with TCA (10%, w/v) and once with ice-cold water. In some experiments, samples were treated for 30 min at 25°C with 50 mM hydroxylamine or 100 mM sodium borate-HCl, pH 9.0, before the second wash. After a final centrifugation (12,000*g*, 15 min, 4°C), the pellet was suspended in 35 μL of solubilization buffer (0.1 M sodium phosphate, pH 5.5, 10 mM DTT, 0.5 mM PMSF, 1% [w/v] SDS, and 10% [v/v] glycerol). A protein assay was then performed according to the method of Schaffner and Weissmann (1973) to apply equal amounts of proteins on each lane of the electrophoresis gel.

Electrophoresis

Proteins were separated by SDS-PAGE in an acidic gel (0.1 M sodium phosphate, pH 5.5, 0.1% [w/v] SDS, 5% acrylamide, and 0.8% bisacrylamide) using a Bio-Rad vertical minigel system (gel dimensions 8.5 × 6 x 1 mm, no stacking gel). The running buffer contained 0.1 M sodium phosphate (pH 5.5) and 0.1% (w/v) SDS. After running the gel was stained with Coomassie blue or dried and exposed

at −80°C with a Kodak X-OMAT LS5 film in an Amersham hypercassette with intensifier screens.

Radioactivity Counting

The radiolabeled proteins were suspended in the solubilization buffer (0.1 M sodium phosphate, pH 5.5, 10 mM DTT, 0.5 mM PMSF, 1% [w/v] SDS, and 10% [v/v] glycerol) and added to 5 mL of a scintillation cocktail (Ready-Safe, Beckman). Radioactivity present in the samples was measured in a Beckman LS 6000 AT scintillation counter.

Protein Assay

Unless otherwise stated protein concentration was determined by the method of Bradford (1976) with BSA as a standard.

Chemicals

Beticolin-1 was purified as described by Milat et al. (1992). The toxin was dissolved in absolute ethanol to a 0.5-mg/mL stock solution. All other chemicals were reagent grade.

RESULTS

Inhibition Kinetics of the Phosphohydrolytic Activity of Purified H^+-ATPase by Beticolin-1

Solubilized Enzyme

When the H^+-ATPase was incubated for 30 min with 1.5 μM beticolin-1 before addition of ATP, the hydrolysis activity decreased by about 50%. This inhibition remained unchanged when the ATP concentration increased from 0.01 to 2.5 mM (Fig. 2A, inset). The Lineweaver-Burk transformation of this kinetic property showed that the K_m of the enzyme (0.060 and 0.058 for control and assay with 1.5 μM beticolin-1, respectively) did not change in the presence of beticolin-1, whereas the V_{max} decreased from 7.1 to 4.0 μmol Pi min^{-1} mg^{-1} protein (Fig. 2A).

Solubilized H^+-ATPase Reconstituted into Liposomes

The purified H^+-ATPase was reconstituted into liposomes made with soybean lipids following the procedure of spontaneous insertion, with a lipid-to-protein ratio of 100 (w/w). The effective insertion of the protein in the liposome membrane was monitored by its ability to transport protons inside vesicles (Simon-Plas et al., 1991). The initial quenching rate of 9-amino-6-chloro-2-methoxyacridine fluorescence induced by 0.5 mM ATP was 12,500% min^{-1} mg^{-1} protein (data not shown).

After reconstitution into liposomes, the enzyme was incubated for 30 min with 4.5 μM beticolin-1. The ATP hydrolysis assays showed that the inhibition caused by the toxin decreased from 46 to 7% when the ATP concentration increased from 0.01 to 1.2 mM (Fig. 2B, inset). The Lineweaver-Burk transformation of this kinetic property showed that the K_m of the enzyme increased 2-fold (from

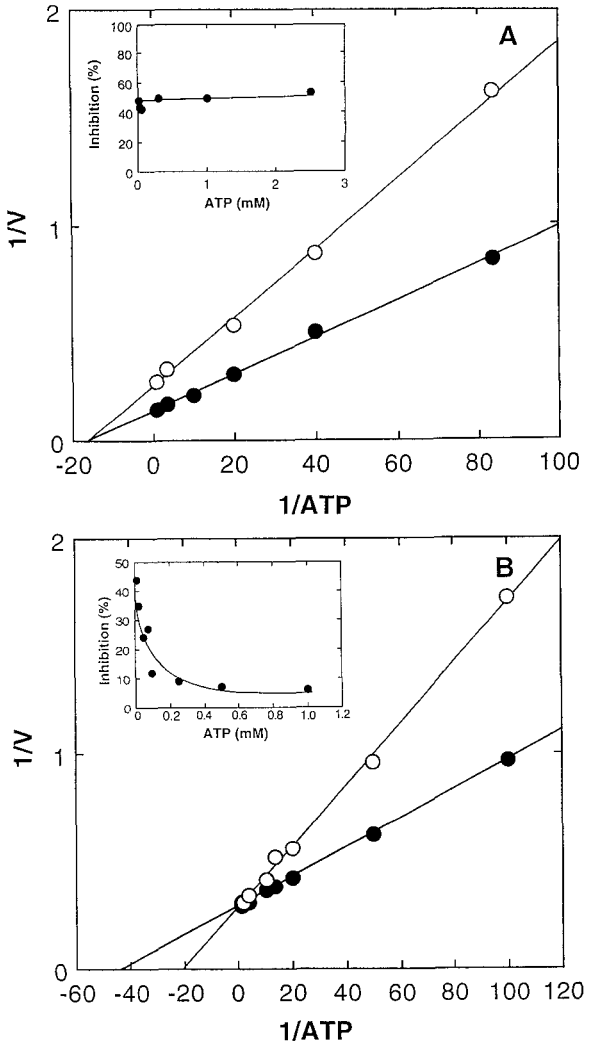

Figure 2. Inhibition of ATP hydrolysis of the purified H^+-ATPase by beticolin-1. A, Solubilized enzyme. Protein (0.5 μg) was incubated for 30 min at room temperature with 1.5 μM beticolin-1 (○, r = 0.997) or with the corresponding amount of ethanol (●, r = 0.998) in a medium (0.5 mL) containing 25 mM Tris-Mes, pH 6.5, 50 mM KCl, 3 mM $MgSO_4$, 2 mM PEP, and 6 units of pyruvate kinase. The reaction was started by adding various amounts of ATP (mM) and allowed to proceed for 30 min at 38°C. 1/ATP, 1/ATP concentration (mM^{-1}). 1/V, 1/specific ATP hydrolysis activity ([μmol Pi min^{-1} mg^{-1} protein]$^{-1}$). Inset, Inhibition (%) versus ATP concentration (mM). B, Solubilized enzyme reconstituted into liposomes. Purified H^+-ATPase was mixed and continuously stirred for 5 s with with liposomes from soybean lipids (lipids/protein = 100, w/w). Aliquots of this preparation containing 0.5 μg of protein were incubated for 30 min at room temperature with 4.5 μM beticolin-1 (○, r = 0.998) or with the corresponding amount of ethanol (●, r = 0.999). The ATP hydrolysis reaction was then allowed to proceed as described in A, with 2 μM gramicidin in the assay medium (0.5 mL). 1/ATP, 1/ATP concentration (mM^{-1}). 1/V, 1/specific ATP hydrolysis activity ([μmol Pi min^{-1} mg^{-1} protein]$^{-1}$). Inset, Inhibition (%) versus ATP concentration (mM). The data were representative of three independent experiments.

0.025 to 0.050 mM), whereas the V_{max} remained unchanged (Fig. 2B).

Detection of the Plasma Membrane H^+-ATPase-Phosphorylated Intermediate

After a 15-s incubation of the purified solubilized enzyme (4°C) with 10 μM [γ-^{32}P]ATP and 1 mM $MgSO_4$ at pH 6.5, the autoradiograph of the gel electrophoresis performed revealed a single 100-kD phosphorylated polypeptide (Fig. 3, lane 1). A 30-min incubation (25°C) of the enzyme with 200 μM orthovanadate before the addition of [γ-^{32}P]ATP strongly decreased the labeling of this polypeptide (Fig. 3, lane 2). Treatments performed after the phosphorylation with hydroxylamine or high pH completely abolished the labeling of this 100-kD band (Fig. 3, lanes 3 and 4).

When the same procedure was applied to the plasma membrane fraction previously washed with Triton X-100 and potassium bromide, three bands were phosphorylated (Fig. 4, lane 1). The most intense corresponded to the only band observed with the purified enzyme.

Effect of Beticolin-1 on the H^+-ATPase-Phosphorylated Intermediate Formation

A protein assay was performed before the sample was loaded on the electrophoresis gel so that the weakening of a band could not be attributed to a loss of proteins. In the same way, results of counting experiments are expressed in cpm/μg proteins (assayed in the counting sample).

Purified Solubilized Enzyme

When 3 μg of this purified H^+-ATPase were preincubated for 30 min at room temperature with various amounts of beticolin-1 (1.5, 3.1, or 6.2 μM), the specific radioactivity of the samples was quite similar (Table I). The specific radioactivity of the samples after incubation with 6.2 μM beticolin-1 was chased by an excess (2 mM) of unlabeled ATP or ADP (Table I).

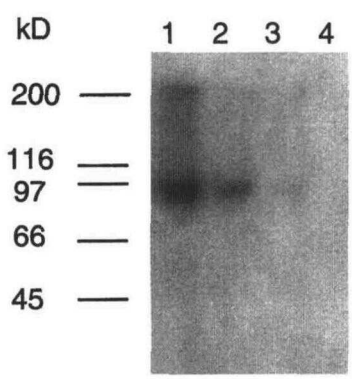

Figure 3. Characterization of the plasma membrane H^+-ATPase phosphorylated intermediate. The phosphorylation medium (0.9 mL) contained 50 mM Tris-Mes, pH 6.5, 50 mM KCl, 1 mM $MgSO_4$, and 3 μg of purified ATPase. The reaction was initiated by 10 μM (12 μCi) [γ-^{32}P]ATP, allowed to proceed for 15 s at 4°C, and stopped by addition of ice-cold TCA at a final concentration of 10%. Lane 1, Control; lane 2, incubation with 200 μM orthovanadate; lane 3, treated with 50 mM hydroxylamine; lane 4, treated with 100 mM sodium borate, pH 9.

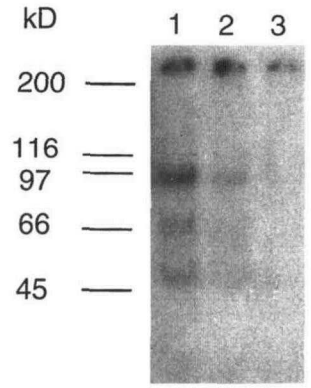

Figure 4. Effect of beticolin-1 on the plasma membrane H^+-ATPase-phosphorylated intermediate. Plasma membranes (40 μg) were preincubated for 30 min with beticolin-1 in 50 mM Tris-Mes, pH 6.5, 50 mM KCl, and 1 $MgSO_4$ at room temperature. Phosphorylation was then performed as described in the legend of Figure 3. Lane 1, Plasma membranes; lane 2, plasma membranes with 3.1 μM beticolin-1; and lane 3, plasma membranes with 6.2 μM beticolin-1.

Purified Enzyme Reconstituted into Liposomes

When the reconstituted enzyme was incubated for 30 min with beticolin-1 (3.1, 6.2, or 9.4 μM), the toxin induced a dose-dependent decrease of the specific radioactivity of the samples (Table II).

Plasma Membrane Fraction Washed with Triton X-100 and Potassium Bromide

The preincubation for 30 min of 40 μg of plasma membranes washed with Triton X-100 and potassium bromide with beticolin-1 (3.1 or 6.2 μM) led to a significant dose-dependent decrease of the phosphorylation of the 100-kD polypeptide (Fig. 4, lanes 1–3). The phosphorylation of the

Table I. *Effect of beticolin-1 on the phosphorylated intermediate level of the solubilized purified H^+-ATPase*

The phosphorylation procedure is described in the legends of Figures 3 and 4. In the chase experiments unlabeled ATP or ADP was added after incubation with [γ-^{32}P]ATP and 30 s before addition of TCA. Results are means ± SE of three independent experiments.

Incubation Medium	cpm/μg Enzyme	% of Control
Control	1495 ± 152	100
Beticolin-1		
1.5 μM	1410 ± 56	95
3.1 μM	1607 ± 145	107
6.2 μM	1542 ± 123	103
6.4 μM + 2 mM ATP	195 ± 27	13
6.4 μM + 2 mM ADP	239 ± 34	16

Table II. *Effect of beticolin-1 on the phosphorylated intermediate level of the purified H^+-ATPase reconstituted into liposomes*

Purified enzyme (3 μg) reconstituted into liposomes (lipid/protein = 100, w/w) was phosphorylated in the absence or the presence of various amounts of beticolin-1, as described in the legends of Figures 3 and 4. Results are means ± SE of three independent experiments.

Incubation Medium	cpm/μg Enzyme	% of Control
Control	1402 ± 53	100
Beticolin-1		
3.1 μM	1023 ± 41	73
6.2 μM	653 ± 111	47
9.4 μM	546 ± 61	39

two other polypeptides (66 and 47 kD) also decreased in the presence of the toxin.

DISCUSSION

To our knowledge this is the first description of the physiological effects of beticolin-1, a purified non-host-specific toxin produced by the fungus *C. beticola*. The determination of structures (Milat et al., 1992, 1993; Ducrot et al., 1994b) and the analysis on different TLC systems lead to the conclusion that this toxin is a purified form of the previously known CBT. The results obtained in this study show clearly that beticolin-1 inhibits directly the plant plasma membrane H^+-ATPase, and the low concentration of toxin necessary to produce a high level of inhibition (micromolar concentrations) is quite consistent with its possible in vivo effect. Previous results concerning the effects of CBT on the ATP-dependent proton transport in corn roots vesicles indicated a competitive inhibition with respect to ATP. Similar results were obtained with beticolin-1 on the same material by monitoring both proton transport and ATP hydrolysis (data not shown). In our study, when highly purified H^+-ATPase was used, the kinetics of ATP hydrolysis inhibition by beticolin-1 were noncompetitive with respect to ATP for the solubilized form (Fig. 2A) and competitive when the enzyme was reconstituted in proteoliposomes (Fig. 2B). Therefore, the kinetics of inhibition of H^+-ATPase by beticolin-1 seem to depend on the membrane environment of the enzyme.

These results raise two questions: (a) what could be the kind of enzyme-toxin relationship involved in this inhibition?, and (b) how do we explain the different kinetics of inhibition observed?

First, although it has been demonstrated that beticolin-1 can form stable chelates with magnesium (Jalal et al., 1992; Ducrot et al., 1994b; Mikes et al., 1994a), it is unlikely that the competitive inhibition induced by beticolin-1 on plasma membrane H^+-ATPase inserted in a membrane structure could be the result of a competition with $MgATP^{2-}$ for the catalytic site of the enzyme. Indeed, there is no structural analogy between the two complexes. Moreover, the hydrophobic feature of beticolin-1 and its accumulation in lipid bilayers has already been demonstrated (Mikes et al., 1994b). Furthermore, when the ATPase was reconstituted into liposomes, a 3-fold higher concentration of beticolin-1 was necessary to produce an inhibition comparable to the one obtained with the solubilized enzyme. This difference could be due to a trapping effect of beticolin-1 by lipids. This characteristic confirms the unlikelihood of a direct interaction between the toxin and the catalytic site located at a hydrophilic region of the protein. Thus, a more probable hypothesis would be that beticolin-1 interacts with a hydrophobic domain of the protein near the lipid bilayer in a way that leads to conformational changes. This type of regulation of the plasma membrane ATPase has already been proposed for lysoPC, a hydrophobic effector leading to a displacement of the regulatory C-terminal domain of the enzyme (Palmgren et al., 1991). In this connection, it has to be noted that preliminary results indicate an antagonistic effect of beticolin-1 and lysoPC on the plasma membrane ATPase: lysoPC is able to prevent the inhibition of ATPase by beticolin-1 at very low concentrations of this lipid, excluding a trapping effect of the toxin (data not shown).

In this context, the different kinetics of inhibition observed when the enzyme is in a solubilized form or inserted in a membrane could be explained by a different accessibility of the hydrophobic domains of the protein to the toxin.

Further studies were conducted to determine what part of the reaction mechanism of the enzyme could be involved in the enzyme-toxin interaction and to explain these different kinetics. It has been demonstrated that, similar to other cation-pumping ATPases of animal and fungal plasma membranes, the plant plasma membrane H^+-ATPase forms a phosphorylated intermediate during its catalytic cycle (Briskin and Leonard, 1982; Scalla et al., 1983; Vara and Serrano, 1983). Incubation of the purified corn root plasma membrane H^+-ATPase with [γ-^{32}P]ATP yielded only one phosphorylated polypeptide of 100 kD (Fig. 3). Phosphorylation of this polypeptide was sensitive to vanadate, hydroxylamine, and alkaline pH, which suggests that the phosphoprotein bond involved is an acyl phosphate characteristic of phosphorylated intermediate of P-type ATPases. A 100-kD polypeptide was also phosphorylated in the plasma membrane fraction washed with detergent (Fig. 3). However, two other polypeptides (66 and 47 kD) were also phosphorylated in this membrane fraction (Fig. 4). These additional peptides were also affected by vanadate but not by hydroxylamine, basic pH, or isotopic dilution, suggesting that they are not acyl-phosphate bonds but probably proteins phosphorylated by protein kinases (not shown). Additional phosphorylated polypeptides with such characteristics have already been found on corn root microsomes (Scalla et al., 1983) or *Schizosaccharomyces pombe* plasma membranes (Amory and Goffeau, 1982).

When the purified solubilized H^+-ATPase was preincubated with 1.5 to 6.2 μM beticolin-1 before the phosphorylation reaction, no modification of the level of phosphorylated intermediate was observed (Table I). However, in similar experimental conditions, a 50% inhibition of the phosphohydrolytic activity of the enzyme was achieved with 1.5 μM toxin and this inhibition was noncompetitive with respect to ATP (Fig. 2A). Such a discrepancy between

the action of an inhibitor on the hydrolysis activity and the phosphorylated intermediate level of the H^+-ATPase has already been reported (Amory and Goffeau, 1982; Vara and Serrano, 1983). According to these authors, it means that such inhibitors cannot affect the steady-state phosphoprotein concentration without affecting the dephosphorylation constant, since in this case they would inevitably produce a parallel inhibition of the ATPase activity and of the phosphorylation level (Amory and Goffeau, 1982).

On the other hand, the dephosphorylation step does not seem to be inhibited since no accumulation of the phosphorylated intermediate was observed in the presence of beticolin-1 (Table I). The fact that a chase by an excess (2 mM) of unlabeled ATP totally discharged the phosphoenzyme (Table I), even in presence of 6.4 μM beticolin-1 (producing about 90% inhibition of the phosphohydrolytic activity), seems to confirm that the dephosphorylation step is not blocked. It has been shown (Briskin, 1988a, 1988b) that (a) the phosphorylated intermediate consists of a mix of at least two forms that are chemically equivalent, named E_1P and E_2P (Fig. 5) by analogy with other transport ATPases, and (b) in the presence of 50 mM KCl (as in our experiments), E_1P and E_2P are present in equivalent amounts. These two forms can be distinguished by their different sensitivities to ADP (Briskin, 1988a): E_1P is rapidly discharged by an excess of unlabeled ADP, regenerating ATP, since E_2P is unaffected by ADP. Because the addition of 2 mM unlabeled ADP for 30 s after the phosphorylation abolished about 85% of the radioactivity (Table I), the phosphorylated intermediate could be mainly in the E_1P form when the solubilized enzyme is treated with 6.4 μM beticolin-1. Therefore, the hypothesis that beticolin-1 might inhibit the solubilized purified H^+-ATPase by blocking the E_1P to E_2P transition (Fig. 5) should be considered.

When the purified H^+-ATPase was reconstituted into liposomes, the formation of the phosphorylated intermediate was affected in a dose-dependent manner by beticolin-1 (Table II). In the same way, the preincubation of a plasma membrane fraction with beticolin-1 led to a decrease of the phosphorylation rate of the 100-kD polypeptide (Fig. 4). This result indicates that when the H^+-ATPase is inserted in a lipid bilayer beticolin-1 inhibits ATP hydrolysis by preventing the formation of E_1P. In the two most recent situations in which beticolin-1 affected the phosphorylated intermediate formation, i.e. purified enzyme reconstituted into liposomes and a plasma membrane fraction, similar concentrations of toxin produced an inhibition of H^+-ATPase phosphohydrolytic activity competitive with respect to ATP (Fig. 2B).

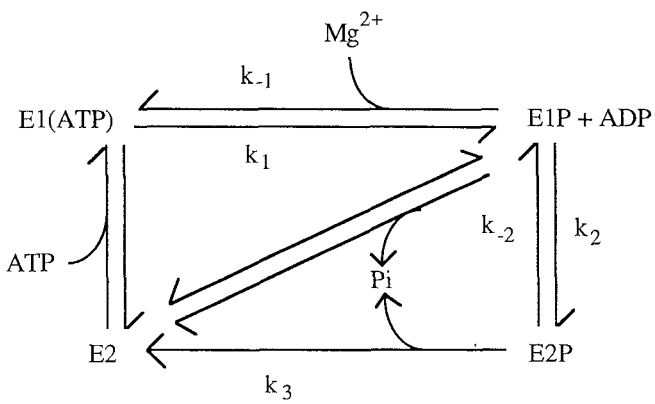

Figure 5. Proposed reaction mechanism for the red beet plasma membrane ATPase (from Briskin, 1988b).

In conclusion, our results demonstrate that in the plant plasma membrane H^+-ATPase is a direct target for beticolin-1. Moreover, it appears that the kinetics of inhibition are different depending on the enzyme environment and that this observation can be correlated with the effect of the toxin on the phosphorylated intermediate of the enzyme. Studies are envisaged to understand better the nature of the enzyme-toxin interaction and to establish some structure-function relationships using different beticolins of known structure and comparing their biological activities.

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