Phosphorylation by Inorganic Phosphate of the Plasma Membrane H\(^+\)-ATPase from Red Beet (Beta vulgaris L.)

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ABSTRACT

The phosphorylation of plasma membrane proteins from red beet (Beta vulgaris L.) by radioactive inorganic phosphate was studied. Only few proteins were phosphorylated, among them was one polypeptide with an apparent molecular weight of about 100,000. The phosphorylation of this protein was decreased when orthovanadate was present in the reaction mixture, or when the phosphorylated protein was treated with hydroxylamine. These facts suggest that this protein is a transport ATPase which is phosphorylated in a carboxyl group during the catalytic cycle. This protein was identified immunologically as the plasma membrane H\(^+\)-ATPase. The phosphorylation level of this enzyme was enhanced by dimethyl sulfoxide, whereas potassium ions did not have a significant effect on this level unless ATP was present. ATP stimulated the phosphorylation by inorganic phosphate. This stimulation was more apparent in the presence of potassium ions.

The plasma membrane H\(^+\)-ATPase is phosphorylated during its catalytic cycle in an aspartic acid \(\gamma\)-carboxyl group (5, 6). This phosphorylation is a characteristic common to all transport ATPases belonging to \('P\)' or \('E1\'-\('E2\)' class, like the Ca\(^{2+}\)-ATPase of the sarcoplasmic reticulum, the plasma membrane Na\(^+\)+K\(^+\)-ATPase of animal cells or the plasma membrane H\(^+\)-ATPase of yeasts (1, 3, 10). The Ca\(^{2+}\) and Na\(^+\)+K\(^+\)-ATPases can be phosphorylated by both ATP and inorganic phosphate (16, 20). However, the yeast enzyme is difficult to phosphorylate by Pi (2). This difficulty has been explained by considering that the rate of phosphoenzyme hydrolysis is greater in the yeast H\(^+\)-ATPase than in the sarcoplasmic reticulum Ca\(^{2+}\)-ATPase (12). There are no reports on the phosphorylation of plant H\(^+\)-ATPases by Pi.

Amory et al. (2) observed that ATP enhanced both the phosphorylation by Pi of the yeast plasma membrane H\(^+\)-ATPase and the exchange of oxygen between phosphate and water catalyzed by this enzyme. These observations indicate that this ATPase can exist in two forms: E1 which is specific for binding ATP and E2 which is the only form able to bind Pi. The E2 form is generated through the hydrolysis of the phosphoenzyme E-P, which in turn is formed by the transfer of a phosphate group from ATP to the enzyme (Fig. 1).

It has also been reported that Pi inhibits the plasma membrane H\(^+\)-ATPase from red beet, but partially, i.e. this enzyme can hydrolyze ATP even extrapolating to infinite Pi concentrations. This inhibition is greatly enhanced in the presence of 24% (v/v) DMSO and potassium ions (14). To explain these findings the existence of two enzyme-phosphate complexes was proposed: the covalent phosphoenzyme complex E-P and a noncovalent complex (E'P) which is formed either by the hydrolysis of E-P or by the interaction of the ATPase with Pi. The noncovalent complex is able to bind and hydrolyze ATP, while the E-P form is not (Fig. 1). The proposed noncovalent E-ATP-Pi complex explains the kinetics observed with the gastric mucosa H\(^+\)+K\(^+\)-ATPase (21) and the plant plasma membrane H\(^+\)-ATPase (our unpublished data), as well as the partial inhibition of these enzymes by phosphate (14, 21). Dimethyl sulfoxide probably shifts the equilibrium between these two complexes toward E-P formation by lowering the water activity of the medium. In the absence of DMSO, E'P predominates while in its presence, the inactive form E-P is the most abundant. Potassium appears to catalyze the interconversion of these two forms (14).

In this work, we observed that inorganic phosphate can phosphorylate a plasma membrane protein from Beta vulgaris, which most probably corresponds to the H\(^+\)-ATPase. This phosphorylation was enhanced by DMSO. We also observed that ATP enhances the phosphorylation by Pi, specially in the presence of potassium ions.

MATERIALS AND METHODS

Plant Material

Red beets (Beta vulgaris L.) were purchased at a local market and kept at 4°C until used, never more than 4 d later.

Plasma Membrane Preparation

Plasma membrane vesicles were prepared and stored following the method of O'Neill and Spanswick (18) as described previously (14).

Membrane Protein Phosphorylation

Plasma membrane proteins were phosphorylated by \([\text{\textsuperscript{32P}}]\) orthophosphoric acid (New England Nuclear, Carrier free) in a reaction medium (50 \(\mu\)L) containing 15 mM Tris/Mes (pH 6.5), 7.5 mM MgSO\(_4\), 30 \(\mu\)Ci \([\text{\textsuperscript{32P}}]\) orthophosphoric acid (about 66.7 \(\text{nm}\)), and the indicated concentrations of DMSO, non-radioactive Pi, ATP (Sigma, Disodium salt, converted to the Tris salt by ion exchange [19]) and KCl. The reactions, carried out at room temperature, were started by the addition of 31 \(\mu\)g of plasma membrane protein, unless otherwise indicated. At the times indicated in the footnotes, the reaction was
stopped adding 0.5 mL of 10% TCA with 2 mM KH₂PO₄. This mixture was left on ice for, at least, 10 min and centrifuged at 8400g for 10 min. The pellet was washed twice with 0.5 mL of stopping solution and once with 0.5 mL of diethyl ether.

**Polyacrylamide Gel Electrophoresis**

The method described by Amory et al. (1) was used with modifications. Protein pellets were dissolved in 30 µL of a solution containing 0.25 M sucrose, 35 mM TDAB, 1.54% (v/v) 2-mercaptoethanol, 0.2 mg/mL xylene cyanol FF as front indicator, and 100 mM KH₂PO₄/H₃PO₄ buffer (pH 4.0). The pellets were suspended in the above solution with the aid of a glass rod and this suspension was subjected to two cycles of freezing and thawing, centrifuged for 30 s in a Beckmann microfuge, and applied to 1.5 mm thick electrophoresis gels.

The separating gel (final volume: 30 mL) contained 10% acrylamide, 0.27% N,N'-methylene-bis-acrylamide (Bis), 2.33 mM TDAB, 37.5 mM KH₂PO₄ buffer adjusted at pH 2.0 with H₃PO₄, 0.077% (w/v) ascorbic acid, and traces of FeSO₄. After degassing under vacuum, the polymerization was started by adding 2.3 mL of 0.03% H₂O₂. The stacking gel (9.05 mL) contained 4.45% acrylamide, 0.012% Bis, 69.7 mM KH₂PO₄/H₃PO₄ buffer (pH 4.0), 2.57 mM TDAB, 0.084% ascorbic acid, and traces of FeSO₄. This gel was polymerized by adding 0.76 mL of 0.03% H₂O₂. The chamber buffer contained 75 mM glycine and 3.72 mM TDAB (pH 3.0 adjusted with H₃PO₄). The electrophoreses were run at room temperature for 4 to 5 h. The gels were dried after washing them in a 1% glycerol solution for 10 min and subsequently exposed to DuPont Cronex film with intensifying screens at -60°C, usually for 9 d. All treatments were repeated at least twice, some of them with different membrane preparations.

**Protein Blotting and Immunodetection of the ATPase**

After placing the gels in the transfer buffer (75 mM glycine, 0.1% [w/v] TDAB, 20% [v/v] methanol, and enough H₃PO₄ to attain a pH of 3.0) for 2 h, the proteins were transferred to nitrocellulose paper at 180 mA overnight in a Hoefer TE 22 transfer cell. In this system the proteins migrate toward the cathode. The nitrocellulose blot was then washed twice with TBS (0.5 mM NaCl and 20 mM Tris [pH 7.5]) for 10 min each time and placed in 3% gelatin in TBS for 1 h. After washing the blot twice (10 min each time) with TTBS, it was placed in 20 mL of 1% (w/v) gelatin in TTBS with 20 µL of a rabbit antibody raised against the plasma membrane H⁺-ATPase from *Arabidopsis thaliana* by Dr. R. Serrano (European Molecular Biology Laboratory, Heidelberg, Germany) for 4 h with gentle agitation. The blot was then washed twice with TTBS and 8 µL of alkaline phosphatase conjugated goat anti-rabbit IgG antibody (Bio-Rad) was applied to the blot in 24 mL of 1% gelatin in TTBS for 4 h. After washing the blot with TTBS as described, it was developed in 20 mL of 0.1 M NaHCO₃ (pH 9.8) with 1 mM MgCl₂, by adding 0.2 mL each of 5-bromo-4-chloro-3-indolyl phosphate (7.5 g/L in dimethylformamide) and nitro blue tetrazolium (15 g/L in 70% aqueous dimethylformamide).

**Protein Determination**

Protein was determined following the method of Bensadoun and Weinstein (4), using bovine serum albumin as standard.

**RESULTS**

The phosphorylation pattern of plasma membrane proteins by ³²Pi is shown in Figure 2. The most conspicuous of the few phosphorylated protein bands observed, had a molecular mass of about 100 kD, estimated by comparing the autoradiographs with stained gels with molecular mass markers. The phosphorylation of this protein was enhanced by the presence of DMSO in the reaction medium. As shown in Figure 2, the highest phosphorylation level was achieved with 40% (v/v) DMSO, although near maximum levels were obtained with a concentration of 24% (v/v) DMSO. Since the phosphorylation of this protein was low and erratic in the absence of DMSO, all the following experiments were carried out with DMSO at a concentration of 24% (v/v). In these conditions a saturation level of about 14 pmol of phosphate bound per mg of protein was estimated by counting the radioactivity in washed protein pellets, without separating the proteins by electrophoresis.

In heat-denatured plasma membranes, the phosphorylation by ³²Pi of the 100 kD protein was not detected (Figs. 3 and 5, lane 1). The radioactivity in this protein disappeared almost completely when the proteins phosphorylated with ³²Pi were treated with 50 mM hydroxyamine for 30 min (Fig. 3). This indicates that the phosphate is bound to the 100 kD polypeptide forming an anhydride bond with a carboxyl group.

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1. Abbreviations: TDAB, Tetradecyltrimethylammonium bromide; TBS, tris-buffered saline; TTBS, TBS with 0.05% (v/v) Tween 20.
Figure 2. Phosphoprotein formation with $^{32}$Pi, in media with different DMSO concentrations. The phosphorylation reactions were carried out with 66.7 mM Pi (with a specific activity of 9000 Ci/mmol), in the solution indicated under "Materials and Methods" containing: 40% (lanes 1–3), 24% (lanes 4–6), or 0% DMSO (lanes 7–8) for 2 min (lanes 1, 4, and 7), 1 min (lanes 2, 5, and 8), or 30 s (lanes 3 and 6). The 100 kD polyepitope that is phosphorylated by $^{32}$Pi is indicated by an arrow. The positions of the origin and the dye front are indicated by the letters o and d, respectively.

Figure 3. Effects of hydroxylamine on the stability of vanadate on the formation of the 100 kD phosphoprotein with $^{32}$Pi. After labeling in 24% DMSO for 2 min, precipitating and washing the proteins as indicated under "Materials and Methods;" proteins in lanes 2 to 5 were incubated for 30 min in 15 μL of 20 mM phosphate buffer (pH 6.5) (lanes 2 and 4, marked –H), or in this buffer with 50 mM hydroxylamine hydrochloride (lanes 3 and 5 [+H]). Double-concentrated electrophoresis sample buffer (15 μL) was added to protein suspensions before running the electrophoresis. Proteins in lanes 6 to 9 were phosphorylated in the presence (lanes 7 and 9 [+V]) or absence (lanes 6 and 8 [–V]) of 0.1 mM sodium orthovanadate. The proteins in lanes 2, 3, 6, and 7 were phosphorylated in the presence of 50 mM KCl. In lane 1, membrane vesicles were heat-denaturated at 100°C for 3 min and phosphorylated as those in lane 8 (HD).

Figure 4. Comparison of the plasma membrane proteins labeled with $^{32}$Pi (A) with those reacting with an antibody raised against the H+-ATPase from A. thaliana (B). Proteins (61 μg) were labeled for 2 min in 150 μL of a medium containing 24% DMSO and 130 μCl (96.3 nm) of $^{32}$Pi. After separating the proteins by electrophoresis, half of the gel was dried and exposed to radiographic film (A). The proteins in the other half, not labeled with $^{32}$Pi but treated otherwise as the labeled proteins, were transferred to nitrocellulose and detected with antibodies as described in "Materials and Methods" (B). The positions and the molecular masses (in kD) of the markers are indicated in the left margin.

phosphorylation of this protein was inhibited by the presence of 0.1 mM orthovanadate in the reaction mixture (Fig. 3). The presence of 50 mM KCl during the reaction did not show any significant effect on the phosphorylation of the 100 kD protein by $^{32}$Pi.

The results described to this point suggest that the 100 kD polypeptide that is phosphorylated by Pi is a phosphoenzyme-forming ATPase, probably the plasma membrane H+-ATPase. To confirm this hypothesis, an antibody against Arabidopsis thaliana plasma membrane H+-ATPase was applied to the plasma membrane proteins separated by electrophoresis and blotted onto nitrocellulose. The protein bands which reacted with the antibody had the same apparent molecular mass that those phosphorylated by Pi (Fig. 4).

A concentration of nonradioactive Pi of 0.1 mM lowered the levels of radioactivity on the 100 kD polypeptide by competing with the $^{32}$Pi for the phosphorylation site (Fig. 5). If this protein were a phosphoenzyme-forming ATPase, as the mentioned results indicate, it would be expected that nonradioactive ATP could also compete for the phosphorylation site, thus decreasing the phosphorylation by $^{32}$Pi. In fact, this decrease in the phosphorylation levels was observed when ATP was included in the reaction medium at a concentration of 5 mM. On the other hand, 0.1 mM ATP caused an increase in the phosphorylation of the 100 kD protein, instead of the expected decrease due to competition. This stimulation by ATP of the phosphorylation by Pi was only observed in the presence of potassium ions (Fig. 5). At high phosphate con-
centrations, this stimulation was appreciated more clearly, even when potassium was absent (Fig. 6).

When the plasma membrane proteins were phosphorylated with 32Pi for 10 min and 1 mM nonradioactive Pi was added afterward, the radioactivity on the 100 kD protein disappeared in less than 30 s (Fig. 7). It was also found that when ATP was added, instead of nonradioactive Pi, an increase in the phosphorylation of this protein was observed after 30 s. This effect was greatly enhanced in the presence of potassium ions. Phosphate and ATP added together caused the same effect than phosphate alone.

**DISCUSSION**

The following observations indicate that the 100 kD polypeptide that is phosphorylated by 32Pi is a phosphoenzyme-forming ATPase, most probably the plasma membrane H+-ATPase: (a) Its apparent molecular mass corresponds to that reported for the H+-ATPase; (b) the protein-phosphate bond formed from 32Pi is broken with hydroxylamine, which indicates that it is an anhydride bond formed with a carboxyl group; (c) Vanadate, a well-known inhibitor of the H+-ATPase, prevents the phosphorylation of this polypeptide; (d) ATP stimulates the phosphorylation with 32Pi of this protein, a phenomenon that has been reported to occur with the yeast plasma membrane H+-ATPase (2). Finally, this polypeptide reacts with an antibody raised against the plasma membrane H+-ATPase from *Arabidopsis thaliana*.

The formation of a covalent phosphoenzyme complex has been shown to occur when ‘P type’ transport ATPases are incubated with ATP (3, 5, 10, 22). On the other hand, only in few ATPases of this type, the phosphorylation with inorganic phosphate has been reported. The sarcoplasmic reticulum Ca2+-ATPase and the plasma membrane Na+K+-ATPases from animal cells have been phosphorylated with Pi in media lacking the cations that these enzymes transport (16, 19). Probably because hydrogen ions cannot be excluded from the medium, the yeast plasma membrane H+-ATPase has proven to be more difficult to phosphorylate with Pi (2); indeed, only minimal amounts of phoshoenzyme have been obtained when the plasma membrane H+-ATPases from plant cells were phosphorylated with Pi (9).

De Meis et al. (11) have shown that the levels of the phosphoenzyme formed with the sarcoplasmic reticulum Ca2+-ATPase and inorganic phosphate are higher when DMSO is present in the reaction medium. This solvent is also able to enhance the inhibition by Pi of the red beet H+-ATPase activity (14). All these results can be explained con-

Figure 5. Effects of nonradioactive Pi and ATP on the 100 kD protein phosphorylation by 32Pi. The phosphorylation reaction was carried out for 1 min in the presence of 24% DMSO and either 0.1 mM phosphate (lanes 3 and 7), 0.1 mM ATP (lanes 4 and 8), 5 mM ATP (lanes 5 and 9), or no additions (lanes 2 and 6). Proteins in lanes 6 to 9 were phosphorylated in the presence of 50 mM KCI. The membrane vesicles in lane 1 were heat-denatured before being treated with 32Pi.

Figure 6. Effect of ATP on protein phosphorylation by 0.5 mM 32Pi. Plasma membrane proteins were phosphorylated in 24% DMSO for 1 min with 32Pi (specific activity 1 Ci/mmol) in the presence (lanes 5–8) or absence (lanes 1–4) of 50 mM KCI. Proteins in lanes 3, 4, 7, and 8 were phosphorylated in the presence of 1 mM ATP.

Figure 7. Dephosphorylation of the 100 kD phosphoprotein formed with 32Pi in 24% DMSO. Plasma membrane vesicles were incubated with 32Pi in a volume of 50 μL. After, at least, 10 min, 10 μL of water (lanes 1 and 5), 12 mM Tris-phosphate (pH 6.5) (lanes 2 and 6), 5 mM ATP (lanes 3 and 7), or phosphate and ATP together (lane 4). Potassium chloride (50 mM) was present during the phosphorylation of proteins in lanes 1 to 4.
sidering that DMSO decreases the water activity of the medium, a condition that makes the hydrolysis of the phosphoenzyme much slower. In this paper, the phosphorylation of the red beet plasma membrane H\(^+\)-ATPase by Pi was made possible by employing DMSO-rich reaction media.

In the presence of 24% DMSO, ATP at a 5 mM concentration decreases the levels of ATPase phosphorylation by Pi, probably because in these conditions ATP phosphorylates the enzyme more efficiently than inorganic phosphate; although a direct stimulation of the dephosphorylation rate by ATP (8) or competition of the phosphate liberated during ATP hydrolysis with the \(^{32}\)Pi cannot be ruled out as explanations. Lower concentrations of ATP produce, in contrast, a stimulation of the phosphorylation by \(^{32}\)Pi. This observation suggests that the sites able to bind a phosphate molecule are increased by the action of ATP. Since the aminoacid sequence reveals only one phosphorylation site per 100 kD H\(^+\)-ATPase polypeptide (15, 23), ATP could increase the number of enzyme molecules able to be phosphorylated. An explanation for this increase has been advanced by Amory et al. (2). These authors considered that E2, the enzyme form that results directly from the hydrolysis of the phosphoenzyme and the subsequent Pi release (and can bind phosphate in consequence) is not the same form that binds to ATP (E1). E2 is slowly converted into E1, thus the production of the phosphoenzyme by the action of ATP indirectly leads to higher E2 levels (Fig. 1).

Nonradioactive ATP could increase the stability of the phosphoenzyme as well as the number of enzyme molecules able to bind Pi. Indeed, two phosphoenzyme conformations have been reported to exist for several ‘P-type’ ATPases (2, 10) including the plant plasma membrane H\(^+\)-ATPase (8, 9): The low-energy conformation (E – Pi in Fig. 1) can be formed by direct phosphorylation with Pi and can be directly hydrolyzed as well; and the high-energy conformation (E ~ P), which is able to donate its phosphate group to ADP, can be formed either by a direct phosphate transfer from ATP or by an energy-requiring transformation of the low-energy phosphoenzyme. If ATP could provide the energy necessary to transform the low-energy conformation into the high-energy one, which is more stable in media with low concentrations of ADP; the steady-state phosphoenzyme levels would be higher in the presence of ATP, as observed. The Pi \(\rightarrow\) ATP phosphate exchange that has been reported to be catalyzed by the yeast plasma membrane H\(^+\)-ATPase and enhanced by DMSO (12), suggests that the high-energy phosphoenzyme could actually be formed.

Finally, almost all the experiments described in this article were performed both in the presence of potassium ions and in their absence. Potassium has been shown to increase the hydrolysis rate catalyzed by the H\(^+\)-ATPase (7, 13), the rate of phosphate loss from the phosphoenzyme (8), and the conversion of the high-energy phosphoenzyme into the low-energy one (9). In addition, it is possible that potassium is also involved in the binding of phosphate since it increases the inhibition of the H\(^+\)-ATPase caused by Pi (14) or vanadate (17). The results in this article indicate that it is unlikely that K\(^+\) has any direct effect on the binding of Pi to the H\(^+\)-ATPase or on phosphoenzyme formation, since no clear effect of potassium on the phosphoenzyme equilibrium levels was observed when ATP was absent. In contrast, in the presence of ATP, the net phosphorylation of the ATPase by Pi is consistently higher in media with potassium. These findings indicate that K\(^+\) could promote the interaction of the ATPase with ATP (13), or to allow the ATP-requiring formation of a more stable phosphoenzyme conformation. From the results in this article, it seems probable that K\(^+\) could exert its stimulatory effect on the ATP hydrolysis rate by promoting the conversion of the high-energy phosphoenzyme into its low-energy form (9); or by increasing the rate of interconversion between the phosphoenzyme E-P and the noncovalent complex E’Pi without altering the equilibrium constant of this reaction (14).

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LITERATURE CITED