Plant Physiol. (1989) 90, 1279–1285
0032-0889/89/90/1279/07/$01.00/0

Received for publication November 28, 1988
and in revised form February 28, 1989

Rapid Cycling of Autophosphorylation of a Ca^{2+}-Calmodulin Regulated Plasma Membrane Located Protein Kinase from Pea

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ABSTRACT

Plasma membrane vesicles from pea (*Pisum sativum* L.) seedlings contain an autophosphorylating calcium-activated protein kinase of relative molecular weight 18,000 (phosphoprotein 18, pp18). Pulse chase analysis revealed that pp18 autophosphorylation exhibited very rapid turnover. pp18 was the only detectable plasma membrane protein to have this property. pp18 has been highly purified by affinity chromatography and the final preparation contains peptides of relative molecular weight 67,000, 48,000, and 18,000. Highly purified pp18 still showed rapid cycling of autophosphorylated phosphate attached to pp18. Turnover of pp18 autophosphorylation was accelerated by ADP, but this effect did not appear to be the back reaction of the kinase since inorganic phosphate accumulation is increased by ADP. A rapid cycling of phosphorylation is an ideal control point in signal transduction.

Protein kinases represent an ubiquitous class of enzymes (13), and the involvement of protein phosphorylation in signal transduction in plants has considerable potential (5). As in animal cells, protein kinases have been discovered that are both calcium and/or calmodulin dependent (17). These have been detected in membrane, nuclear, and soluble fractions. Additionally, extracellular signals such as red light, gravity, or ABA have been implicated as possibly modifying cytosolic calcium levels (9, 19) as part of a signal transduction process.

In an initial report we identified a membrane-bound, calcium-regulated protein kinase from pea bud tissue (11). On a specific activity basis, calcium-regulated protein kinase activity is fivefold higher in the plasma membrane when compared to microsomal membranes (2). Using the solubilization procedure of Venis (22), approximately 15 to 30% of the protein kinase activity was solubilized, with retention of calcium activation. The same protein kinase is solubilized from microsomal membranes by this procedure as is solubilized from purified plasma membrane preparations (2).

By use of dilution kinetics, nondenaturing PAGE, and renaturation after SDS-PAGE, it was shown that phosphorylation of solubilized pp18[1] had the characteristics of intramolecular autophosphorylation (3). Labeling of intact pea bud plasma membrane using [γ-^{32}P]ATP revealed that pp18 phosphorylation was complete in 15 to 30 s, whereas other proteins increased their labeling for a 4 to 5 min period (3).

In this paper, evidence is provided to suggest that autophosphorylation of pp18 is a rapid cycling of phosphate, attached to serine residues, in both the plasma membrane and in a very highly purified pp18 preparation. This may account for the unusual kinetics of plasma membrane labeling. The rather unusual behavior of this enzyme, along with calcium/calmodulin activation and cellular location, reinforces the notion that it is important in signal transduction and regulation.

MATERIAL AND METHODS

Plant Material

Peas (*Pisum sativum* L. cv Feltham First) were grown in moist vermiculite in the dark at approximately 20°C for 12 d. The experimental material was the bud which contains the unexpanded fourth and fifth leaves.

Chemicals

[γ-^{32}P]ATP (specific activity approximately 185 TBq·mmol^{-1}) was obtained from Amersham International plc (Amersham, Bucks, U.K.). Bovine calmodulin was obtained from Calbiochem (Bishops Stortford, U.K.). Other chemicals were obtained from Sigma London Chemical Co. (Poole, Dorset, U.K.) or British Drug Houses (Poole, Dorset, U.K.).

Membrane Isolation and Solubilization

Microsomal membranes were isolated (12) and washed before isolation of plasma membrane by aqueous polymer two phase partitioning as previously described (2). Percentage distribution (with recovery) of protein and marker enzyme activities for contaminants of the plasma membrane rich fraction were as follows: protein 9% (67%), NADH Cyt *c* reductase 3% (53%), latent inosine diphosphatase 3% (68%), and Cyt *c* oxidase 1% (72%).

Microsomal membranes were solubilized as described previously (12) using the procedure of Venis (22), *i.e.* acetone precipitation of membrane proteins and resuspension in an aqueous buffer. The precipitated proteins were taken up in resuspension buffer (0.3 mM sorbitol, 6 mM Tris, 6 mM Mes, 0.2 mM Na_2EDTA [pH 7.2]) with an additional 10 mM $MgCl_2$. Insoluble material was removed by ultracentrifugation at 40,000*g* for 30 min.

[1] Abbreviation: pp18, phosphoprotein of M_r 18,000.

ATP Agarose Affinity Chromatography

ATP agarose, linked to 4% beaded agarose via a 6 carbon spacer to carbon No. 8, was obtained from Sigma. All solutions and the column were maintained at 4°C. Solubilized microsomal membranes (2 mL) in resuspension buffer plus 10 mM $MgCl_2$ were added to the surface of the 1 mL ATP agarose bed and 20 × 1 mL fractions collected from the column wash of the same buffer. At this stage, all buffer had been drained from the surface of the column. After addition of resuspension buffer plus 20 mM Na_2EDTA and incubation for approximately 1 min, further 4 × 1 mL fractions were collected.

Equivalent purification could be obtained with elution using ATP in resuspension buffer with 10 mM $MgCl_2$. However, this was not used routinely since it led to prior phosphorylation of pp18.

Protein Kinase Assays

Assays were performed exactly as described by Roskoski (18) on Whatman P81 phosphocellulose strips (1 × 2 cm). Incorporated radioactive phosphate was determined by Cerenkov counting of each strip in 5 mL of H_2O. [γ-^{32}P]ATP concentrations range from 0.2 to 0.02 μM.

Gel Electrophoresis

SDS-PAGE of labeled proteins was carried out using 12% gels either 6 × 10 × 0.15 cm (mini gels) or 16 × 20 × 0.1 cm in size and bands detected by autoradiography. Sample buffer contained 4.7% SDS, 10% 2-mercaptoethanol, 20% glycerol, and 125 mM Tris (pH 6.8).

Paper Chromatography

Ascending paper chromatography was performed using Whatman No. 1 formed into cylinders (17 cm high). The paper was prewashed by ascending 10 mM Na_2EDTA and rinsed in deionized H_2O. Separation of ATP and Pi was performed in one or two dimensions. For separation in two dimensions, Propan-2-ol:ethanol:H_2O:triethylamine (30:30:39:1) in the first dimension was followed by butanol:propanoic acid:H_2O:0.1 M Na_2EDTA (325:180:237:8) in the second dimension. Standards of ATP and Pi were detected by fluorescence in UV light and acid molybdate (7), respectively.

Protein Estimation

The method of Bearden (1) was employed with absorbance measurement at a single wavelength (595 nm) and bovine serum albumin as standard.

RESULTS

pp18 Autophosphorylation during *in Vitro* Plasma Membrane Labeling Shows High Rates of Turnover

The data in Figure 1 show the effect of pulse chase conditions on the autophosphorylation of plasma membrane bound pp18 and the phosphorylation of other membrane bound proteins in SDS-PAGE separations. A plasma membrane preparation was incubated for 60 s with 50 nM [γ-^{32}P]ATP in the absence (lanes a–d) or presence (lanes e–h) of approximately 100 μM free calcium ions. The labeled plasma membrane preparation was then chased for a further 30 s with small aliquots of buffer with (lanes d and h) or without (lanes b and f) 1 μM unlabeled ATP. The establishment of pulse chase conditions led to almost total loss of the label on the doublet of bands with electrophoretic mobility of M_r approximately 18,000. These are marked by arrows. Such a doublet of bands at M_r approximately 18,000 is characteristic of the plasma membrane (2). No other phosphorylated plasma membrane protein responded in the same way to the pulse chase conditions, although some slight loss of label was observed from a peptide with an M_r of approximately 65,000. In all other phosphorylated petides a general increase in labeling occurred through the 30 s chase period (compare lanes a and b, or e and f, or g and h).

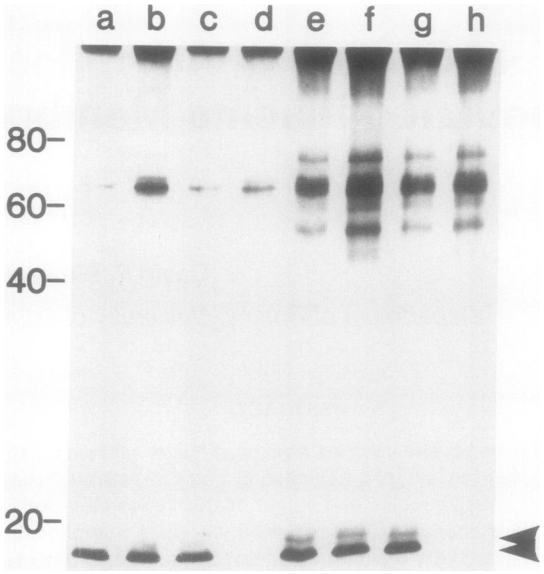

Figure 1. [γ-^{32}P]ATP/unlabeled ATP pulse chase anlalysis of plasma membrane by SDS-PAGE. Purified plasma membrane was labeled for 1 min at 0°C using 50 nM [γ-^{32}P]ATP in the presence (lanes e–h) and absence (lanes a–d) of approximately 100 μM free calcium ions. An aliquot was removed and the reaction stopped by addition of an equal volume of hot sample buffer (lanes a, c, e, and g) and heated for 10 min at 100°C. The remaining membrane was subjected to a chase of membrane resuspension buffer with (lanes d and h) or without (lanes b and f) 1 μM unlabeled ATP. After 30 s the reactions were terminated as above and all fractions separated by SDS-PAGE. The dried down gel was then subjected to autoradiography. Arrows indicate the position of pp18 bands. Numbers on left are mol wt × 10^{-3} based on standard proteins.

Several explanations could be made of these data. There could be a selective phosphatase present in the plasma membrane removing autophosphorylated phosphate from pp18. Alternatively, pp18 could be a phosphorylated enzyme intermediate, or the loss of phosphate could occur from substrates overlying pp18 on the gel, or pp18 could possess both autophosphorylation and phosphatase activities. These four possibilities could be resolved by (a) extensive purification of

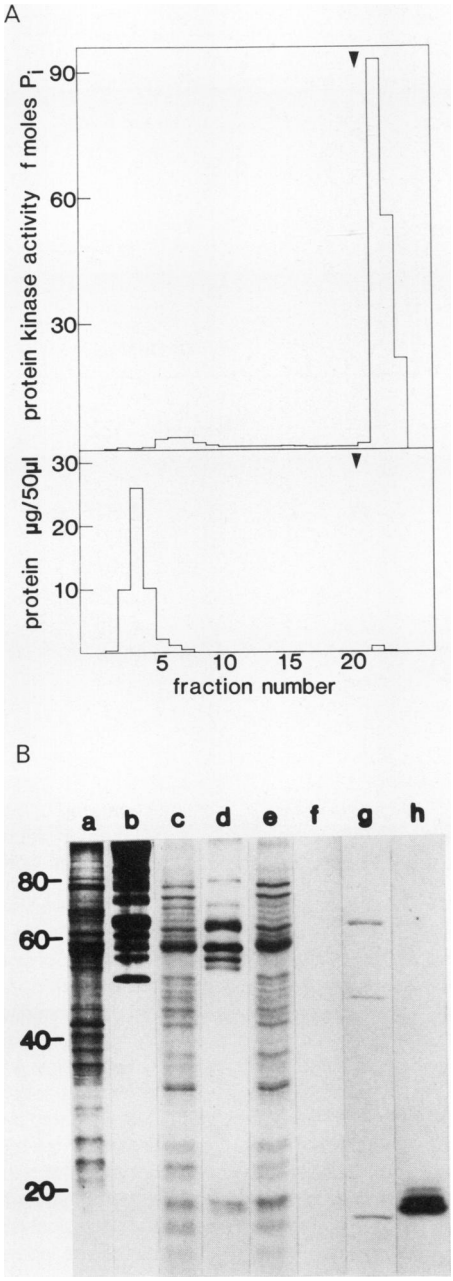

Figure 2. A, Purification of pp18 from solubilized microsomal membranes by ATP agarose affinity chromatography. Two mL of a solubilized microsomal membrane preparation in resuspension buffer plus 10 mM $MgCl_2$ were loaded onto a 1 mL bed of ATP agarose and washed with a further 20 mL of the same buffer. After collection of 20 × 1 mL fractions, the elution buffer was changed to resuspension buffer plus 20 mM Na_2EDTA and a further 4 × 1 mL fraction collected (changeover marked by arrows). Protein in 50 µL of each fraction was estimated by the method of Bearden (1). Protein kinase activity in 15 µL aliquots was estimated by the method of Roskoski (18). B, SDS-PAGE analysis of pp18 purification. Aliquots of microsomal membrane (lanes a and b), solubilized microsomal membrane (lane c and d), the ATP agarose flow-through (lane e and f), and the final EDTA containing eluate from the ATP agarose column (lanes g and h) were separated by SDS-PAGE. Lanes a, c, e, and g represent Coomassie blue stained gels. Lanes b, d, f, and h represent the endogenous protein phosphorylated by incubating the fractions in [γ-^{32}P]ATP and detected by autoradiography. In all cases, samples were heated for 10 min at 100°C with an equal volume of sample buffer prior to SDS-PAGE separation. Protein kinase initial velocity specific activities are (pmol Pi/min/µg protein); lane b, 0.77; lane d, 2.60; lane f, 0.73; lane h, 550.00. Numbers on left are mol wt × 10^{-3} based on standard proteins.

pp18 and (b) determining the phosphoamino acid(s) in pp18 undergoing rapid turnover.

Purification of pp18 by ATP Agarose Affinity Chromatography

In a previous paper (2), we described a limited purification of pp18 by nondenaturing PAGE with electroelution from gel slices. However, this procedure gave only very small amounts of enzyme, insufficient to obtain a reasonable estimate of purity by SDS-PAGE. The solubilized enzyme has now been purified by affinity chromatography on ATP agarose and the relevant data is shown in Figure 2.

Routinely, etiolated pea bud material (100 g fresh weight, 375 mg protein) was used to prepare a microsomal membrane preparation (75 mg protein). Microsomal membrane has been used in preference to purified plasma membrane so as to avoid the loss of plasma membrane as a consequence of its further purification by phase partitioning. After solubilization (as described in "Materials and Methods"), the clarified extract (2 mL containing approximately 2.7 mg protein in resuspension buffer plus 10 mM $MgCl_2$) was loaded onto the 1 mL ATP agarose column bed. The column was then washed with 20 mL of the same buffer, and the flow-through fractions were collected. Bound material was eluted from the column by washing with resuspension buffer containing 20 mM Na_2EDTA. The EDTA eluate contained 36 µg of protein in 2 mL and 95% of the eluted protein kinase activity. Recoveries of total protein from the ATP agarose column were generally approximately 70%. Figure 2A shows profiles of protein and protein kinase activity from such an ATP agarose purification procedure. Figure 2B illustrates the overall purification in qualitative terms. Although the specific activity of the final enzyme is 714-fold higher than that of the microsomal membrane, this may represent a considerable underestimate of the level of purification obtained for several reasons.

The microsomal membrane fraction contains several calcium-dependent, protein kinases (12). Only the protein kinase localized in the plasma membrane seems to be solubilized by the acetone procedure used here. Thus, the specific activity of the protein kinase is anomalously changed by selective solubilization. The number of endogenous substrates changes drastically in the process of solubilization (compare lanes b and d of Fig. 2B). This may seriously reduce sites available for phosphorylation, lowering the measured specific activities. Upon further purification on ATP agarose the remaining substrates, other than pp18, are eliminated (*cf.* lanes d and h of Fig. 2B), thus again anomalously altering the specific activity of the protein kinase fraction.

We have attempted to improve estimates of the protein kinase purification by using exogenously added substrates. Histone H1, one of the few proteins phosphorylated, is the

most suitable exogenous substrate. It can account for up to 30% of the total esterified phosphate in incubations of the microsomal membrane with [γ-^{32}P]ATP, but on solubilization this figure drops to less than 5%. This figure rises to only 20% with the final purified pp18 fraction. Autophosphorylation-induced reduction in other substrate phosphorylation has been observed for this and other protein kinases (3, 10). Attempts to estimate accurately the degree of purification using phosphorylation of bands in the 18 kD region of gels and histone attached to beaded agarose have proved unsuccessful.

Figure 2B, lane g, shows the final enzyme preparation to contain three detectable peptides, of M_r 67,000, 48,000, and 18,000. The M_r 18,000 component was previously identified as the catalytic subunit (3). Additionally, a copurifying lipid, thought to be lysophosphatidylinositol, is also present in this preparation (4). The M_r 67,000 and 48,000 peptides do not appear to be absolutely necessary for autophosphorylation since phosphorylation occurs on protein kinase renatured after SDS-PAGE (3). However, it is likely that the M_r 67,000 peptide is the calmodulin binding peptide associated with the protein kinase (M Collinge, unpublished results). Attempts to separate the M_r 18,000 peptide from the other two peptides using histone H1-agarose, iminodiacetic acid Fe^{3+}, and Con A affinity chromatography have not been successful. In addition, pp18 adhered only weakly to calmodulin-coupled columns that could have been an alternative means of further purification. On size exclusion separation on HPLC, the apparent mol wt of protein kinase activity is approximately 80,000 and is probably an aggregate. The M_r 67,000 and 48,000 peptides are still detectable in the peak protein kinase containing fractions. This may be the result of the solubilization procedure inducing aggregation or it may represent the natural enzymic state in the plasma membrane.

When this preparation of pp18 is labeled using [γ-^{32}P]ATP, at least three labeled bands could be detected, with calculated M_r of 18,300, 18,700, and 18,900 (Fig. 2B, lane h). These are higher than that for the 'unphosphorylated' peptide (M_r 18,000), as isolated prior to *in vitro* autophosphorylation (Fig. 2B, lane g). Phosphorylation induced increases in mol wt have been previously reported for another protein kinase (23). The M_r 18,300 and 18,900 components can be seen in the autoradiograph presented in Figure 1. Detection of the minor bands was variable, depending on gel loading and length of autoradiograph exposure. Autophosphorylation of pp18 in the highly purified fraction can be increased up to threefold by added calcium and calmodulin as previously reported for relatively impure preparations (2). Partial protease digestion using *Staphylococcus aureus* V8 protease and SDS-urea gel separations produced five labeled fragments (M_r ranging from 15,300–5,500), but phosphorylation of all of these seemed equally elevated by calcium and calmodulin.

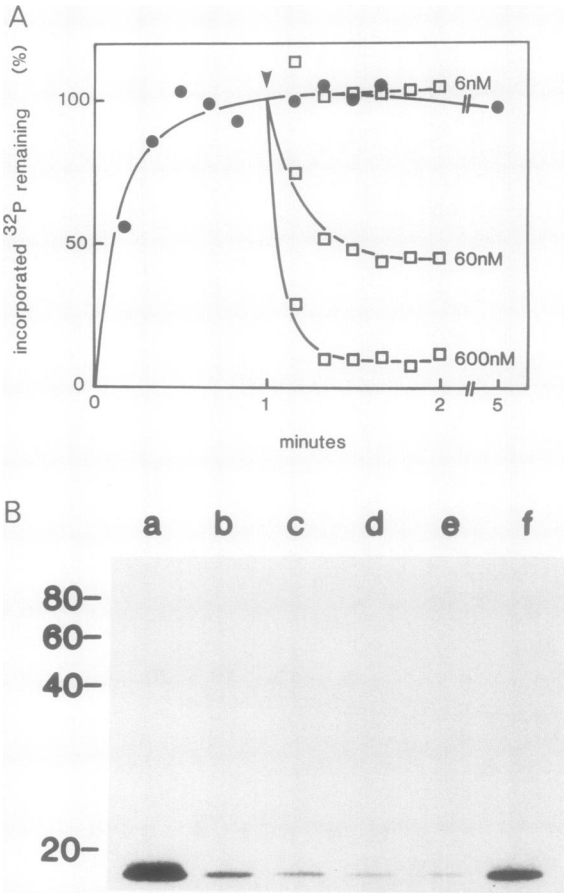

Figure 3. A, Pulse chase analysis of ATP agarose affinity chromatography purified pp18 by phosphocellulose assay. Purified pp18 was labeled using 20 nM [γ-^{32}P]ATP in the presence of approximately 100 μM free calcium ions and 3 μM bovine calmodulin at 0°C. At the times indicated, aliquots were removed to 1 × 2 cm phosphocellulose strips and assayed for kinase activity according to Roskoski (18). At the 1 min point a H_2O chase with 6, 60, or 600 nM unlabeled ATP (pH 7.2) (□) or H_2O was added. Aliquots were removed to phosphocellulose strips as above. Incorporated phosphate was estimated by Cerenkov counting of each strip in 5 mL H_2O and corrected for the slight dilution where appropriate. Results have been normalized about the 1 min point. (●) Indicates the level of labeling both before and after the H_2O chase. B, [γ-^{32}P]ATP/unlabeled ATP pulse chase analysis of highly purified pp18 using SDS-PAGE. Purified pp18 was labeled with [γ-^{32}P]ATP for 2 min at 4°C in the presence of approximately 100 μM free calcium ions and 3 μM bovine calmodulin. An aliquot was removed and the reaction stopped, as described below, prior to separation by SDS-PAGE (lane a). A chase of H_2O with (lanes b–e) or without (lane f) unlabeled ATP at pH 7.2 was then added (final concentration approximately 0.1 μM). Reactions were terminated after 10, 20, 30, and 40 s (lanes b, c, d, and e + f, respectively) by addition of an equal volume of sample buffer and heating for 10 min at 100°C. After separation by SDS-PAGE, the dried down gel was autoradiographed. Numbers on left are mol wt × 10^{-3} based on standard proteins.

Highly Purified pp18 Autophosphorylation Still Shows Rapid Turnover

Figure 3 shows that the highly purified pp18 still showed a rapid cycling of autophosphorylated phosphate. Figure 3A shows the enzyme prelabeled with [γ-^{32}P]ATP and then chased with different concentrations of unlabeled ATP at 1 min. Esterified phosphate was estimated by the Roskoski method on phosphocellulose strips (18). The rate of loss of label is as fast as that in the plasma membrane, suggesting that if loss in the membrane results from specific phosphatase activity then this activity has copurified with the enzyme.

Figure 3B shows an equivalent pulse-chase analysis using 60 nM unlabeled ATP and analysis of the products by SDS-PAGE and autoradiography. pp18 labeling was dramatically reduced in the first 10 s as in Figure 3A.

As a further illustration of the turnover of phosphorylation, an analysis of the steady state plateau of pp18 autophosphorylation was performed. Aliquots from a [γ-^{32}P]ATP-labeled pp18 preparation were removed at intervals during an extended incubation, and ATP/Pi was separated by ascending paper chromatography. After localization, the appropriate regions were excized and radioactivity estimated by Cerenkov counting. A net decrease in [γ-^{32}P]ATP and equivalent production of ^{32}Pi was observed, indicative of a turnover of phosphorylation (not shown).

ADP Can also Initiate Loss of Autophosphorylated Phosphate

Equivalent experiments to Figure 3A, but using an unlabeled ADP chase, were performed. Again the chase conditions led to a rapid loss of autophosphorylated phosphate, but at equivalent concentrations ADP leads to much lower plateau levels than ATP (not shown). The two-dimensional paper chromatography separations of the products of the ADP chase revealed a release of ^{32}Pi, illustrating that the loss of labeling is operating via a mechanism other than the back reaction of the kinase (Fig. 4). The highly purified pp18 preparation was prelabeled using [γ-^{32}P]ATP for 1 min. It was then chased for a further minute with (Fig. 4B) or without (Fig. 4A) 16 nM unlabeled ADP. There is clearly an increase in the level of ^{32}Pi (Fig.4B) compared to the control chase (Fig. 4A) implying that ADP has directly accelerated loss of phosphate. In the absence of enzyme both ATP and ADP chases had no effect on the level of phosphate. The possible mechanisms to explain this reaction are currently under investigation.

Highly Purified pp18 Autophosphorylates on Serine Residues

In a previous paper (2), we demonstrated the presence of phosphoserine in a region of a nondenaturing PAGE separation known to contain autophosphorylated pp18. Since rapid cycling of phosphate on serine is not commonly reported, it was felt necessary to clarify the amino acid(s) involved in autophosphorylation on highly purified pp18. Figure 5A shows pp18 labeled using [γ-^{32}P]ATP for 2 min, precipitated and hydrolyzed for 4 h at 100°C in 6 M HCl. The hydrolysate was separated in two dimensions and comigrating standards detected by ninhydrin staining. Phosphoserine was the only detectable phosphoamino acid in an autoradiograph of the separation. Both base and proteolytic digests of autophosphorylated pp18 failed to reveal any phosphohistidine, phospholysine, phosphoarginine, or phosphotyrosine upon ascending paper chromatography.

Further confirmation that serine is a phosphorylation site was shown by separating labeled pp18 by SDS-PAGE and subjecting the gel to alkali treatment according to the method developed by Cheng and Chen (8). There was a complete loss of label in response to the alkali treatment (Fig. 5B, lane b) when compared to the untreated gel (lane a). However, Coomassie staining of all proteins was recovered upon the return to low pH. This alkali lability of phosphorylation suggested that histidine, lysine, arginine (20, 21), or tyrosine phosphates were insignificant constituents of labeled pp18. Treatment of labeled pp18, prior to SDS-PAGE, with 100 mM hydroxylamine failed to eliminate the attached phosphate.

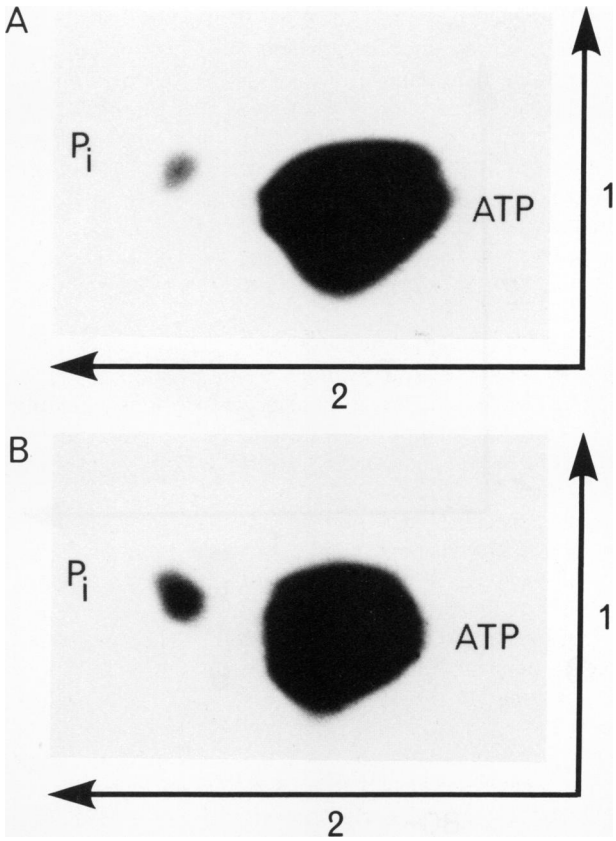

Figure 4. Release of inorganic phosphate by prelabeled pp18 in response to an ADP chase. Purified pp18 was labeled for 1 min as described in the legend to Figure 3A. A chase with (B) or without (A) ADP at pH 7.2 (final concentration 16 nM) was then performed for 1 min. The reaction products were separated by two-dimensional, ascending, paper chromatography. The dried paper was autoradiographed and only the relevant portion is shown. Arrows indicate dimension of separation: 1st, propan-2-ol:ethanol:H_2O:triethylamine (30:30:39:1); 2nd, butanol:propanoic acid:H_2O:0.1 M Na_2EDTA (325:180:237:8).

It was considered that the previously reported associated phosphoinositide (4) might somehow be present in the M_r 18,000 component during SDS-PAGE. However, numerous attempts to extract phosphoinositides from the autophosphorylated M_r 18,000 region were unsuccessful.

DISCUSSION

The evidence provided in this paper is consistent with the notion that autophosphorylation of pp18, a plasma membrane-bound protein kinase, is a rapid cycling of phosphate on serine residues. The dephosphorylating activity seems to copurify with the protein kinase. Purified pp18 still contains two other peptides as well as the catalytic peptide, pp18. This phosphatase-like activity could be on any of these peptides.

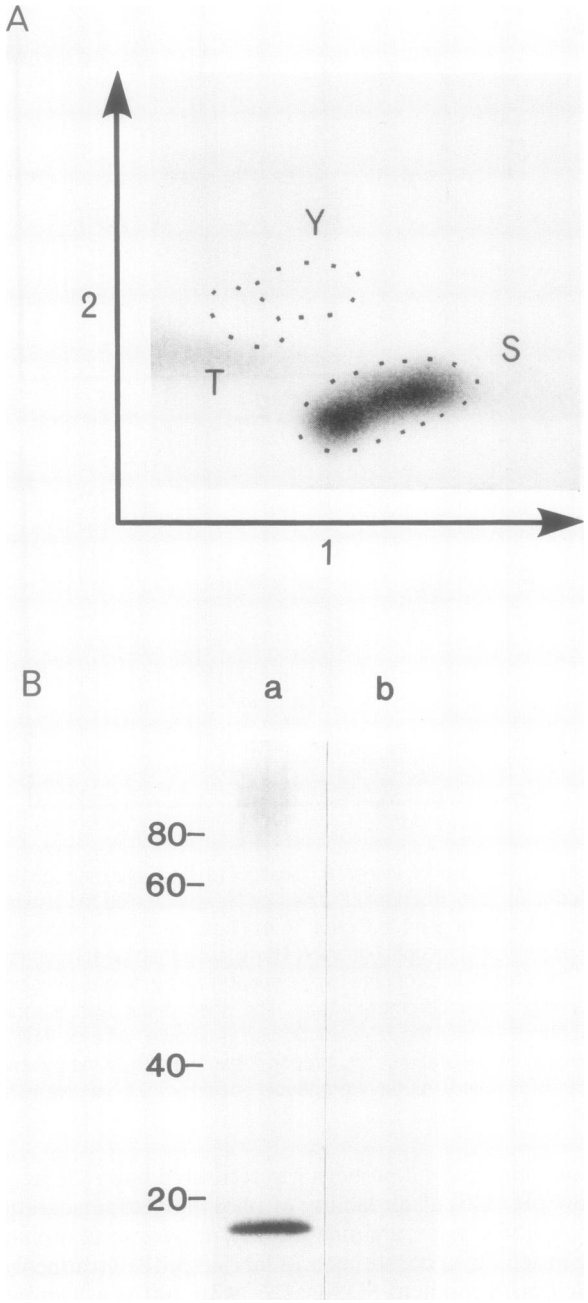

Figure 5. Phosphoamino acid analysis of ATP agarose affinity chromatography purified pp18 by acid hydrolysis. Purified pp18 was labeled using [γ-^{32}P]ATP for 2 min at 4°C in the presence of approximately 100 μM free calcium ions and 3 μM bovine calmodulin. The washed trichloroethanoic acid-precipitated pellet was hydrolyzed for 4 h at 100°C in 6 M HCl and dried down over NaOH pellets. A two-dimensional (indicated by arrows) separation of the hydrolysate was performed and the 100 μm cellulose thin layer plate autoradiographed. First dimension: pH 1.9 electrophoresis, 500 V for 3.5 h; 2nd dimension: ascending chromatography in 2-methylpropanoic acid:ammonia (0.5 M) (5:3). Positions of ninhydrin stained internal phosphoamino acid standards are indicated; S, phosphoserine; T, phosphothreonine; Y, phosphotyrosine. Only the relevent portion of the separation is shown. B, Alkali lability of pp18 autophosphorylation observed after SDS-PAGE. Purified pp18 was labeled as described in the legend to Figure 5A and the reaction terminated by addition of an equal volume of sample buffer and heating at 100°C for 10 min. After separation of equal portions of the preparation by SDS-PAGE the gel was Coomassie stained. One-half of the gel (lane b) was incubated for 30 min at 50°C in 2 M NaOH and Coomassie staining was recovered by returning the gel to storage solution. The dried-down gel halves were then autoradiographed together. Numbers on left are mol wt $\times 10^{-3}$ based on standard proteins.

Attempts to eliminate the two higher mol wt peptides by further purification have not been successful. Separation could be obtained by SDS-PAGE and kinase activity is retained after using this method (3). However, electroelution of pp18 from gels after SDS-PAGE led to purification of the M_r 18,000 peptide, but with loss of kinase activity.

The turnover of phosphate on pp18 is a genuine cycling since Pi accumulates as ATP declines. Thus, a steady state of autophosphorylation is quickly reached (Fig. 3A). With continued incubation, there appears to be an acceleration of ATP loss and Pi accumulation. This may simply reflect the slow accumulation of ADP in the assay medium. Inclusion of ADP in the incubation mixture leads to an accelerated loss of autophosphorylated [^{32}P]-phosphate. The steady state of autophosphorylation may then, in some way, reflect the ratio of ATP to ADP.

Three possible mechanisms could explain the ADP induced loss of attached [^{32}P]phosphate: (a) ADP activation of the phosphatase-like component of the reaction, (b) ADP inhibition of autophosphorylation, or (c) [^{32}P]phosphate could be replaced with [^{31}P]phosphate from the ADP. These possibilities have not as yet been distinguished, although examples of the latter two already exist in the literature. First, isocitrate dehydrogenase kinase, a key metabolic regulatory enzyme in bacteria, has both kinase and phosphatase activities (15, 16). The phosphatase component is activated by ADP. Second, pyruvate Pi dikinase can be inactivated via phosphorylation of a threonine residue from the β-phosphate of ADP (6).

The calcium dependence of pp18 and its plasma membrane location imply a role in signal transduction. The rapid cycling of autophosphorylatable phosphate reinforces this conclusion. The pp18 preparation, and its behavior in the plasma membrane, has two of the properties stipulated by Koshland (14) to make it zero order ultrasensitive. That is, it exhibits intramolecular autophosphorylation and phosphatase-like activities, with a rapid turnover of phosphorylation in the plasma membrane. Calcium and calmodulin accelerate the initial rate of autophosphorylation, the kinase activity (2); but we have not determined whether these inhibit dephosphorylation (the phosphatase-like activity) as required by the Koshland model. The first discovered example of zero order ultrasensitivity was that of isocitrate dehydrogenase kinase/phosphatase to which pp18 autophosphorylation has some similarity.

pp18 has considerable regulatory potential; future work will have to try to establish the reality of that potential in the membrane and whole cell.

ACKNOWLEDGMENTS

We acknowledge the support of the Agricultural and Food Research Council Cell Signalling and Recognition Program.

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