Protein Phosphorylation – Dephosphorylation in the Cytosol of Pea Mesophyll Cells

Ruth Hracky and Jürgen Soll

Botanisches Institut der Universität München, Menzinger Straße 67, D-8000 München 19, Bundesrepublik Deutschland

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Soluble protein kinase and protein phosphatase activities were localized in the cytosol of pea mesophyll cells using protoplasts fractionation techniques. The molecular weights of the phosphorylated cytosolic proteins, as determined by polyacrylamide gel electrophoresis, were 68, 55, 46, 38, 36, 30, 22 and 12 kDa. Histone and, to a much lesser extent, casein but not phosvitin were accepted as exogenous substrates. In every case serine served as acceptor amino acid for the phosphate residue. The protein phosphorylation activity had an alkaline pH optimum, and showed no response to varying Mg^{2+}, Ca^{2+}, P_i, cyclo-AMP or calmodulin concentrations. The kinase activity was competitively inhibited by ADP and pyrophosphate with apparent K_i values of 0.5 and 0.17 mM, respectively. High ATP concentrations (1–4 mM) resulted in a strong decrease of radioactivity in the ^{32}P labeled proteins. It is proposed that the ratio of protein phosphorylation to protein dephosphorylation is regulated by the ATP to ADP ratio in the cytosol.

Introduction

Many biochemical processes are ultimately controlled by phosphorylation-dephosphorylation of regulatory proteins. Even though a lot of information has accumulated from work in animals and microorganisms (for a review see ref. [1]) much less information is available for plant tissues. Of the protein kinases described so far in plant cells most are membrane bound and located either in the chloroplast [2–4] or at the plasma membrane [5]. Soluble protein kinases have been localized in the chloroplast stroma [6] and in the supernatant of carrot cell culture homogenates [7]. To further study protein kinase distribution in plant cells we used protoplast isolation and fractionation techniques. We report here on the presence and partial characterization of protein kinase and protein phosphatase activities in the cytosol of pea mesophyll cells.

Materials and Methods

(γ-^{32}P)ATP (111 TBq mol^{-1}) was from Amersham-Buchler (Braunschweig, FRG). Histone III-S and calmodulin from spinach were purchased from SIGMA-Chemicals. Enzymes for the protoplast isolation were either from Calbiochem (Cellulysin) or from SERVA (Macerozym R-10). Pea plants (*Pisum sativum*, cv. Rosa Krone, Bayerische Futtersaatbau, Munich, FRG) were grown on vermiculite in the green house for 10–12 days. All other chemicals were purchased from commercial sources and were of reagent quality.

Protoplasts were isolated from 20 g of pea leaves essentially as described in [8] except that 0.2% polyvinylpyrrolidone-40 was used. After maceration of the leaves for 2 h at 30 °C in the light, protoplasts were collected by centrifugation at $100 \times g$ for 5 min and further purified on a discontinous silicasol gradient (0-, 20-, 30%, v:v, Percoll in isolation medium, $200 \times g$, 10 min) [8]. Intact purified protoplasts were collected from the 0–20% Percoll gradient interphase. They were washed free of Percoll and recovered by centrifugation at $100 \times g$ for 5 min. Cytosol was obtained by breaking the protoplasts by repeated passages of the suspension through a small syringe outlet (aperture 600 µm). Chloroplasts were separated by centrifugation at $1000 \times g$ for 5 min. Membrane particles were removed from the cytosolic fraction by centrifugation at $100{,}000 \times g$ for 1 h. Protein phosphorylation was assayed by following the incorporation of ^{32}P from (γ-^{32}P)ATP into the trichloracetic acid and ethanol insoluble fraction as in [4]. Standard incubations contained 50 mM N-Tris(hydroxy-methyl)methylglycine-KOH pH 7.6, 4 mM $MgCl_2$, 300 µM ATP (containing $0.5–1 \times 10^5$ Bq(γ-^{32}P)ATP) and where indicated 100 µg Histone III-S, in a final volume of 100 µl. Enzyme assays were carried out at 22 °C for 15 min.

Reprint requests to Dr. J. Soll.

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^{32}P incorporation was either analyzed by SDS-PAGE on 7.5–15% polyacrylamide gels [4, 9] or by liquid scintillation counting. Autoradiography was done at −80 °C using an intensifying screen (MR-600, Agfa-Gaevert). Protein was estimated by the method in [10] using bovine albumin as reference.

Results

Initial experiments demonstrated high protein kinase activity in the cytosolic fraction of pea mesophyll cells which was different from the activity in the soluble chloroplast stroma (Fig. 1). Proteins phosphorylated in the cytosol had a molecular weight of 68, 55, 46, 38, 36, 30, 22 and 12 kDa. The phosphorylated band at 61 kDa is due to stromal contaminations (compare Fig. 1, lane B, D). Contamination of the cytosolic fraction by soluble proteins from mitochondria, chloroplasts and peroxisomes was assayed using soluble marker enzymes for each compartment, *e.g.* isocitrate dehydrogenase (E.C. 1.1.1.41) reversible glyceraldehyd 3-phosphate dehydrogenase (E.C. 1.2.1.13) and hydroxypyruvate reductase (E.C. 1.1.26), respectively [11, 12, 13]. Usually crosscontamination were about 10% by mitochondria and chloroplasts, and less than 5% by peroxisomes. Histone III-S (1 mg/ml) can serve as acceptor protein for the cytosolic kinase activity (Fig. 1, lane A) and to a much lesser extent also for the stromal protein kinase (Fig. 1, lane C). The cytosolic protein kinase activity accepts casein (1 mg/ml) only very poorly and phosvitin (1 mg/ml) not at all. The presence of histone in the phosphorylation assay results in a 10-fold increase in ^{32}P incorporation over the control value (Fig. 1, lane A, B) (casein 2–3-fold increase). When the mixture of phosphorylated cytosolic proteins is hydrolyzed and the phosphoaminoacids are analyzed by high voltage electrophoresis on silica gel plates [14] only phosphoserine is labeled, no phosphotyrosine and phosphothreonine is found (data not shown). When histone or casein were included in the incubation mixture phosphoserine was also the only labeled amino acid.

Further attempts were made to characterize the enzyme properties of the cytosolic protein kinase activity. Cytosolic protein phosphorylation exhibited an alkaline pH optimum in the broad range between 7.6 and 12 (Fig. 2). It showed a sharp loss of activity

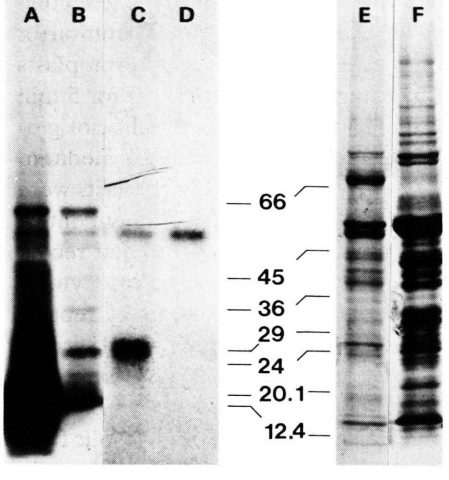


Fig. 1. SDS-PAGE of cytosolic and soluble chloroplast proteins from pea and their phosphorylation products. Lane A–D autoradiograms of phosphorylated proteins. A, 150 μg cytosolic protein + histone; B, the same as in A-histone; C, 136 μg soluble chloroplast protein + histone; D, the same as in C-histone; E, F, Coomassie brilliant blue stain of cytosolic proteins (205 μg) and chloroplast soluble proteins (408 μg). Molecular weight markers (in kDa); bovine serum albumin (66); egg albumin (45); glycerin-3-phosphate dehydrogenase (36); carbonic anhydrase (29); trypsinogen (24); soybean trypsin inhibitor (20.1); cytochrome *c* (12.4).

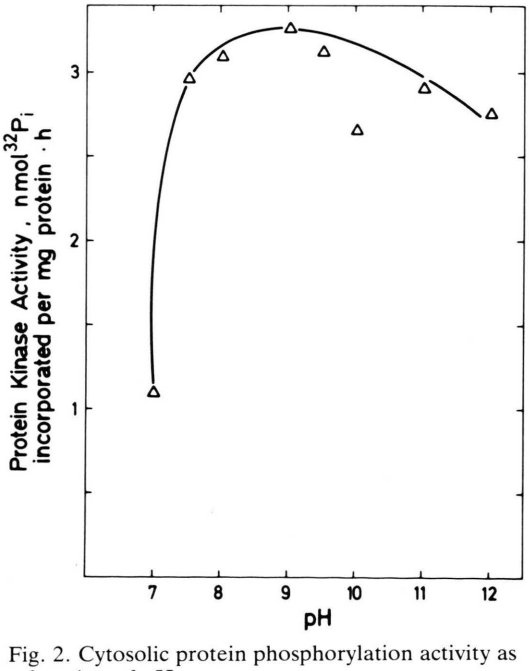
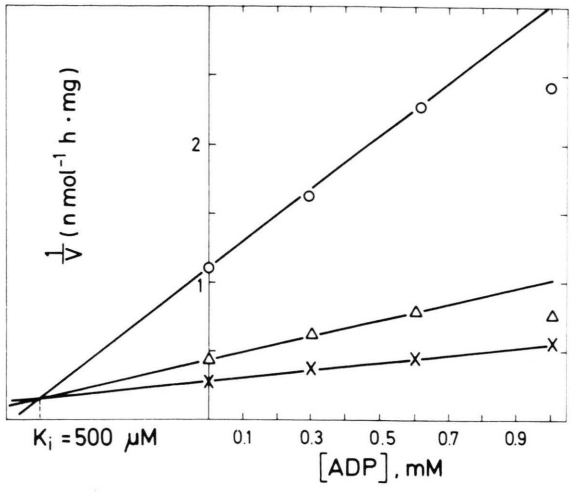


Fig. 2. Cytosolic protein phosphorylation activity as a function of pH.

at neutral and acidic pH (Fig. 2 and data not shown). Protein phosphorylation was essentially independent of Mg^{2+} (0–10 mM $MgCl_2$) and inhibited by Mn^{2+} (50% inhibition at 1.1 mM $MnCl_2$). These experiments were performed in the presence of 0.5 mM EDTA. Others [7] have described the stimulation of soluble protein kinases in plant tissues by Ca^{2+} and calmodulin. In our hands the inclusion of Ca^{2+} (3–10 μM) and calmodulin (7 μM) did not result in any significant increase in ^{32}P incorporation (stimulation between 10–15%). Furthermore cyclo-AMP (1–100 μM) had no effect on the ^{32}P incorporation. Various nucleoside diphosphates and monophosphates were tested to determine their influence on the ^{32}P incorporation. AMP, CMP, GMP and UMP were without effect (0.3 mM ATP, 0.6 mM nucleoside monophosphates). In contrast, ADP, GDP and CDP competively inhibited the phosphorylation to a similar extent (data not shown). The apparent K_i value for ADP is 500 μM (Fig. 3A). PP_i was a more potent inhibitor with an apparent K_i value of 170 μM (Fig. 3B). Phosphate (0.1–10 mM) did not affect the ^{32}P incorporation.

Protein phosphorylation as a function of ATP concentrations increased up to about 0.6 mM ATP. Higher ATP concentrations of the same specific activity produced a strong decrease of ^{32}P incorporation (Fig. 4). It was possible to show, that high ATP concentrations (1–3 mM) did not inhibit the protein kinase. This was demonstrated using an enzyme preparation which was repeatedly frozen (−20 °C) and thawn (4 times). The treatment resulted in an almost complete loss of protein phosphatase activity while the protein kinase showed a normal saturation be-



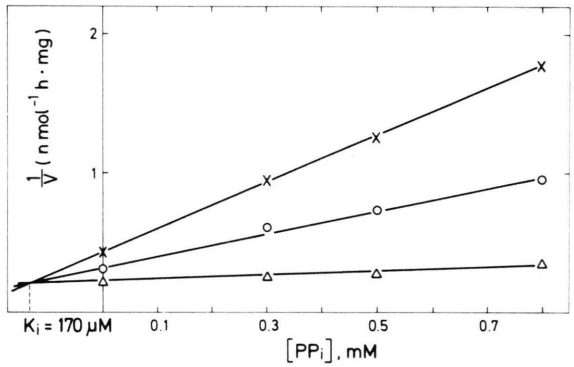


Fig. 3. Competitive inhibition of cytosolic protein phosphorylation by ADP (A) and sodium pyrophosphate (B). The K_i was determined by a Dixon plot using the following ATP concentrations; A (x, 0.5 mM, △, 0.3 mM; ○, 0.05 mM); B (△, 0.5 mM); ○, 0.3 mM; x, 0.05 mM).

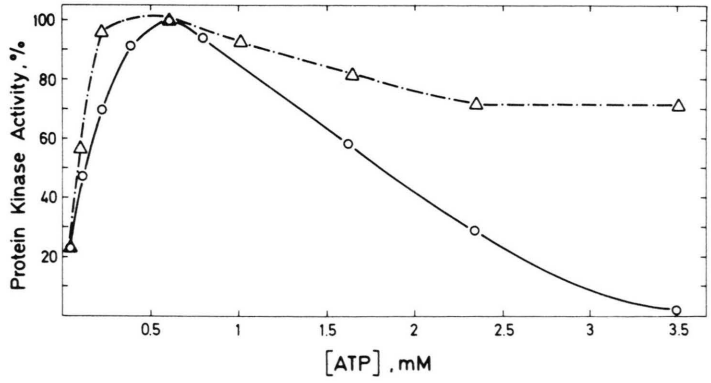


Fig. 4. ^{32}P incorporation into cytosolic proteins in the presence of histone as a function of ATP concentration. Cytosol was stored at −196 °C prior to the assay for 2 days (○); cytosol was stored as above but frozen at −20 °C and thawn for 4 times prior to the enzyme test (△).

haviour with respect to ATP ($S_{0.5}$ 0.3 mM; V_{max} 38 pmol of $^{32}P \cdot bound \cdot mg^{-1} - min^{-1}$) (Fig. 4). The results in Fig. 4 were further investigated by a different approach. Cytosolic proteins were phosphorylated for 15 min using 0.3 mM ATP, then 3 mM ATP was added and the dephosphorylation was followed over a period of 30 min. Analysis of the phosphorylation pattern by SDS-PAGE with subsequent autoradiography showed an almost complete dephosphorylation after 30 min in the presence of 3 mM ATP (Fig. 5). The phosphatase activity was inhibited by the simultaneous inclusion of ADP (3 mM) in the assay (Fig. 5). To exclude the exchange of covalently

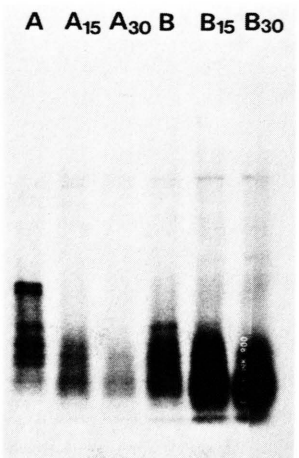


Fig. 5. Stimulation of protein dephosphorylation by ATP and its inhibition by ADP. 190 μg of cytosolic protein were incubated in the standard incubation mixture (0.3 mM ATP) for 15 min (A, B). Then 3 mM ATP of the same specific radioactivity was added and the dephosphorylation was checked after an additional 15 and 30 min (A_{15}/A_{30}). B_{15}/B_{30} as above, but 3 mM ATP + 3 mM ADP were added after the initial phosphorylation period. The phosphorylated protein mixture was analyzed by SDS-PAGE and subsequent autoradiography as described in Material and Methods.

bound phosphate residues during the course of the experiment the ATP added to activate the phosphatase was of the same specific radioactivity as for the preincubation to phosphorylate cytosolic proteins.

Discussion

The results presented in this paper demonstrate for the first time a protein phosphorylation-dephosphorylation system in the cytosol of leaf mesophyll cells. The data do not allow to decide whether only one protein kinase and one protein phosphatase is present in the cytosol or whether multiple kinases and phosphatases are localized in this cell compartment. The cytosol used in our study was about 80% pure and only slightly contaminated by soluble proteins from organelles, as demonstrated in Fig. 1. Soluble protein kinases have previously been localized i) in the stroma of chloroplasts from spinach [6], ii) in the supernatant of homogenates from non green carrot tissue cultures [7, 15], iii) in the soluble protein fraction from zucchini hypocotyl hooks and stems [16]. The cytosolic protein kinase activity described in this paper has a characteristic phosphorylation pattern, which is distinct from that of other soluble protein kinases (compare Fig. 1, references [6, 7, 16]). It also differs in its biochemical behaviour, *e.g.* it is not Ca^{2+} and calmodulin dependent in our hands [7, 16]. Interestingly, the protein kinase shows a broad alkaline pH optimum with a strong decrease in activity in the neutral and acidic range; this phenomenon is more common for stromal proteins and indicates a possible regulatory site of the enzyme. Furthermore the degree of protein phosphorylation is dependent on the ATP/ADP ratio. We have therefore determined the characteristics of ^{32}P incorporation at 300 μM ATP, an ATP level at which the protein phosphatase is presumably not active. High ATP concentrations do not inhibit the protein phosphorylation they activate a very potent protein phosphatase (Fig. 5). In turn protein dephosphorylation is inhibited by ADP more strongly than protein phosphorylation which shows only very sluggish inhibition or no inhibition at all at equimolar ATP/ADP ratios.

It is not yet clear which regulatory function this important novel system fulfills in the cytosol. Clearly more work is needed to purify its components and to elucidate their functions.

Acknowledgements

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