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REGULATION OF C_4 PHOTOSYNTHESIS : REGULATION OF PYRUVATE,P_i DIKINASE BY ADP-DEPENDENT PHOSPHORYLATION AND DEPHOSPHORYLATION

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SUMMARY: Pyruvate,P_i dikinase in extracts of chloroplasts from mesophyll cells of Zea mays is inactivated by incubation with ADP plus ATP. This inactivation was associated with phosphorylation of a threonine residue on a 100 kDa polypeptide, the major polypeptide of the mesophyll chloroplast stroma, which was identified as the subunit of pyruvate,P_i dikinase. The phosphate originated from the β-position of ADP as indicated by the labelling of the enzyme during inactivation in the presence of [β-^{32}P]ADP. During inactivation of the enzyme up to 1 mole of phosphate was incorporated per mole of pyruvate,P_i dikinase subunit inactivated. ^{32}P label was lost from the protein during the P_i-dependent reactivation of pyruvate,P_i dikinase.

Pyruvate,P_i dikinase (EC 2.7.9.1) catalyses the synthesis of phosphoenolpyruvate, the primary CO_2 acceptor of C_4 photosynthesis, by the following reaction : pyruvate + ATP + P_i $\rightleftarrows$ PEP + AMP + PP_i (1). The enzyme undergoes rapid activation when leaves are illuminated and inactivation in the dark (2,3) and these processes are considered to be accounted for by a P_i-dependent activation and an ADP plus ATP-dependent inactivation of the enzyme that can be demonstrated in vitro and are catalysed by protein factors present in chloroplasts (2, 4-6). Recently, it was demonstrated that the proteins catalysing activation and inactivation are apparently identical (7).

The present study aimed to provide information about the molecular mechanism of inactivation of pyruvate,P_i dikinase by ADP plus ATP and its subsequent reactivation by P_i. We present evidence that inactivation is due to phosphorylation of an enzyme threonine residue and reactivation is accompanied by loss of this phosphate. In contrast to other protein phosphorylation reactions the protein phosphate originates from the β-position of ADP.

Abbreviations: SDS, sodium dodecyl sulfate; AP_5A, P^1, P^5-di(adenosine-5'-) pentaphosphate.

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METHODS

The source of tissue (Zea mays leaves, variety Dekalb 805A), biochemicals and reagent enzymes was as previously described (4). Radiochemicals used were [γ-^{32}P]ATP from New England Nuclear and [α-^{32}P]ATP, [2-^{3}H]ATP and [2-^{3}H]ADP from Radiochemical Centre, Amersham. [β-^{32}P]ADP and [β,γ-^{32}P]ATP were prepared from [γ-^{32}P]ATP by exchange reactions catalysed by adenylate kinase ([γ-^{32}P]ATP incubated with 2 mM ADP, 2 mM $MgCl_2$ plus 2 units of adenylate kinase at pH 7.5 until radioactive equilibrium reached). Products were separated on a DEAE Sephadex A-25 column eluted with a 0.1 to 1 M triethylammonium bicarbonate gradient pH 7.7. Purity and the position of ^{32}P labelling in ATP and ADP was checked by polyethyleneimine cellulose thin layer chromatography (developing solvent 0.5 M acetic acid with 1M LiCl) and by the transfer of label to glucose 6-P in the presence of hexokinase.

Washed intact mesophyll chloroplasts from Zea mays leaves and extracts consisting of soluble protein from these chloroplasts were prepared as previously described except that bovine serum albumin was omitted from wash buffer and the medium used for the freeze-thaw extraction of chloroplasts (5). Pyruvate,P_i dikinase was purified from chloroplast extracts to near homogeneity by $(NH_4)_2SO_4$ precipitation and fractionation on columns of Sephacryl S-300 and Amicon Matrex Blue A (A.R. Ashton, J.N. Burnell and M.D. Hatch, to be described in detail elsewhere).

Reaction mixtures for following activation and inactivation of pyruvate, P_i dikinase were as previously described (5) except where otherwise specified in the text. For gel electrophoresis the reactions were stopped by precipitating protein by the addition of acetone (to 80% (v/v)) and then running samples of the resuspended protein on 12.5% SDS-polyacrylamide gels as described by Läemmli (8). Protein was located by Coomassie blue staining and ^{32}P label by autoradiography using preflashed X-ray film with a fluorescent intensifying screen at -80°C. For quantitation bands were cut out and measured by Cerenkov radiation counting. Gels for reactions run with ^{3}H-labelled nucleotides were cut into segments and measured by scintillation counting. Gradient SDS-polyacrylamide gel electrophoresis and two-dimensional isoelectric focussing-SDS-polyacrylamide gel electrophoresis were conducted as described by O'Farrell (9).

Pyruvate,P_i dikinase was assayed as described by Chapman and Hatch (4) and protein was determined with Coomassie blue (10) with bovine serum albumin as a standard. Specific activity is expressed as units (µmol of phosphoenolpyruvate formed per min) per mg protein.

RESULTS AND DISCUSSION

The major protein of mesophyll chloroplast extracts comigrates with highly purified pyruvate,P_i dikinase separated by electrophoresis on SDS 10-16% gradient polyacrylamide gels (Fig. 1A, molecular mass 94 kDa), on SDS 12% polyacrylamide gels (molecular mass about 100 kDa, see Fig. 1B) and two-dimensional isoelectrofocussing-SDS acrylamide gel electrophoresis (Fig. 2). Sugiyama (11) reported earlier that pyruvate,P_i dikinase is a tetramer consisting of four identical subunits with a molecular mass of about 95 kDa.

When pyruvate,P_i dikinase in chloroplast extracts was inactivated in the presence of ADP plus ATP variously labelled with ^{32}P and ^{3}H substantial

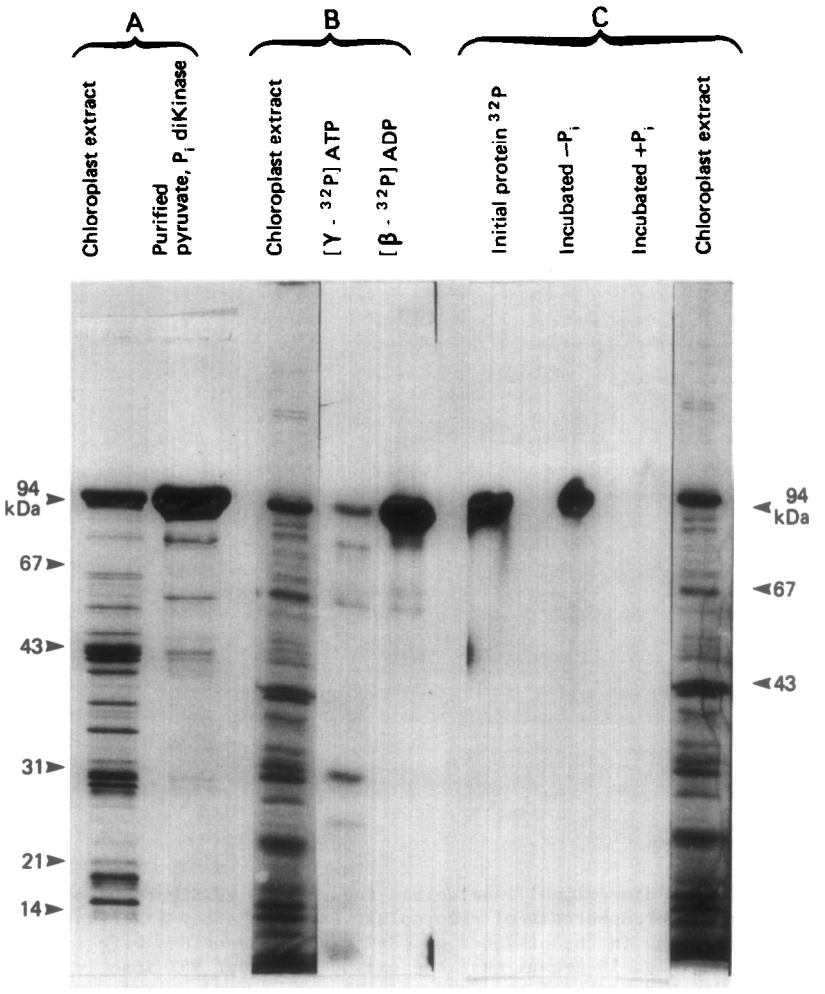


Figure 1A. Coincidence of a 100 kDa protein of mesophyll chloroplast extracts with the subunit of purified pyruvate,P_i dikinase after gradient SDS-polyacrylamide gel (10-16%) electrophoresis. Protein was located by Coomassie blue staining.

Figure 1B. Labelling of the 100 kDa protein band in chloroplast extracts (separated by 12.5% SDS-polyacrylamide gel electrophoresis) during inactivation of pyruvate,P_i dikinase in reaction mixtures (see Ref. 5) including chloroplast stromal extract, 80 μM ADP and 80 μM ATP labelled as shown with either [β-^{32}P]ADP (specific activity 2.2 x 10^5 cpm/nmole) or [γ-^{32}P]ATP (specific activity 3.8 x 10^5 cpm/nmole) and 200 μM AP_5A to inhibit adenylate kinase activity (see Ref. 5). For this experiment samples were from a reaction in which pyruvate,P_i dikinase was inactivated by more than 90% in 29 min before reactions were stopped. Other details are described in the Methods section. The first track shows soluble chloroplast protein in the reaction containing [γ-^{32}P]ATP (developed with Coomassie blue) included for comparison with the radioautograms shown in tracks 2 and 3.

Figure 1C. Orthophosphate-dependent de-labelling of ^{32}P-labelled pyruvate,P_i dikinase during activation of the enzyme. Enzyme (labelled during inactivation under conditions described for Fig. 1B) was treated on Sephadex G-25 to remove nucleotides and then incubated either with no other additions or with 2 mM P_i for 85 min. During this time about 100% of the original enzyme activity was recovered in the reaction containing P_i. Tracks 1 to 3 are radioautograms and track 4 is Coomassie blue developed chloroplast protein for samples subject to SDS - 12.5% polyacrylamide electrophoresis (see Methods for other details).

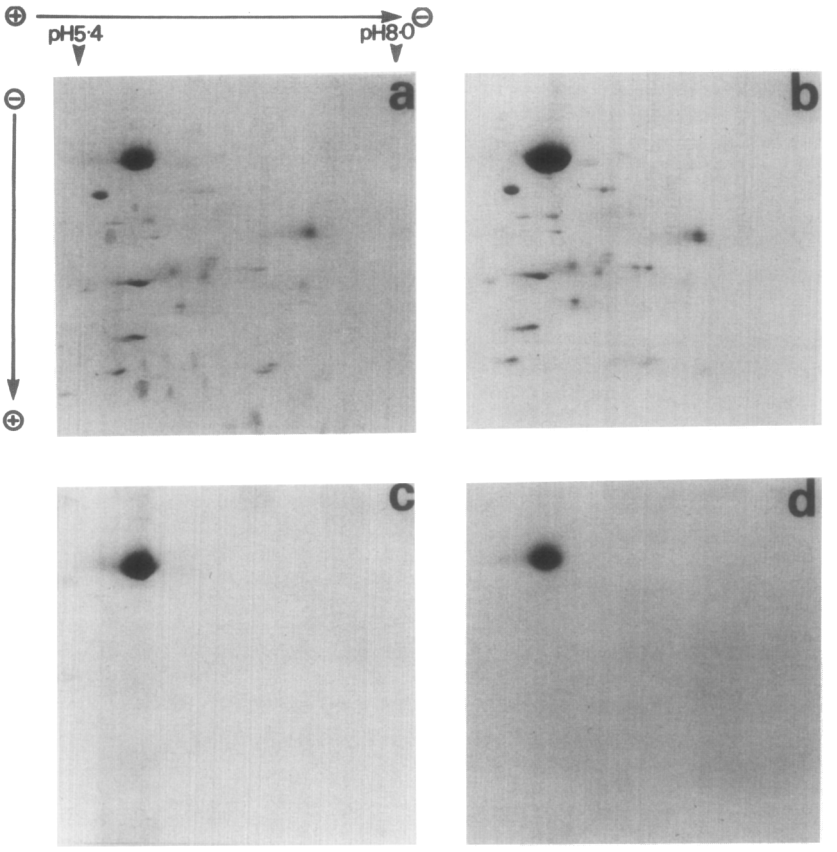
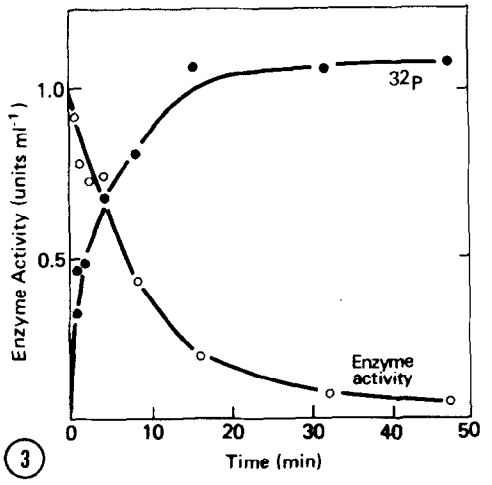


Figure 2. Two dimensional isoelectric focussing - gradient SDS polyacrylamide gel electrophoresis of chloroplast extracts after inactivation of pyruvate,P_i dikinase in the presence of [β-^{32}P]ADP. Reactions were essentially as described for Fig. 1B except that the enzyme was about 95% inactivated in 64 min. The diagrams are (a) the Coomassie blue developed protein pattern for the soluble chloroplast extract after inactivation in the presence of [β-^{32}P] ADP, (b) protein pattern for chloroplast extract supplemented with purified pyruvate,P_i dikinase, (c) purified pyruvate,P_i dikinase, (d) radioautogram of the gel described in (a).

labelling of the 100 kDa protein band only occurred from [β-^{32}P]ADP (Fig. 1B). Under the same conditions little or no label (on a molar basis 2% or less relative to [β-^{32}P]ADP) was incorporated into the 100 kDa protein in the presence of [γ-^{32}P]ATP (Fig. 1) or [β,γ-^{32}P]ATP, [α-^{32}P]ADP and ATP and [2-^{3}H]ADP and ATP (results not shown). Most of the ^{32}P incorporated into protein during the inactivation of pyruvate,P_i dikinase in the presence of [β-^{32}P]ADP appeared in a single spot after two-dimensional gel electrophoresis that coincided with the 100 kDa band in chloroplast extracts and with purified pyruvate,P_i dikinase (see Fig. 2).



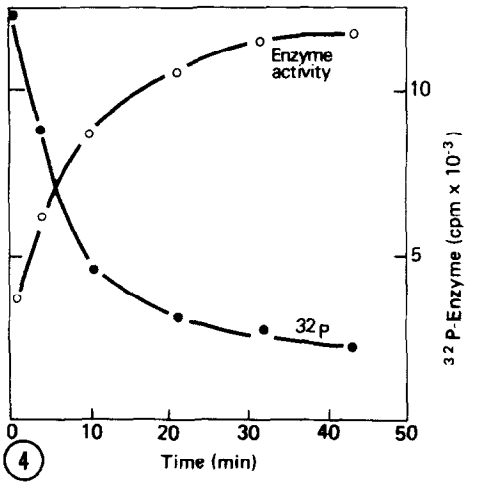


Figure 3. (left). Relation between inactivation of pyruvate,P_i dikinase and incorporation of label from [β-^{32}P]ADP. The inactivation reaction (see Ref. 5) included 0.1 mM ATP, 0.2 mM AP_5A, 0.1 mM [β-^{32}P]ADP (2.0 x 10^7 cpm) and chloroplast extract in a total volume of 2.45 ml. At intervals 25 µl aliquots were assayed for pyruvate,P_i dikinase activity and 250 µl aliquots were precipitated with 4 volumes of acetone. The precipitated proteins were separated by SDS-polyacrylamide gel electrophoresis as described in Methods. The pyruvate,P_i dikinase band was located by autoradiography, the band excised, and the radioactivity measured.

Figure 4. (right). Time-course of loss of label from pyruvate,P_i dikinase during the P_i-dependent activation of ^{32}P-labelled inactive enzyme. Pyruvate, P_i dikinase labelled during inactivation in the presence of [β-^{32}P]ADP was treated on column of Sephadex G-25 and then incubated with 2 mM P_i (see Fig. 1C). At intervals samples of this reaction were removed for analysis of protein-^{32}P and enzyme activity (see Fig. 3).

Fig. 3 shows that the labelling of pyruvate,P_i dikinase during inactivation in the presence of [β-^{32}P]ADP was reciprocally related to the changing level of enzyme activity. Likewise, the orthophosphate-dependent activation of ^{32}P-labelled inactive pyruvate,P_i dikinase was linked with the loss of ^{32}P label from the protein (Fig. 4). Based on the maximum specific activity we observed for pyruvate, P_i dikinase at 25°C of about 20 units mg^{-1} protein this represented an incorporation of about 1.1 mole of ^{32}P orthophosphate per mole of enzyme subunit (assuming four identical subunits, see Ref. 11). The specific activity was determined in chloroplast extracts (adjusted by determining the proportion of the total stromal protein in the 100 kDa pyruvate,P_i dikinase band assessed from densitometer scans after SDS polyacrylamide electrophoresis) and on purified enzyme. The proportion of total chloroplast soluble protein in the 100 kDa band was about 19% (varied between 17% and 22%) and the

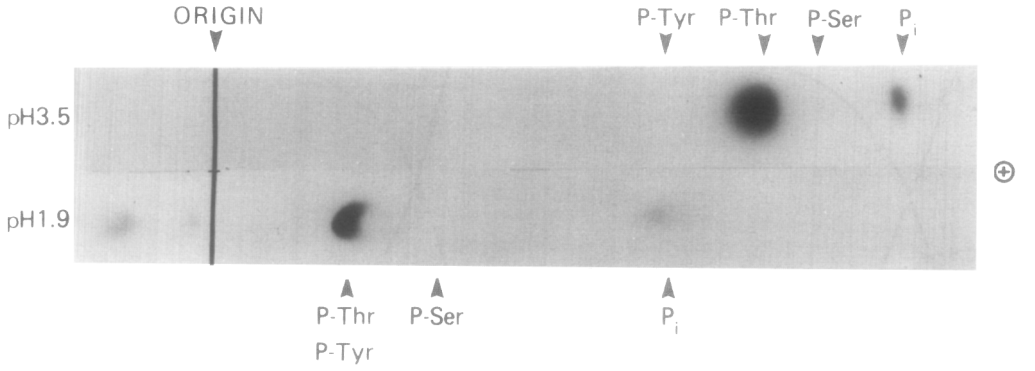


Figure 5. Labelled products formed by acid hydrolysis of ^{32}P-labelled inactive pyruvate,P_i dikinase. The area of a polyacrylamide gel containing radioactive enzyme (100 kDa band) was excised, dispersed and then hydrolysed in 6N HCl at 110°C for 2 h. The filtrate containing more than 95% of the original ^{32}P was evaporated to dryness then redissolved in a solution containing the markers phosphoserine, phosphothreonine and phosphotyrosine. Samples were subject to high voltage paper electrophoresis with various markers in adjacent channels at either pH 1.9 (2.5% formic acid; 7.8% acetic acid, 3500 volts, 60 min) or pH 3.5 (0.5% pyridine; 5% acetic acid, 2000 volts, 60 min). After radioautography and development of markers the radioactive spots were excised and counted by Cerenkov radiation counting. For the pH 1.9 run 60% of the total ^{32}P was located in the phosphothreonine region and 19% in orthophosphate. For the pH 3.5 treatment 76% of the radioactivity ran with the phosphothreonine marker and 24% with orthophosphate.

specific activity ranged between 8 and 20 units mg^{-1} protein. For purified enzyme specific activities of up to 19 units mg^{-1} protein were observed but most often lower values were obtained, including values similar to that recorded earlier by Sugiyama (11) of about 5 units mg^{-1} protein. The instability of purified pyruvate,P_i dikinase could contribute to the lower values observed.

Analysis of the products of acid hydrolysis of the protein labelled from [β-^{32}P]ADP indicated the label was located on a threonine residue. The relatively high resistance of this phosphate derivative to acid hydrolysis (only about 30% hydrolysed in 6N HCl at 110°C for 2 hr) excludes various derivatives including acyl phosphates and N-phosphoryl compounds such as phospholysine and phosphohistidine (12). Following paper electrophoresis of acid hydrolysis products most of the ^{32}P coincided with the phosphothreonine marker (Fig. 5). Essentially all the remaining label appeared with orthophosphate; no label was detected with either phosphoserine or phosphotyrosine after electrophoresis at pH 3.5.

Our data are consistent with the major polypeptide of chloroplast stromal extracts (separated on SDS-polyacrylamide gels; molecular mass 100 kDa) being the subunit of pyruvate,P_i dikinase. During ADP plus ATP-mediated inactivation of pyruvate,P_i dikinase in chloroplasts ^{32}P is incorporated from [β-^{32}P]ADP into this 100 kDa polypeptide. Our conclusion is that inactivation is caused by phosphorylation of a particular threonine residue on the enzyme. Activation is a P_i-dependent process (1, 4) and if the above conclusion is correct it follows that during incubation with P_i to activate the ^{32}P-labelled inactive enzyme this label should be removed from the protein. As shown in Fig. 1C label initially present in the inactive enzyme is lost during incubation with P_i; there was no loss of label during incubation for a comparable period without P_i or with 10 mM pyruvate (results for the latter not shown). Fig. 4 shows there is an inverse relationship between pyruvate,P_i dikinase activity and ^{32}P label on the enzyme during P_i-dependent activation.

Notably, pyruvate,P_i dikinase would be phosphorylated from the β-phosphate residue of ATP during the course of catalysis (13) and for the bacterial enzyme at least this occurs on a histidine residue (14). Besides the fact that enzyme phosphorylation occurring during inactivation is specifically derived from the β-phosphate group of ADP there are several other observations that distinguish this process from catalytic phosphorylation. For instance, the enzyme-phosphate derived from ADP is not removed by incubating with pyruvate (the catalytic phosphate is transfered to give phosphoenolpyruvate). In addition, there is no inactivation of pyruvate,P_i dikinase, and no labelling from [β-^{32}P]ADP, if chloroplast extracts are treated to inactivate the regulatory protein essential for catalysing enzyme inactivation (see Ref. 15).

Previous studies have established that the light-dark mediated activation and inactivation of pyruvate,P_i dikinase in leaves is most likely accounted for by an ADP plus ATP-dependent inactivation and a P_i-dependent reactivation of this enzyme, both demonstratable in <u>in vitro</u> systems (see Introduction).

The present studies provide evidence that inactivation of pyruvate,P_i dikinase results from a phosphorylation of the enzyme by ADP and that P_i-dependent reactivation is accompanied by dephosphorylation. The operation of ADP as a phosphate donor is apparently unique amongst protein phosphorylation reactions and indeed is rare amongst phosphotransferase reactions in general (the reaction catalysed by adenylate kinase is one example). The exact role of ATP in the inactivation process and the mechanism of activation (hydrolysis or phosphorolysis) remain to be determined.

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