Vol. 118, No. 1, 1984 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 13, 1984 Pages 65-72

REGULATION OF C_4 PHOTOSYNTHESIS : CATALYTIC PHOSPHORYLATION AS A PREREQUISITE FOR ADP-MEDIATED INACTIVATION OF PYRUVATE,P_i DIKINASE

J.N. Burnell and M.D. Hatch

Division of Plant Industry, CSIRO, GPO Box 1600,
Canberra, A.C.T. 2601 Australia

Received November 10, 1983

Evidence is provided that the role of ATP in the ADP plus ATP-dependent inactivation of pyruvate,P_i dikinase is to catalytically phosphorylate the enzyme. Only this phosphorylated form of the enzyme is susceptible to inactivation by reacting with ADP. Phosphoenolpyruvate, which also phosphorylates pyruvate,P_i dikinase during catalysis, can replace the ATP-requirement for inactivation.

Pyruvate,P_i dikinase (EC 2.7.9.1) catalyses the synthesis of phosphoenolpyruvate, the primary CO_2 acceptor in C_4 photosynthesis, from pyruvate (1). In leaves this enzyme is activated in the light and inactivated in the dark (2,3). Pyruvate,P_i dikinase in chloroplast extracts is inactivated by a process requiring ADP and trace amounts of ATP (4); both enzyme inactivated in this way and enzyme isolated from darkened leaves is reactivated in a process requiring P_i (5,6). The activation and inactivation processes are catalysed by the same protein (7).

Recently we demonstrated that ADP plus ATP-dependent inactivation of pyruvate,P_i dikinase results from the phosphorylation of an enzyme threonine residue from the ß position of ADP (8,9) and that activation results from the phosphorolytic cleavage of the threonine-bound phosphate (9). This paper provides information on the ATP-requirement for inactivation. We present evidence that ATP is required to phosphorylate pyruvate,P_i dikinase and that this catalytically phosphorylated form of the enzyme is the sole substrate for the ADP-mediated inactivation.

METHODS

The source of tissue (Zea mays), biochemicals, radiochemicals and reagent enzymes was as previously described (8), [β-^{32}P]ADP was prepared

0006-291X/84 $1.50

and isolated as described by Ashton & Hatch (8). [β-^{32}P]ATP was synthesized from [β-^{32}P]ADP and phosphoenolpyruvate using pyruvate kinase and linking the reaction with lactate dehydrogenase and NADH. Reaction mixtures contained 0.1 µmole [β-^{32}P]ADP (5.6×10^8 cpm), 0.2 µmol phosphoenolpyruvate, 0.2 µmol $MgCl_2$, 0.5 µmol KCl, 1.0 µmol Hepes-KOH, pH 7.6, 0.1 µmol NADH, 5 units of pyruvate kinase (from rabbit muscle) and 2 units of lactate dehydrogenase (from beef muscle) in a total volume of 0.1 ml. Following incubation at 25°C for 60 min [β-^{32}P]ATP was separated from the reaction mixture on a DEAE-Sephadex A-25 column by elution with a 0.05 to 0.5 M triethylammonium bicarbonate gradient, pH 7.7. [^{32}P]Phosphoenolpyruvate was synthesized from [γ-^{32}P]ATP and oxaloacetate using phosphoenolpyruvate carboxykinase isolated from bundle sheath cells of Chloris gayana (10) in a reaction containing 1 nmol [γ-^{32}P]ATP (2.9×10^3 C_i mmole^{-1}), 0.1 µmol oxaloacetate, 1 µmol Hepes-KOH, pH 8.0, and 0.1 unit of phosphoenolpyruvate carboxykinase in a total volume of 0.1 ml. Following incubation at 25°C for 15 min the product was separated from [γ-^{32}P]ATP on a DEAE-Sephadex A-25 column eluted with a 0.05 M to 0.5 M triethylammonium bicarbonate gradient, pH 7.7.

Pyruvate,P_i dikinase regulatory protein (PDRP) was partially purified by mixing blue dextran (5 mg/ml) with chloroplast extracts prepared as described previously (4) and running through a Sephacryl S-300 column equilibrated with 50 mM Tris-HCl, pH 8.3, 5 mM $MgCl_2$, 2 mM P_i and 5 mM dithiothreitol. Inactivation and activation of pyruvate,P_i dikinase were assayed as described previously (9) except that the ADP used was purified by chromatography on Dowex-1 as previously described (11); the original ADP contained 1.7% ATP and the purified ADP contained < 0.01% ATP. Pyruvate,P_i dikinase was purified from maize leaves as described previously (12).

Purified pyruvate,P_i dikinase was catalytically phosphorylated by incubating at 25°C for 20 min with either 2.5 mM phosphoenolpyruvate, 5 mM $MgCl_2$, 5 mM $(NH_4)_2SO_4$ and 10 mM Hepes-KOH buffer, pH 7.8, or by incubating with 1 mM ATP, 1 mM P_i, 5 mM $MgCl_2$ and 10 mM Hepes-KOH buffer, pH 7.8. Pyruvate,P_i dikinase was separated from phosphoenolpyruvate, ATP and orthophosphate by Dowex 1-OH ion exchange chromatography as described previously (9).

Gel electrophoresis of acetone precipitated protein was conducted as described by Laemmli (13) except that the pH of the "final sample buffer" was raised from 6.8 to 7.8 and the pH of the stacking gel was increased from 6.8 to 8.3. Proteins were dissociated by heating in the "final sample buffer" for three min at 60°C. These modifications were incorporated to prevent hydrolysis of the acid-labile but alkali-stable phosphohistidine residue.

RESULTS AND DISCUSSION

Purified pyruvate,P_i dikinase is rapidly and completely inactivated during incubation in the presence of ADP, ATP and the regulatory protein but there is little inactivation if ATP is omitted and glucose and hexokinase are included (to remove traces of ATP [4,14]) (Fig. 1). Similar results have been reported during earlier studies on pyruvate,P_i dikinase inactivation in chloroplast extracts (4). We now report that following pretreatment of the enzyme with ATP plus P_i and then removing these metabolites, substantial inactivation occurs during incubation with ADP plus glucose and hexokinase (Fig. 1). Since this effect depended on both ATP and P_i (Fig. 2 shows the

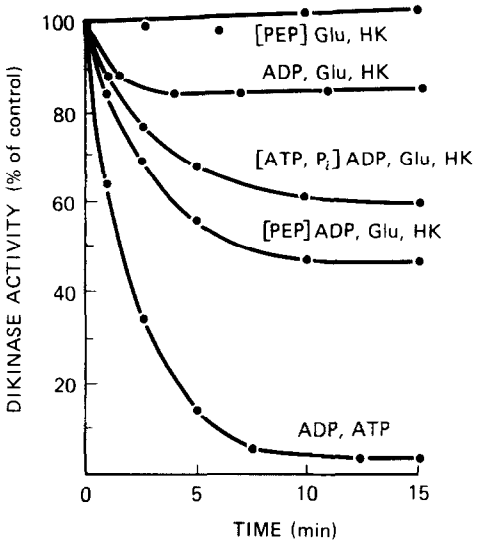

Fig. 1. Effect of phosphoenolpyruvate-and ATP plus P_i-pretreatment of pyruvate,P_i dikinase on the nucleotide requirement for inactivation of pyruvate,P_i dikinase.

Purified pyruvate,P_i dikinase was incubated with either phosphoenolpyruvate or ATP and P_i (as shown in square brackets in the figure) and then separated from the phospho anions by Dowex ion exchange chromatography (see METHODS). Pyruvate,P_i dikinase was then incubated in a basic medium containing 50 mM Tris-HCl buffer, pH 8.3, 0.1 mM P_i, 5 mM $MgCl_2$, 2 mM dithiothreitol, 0.1 mg/ml BSA and partially purified PDRP. Additions as indicated were 2 mM ADP, 2 mM glucose (Glu), 6 units hexokinase (HK) and 0.1 mM ATP.

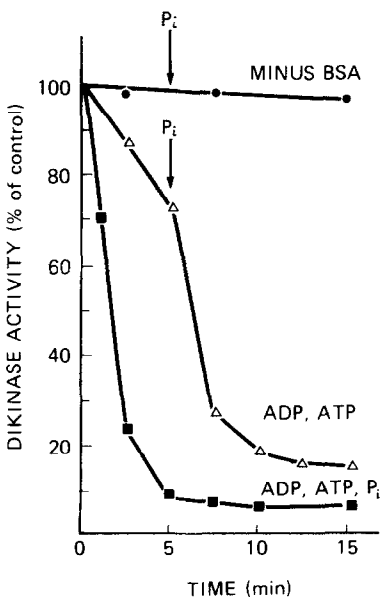

Fig. 2. Effect of P_i on ADP plus ATP-dependent inactivation.

Partially purified PDRP was run through a column of Dowex 1-OH^- to remove P_i and added to a basic incubation mixture as described for Fig. 1. Additions and deletions to the incubation mixtures were made as indicated in the figure at the same concentrations as specified in Fig. 1.

P_i-dependent stimulation of ADP plus ATP-dependent inactivation) it seemed possible that phosphorylation of the enzyme at its catalytic site was critical for inactivation (see 15,16). This follows from the observation that pyruvate,P_i dikinase from sugar cane catalyses the conversion of pyruvate to phosphoenolpyruvate via two partial reactions involving a phosphorylated enzyme (E-P) intermediate (15) viz;

$$E + ATP + P_i \rightleftharpoons E\text{-}P + AMP + PP_i \quad (1)$$

$$E\text{-}P + \text{pyruvate} \rightleftharpoons E + \text{phosphoenolpyruvate} \quad (2)$$

In further support of the above view it was shown that pretreatment of the enzyme with phosphoenolpyruvate, which would also catalytically phosphorylate the enzyme (via reversal of reaction 2), allowed substantial subsequent inactivation in the presence of ADP plus glucose and hexokinase (Fig. 1). In addition, these effects of ATP plus P_i or phosphoenolpyruvate pretreatment could be reversed by exposure of the enzyme to pyruvate (Fig. 3). Pyruvate would remove phosphate attached to the catalytic site of the enzyme (via reaction 2). If catalytic phosphorylation of the enzyme is a

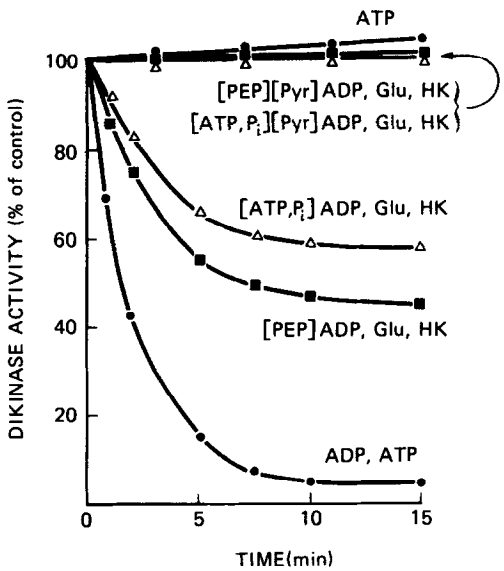

Fig. 3. Effect of pyruvate-treatment on the inactivatability of phosphoenolpyruvate- and ATP plus P_i-pretreated pyruvate,P_i dikinase. Following pretreatment by phosphoenolpyruvate or ATP and P_i (see Legend to figure 1), pyruvate,P_i dikinase was incubated with 5 mM pyruvate at 25°C for 20 min. After removing the pyruvate by Dowex ion exchange chromatography the pyruvate,P_i dikinase was incubated in the basic medium with additions as described in the Legend to figure 1.

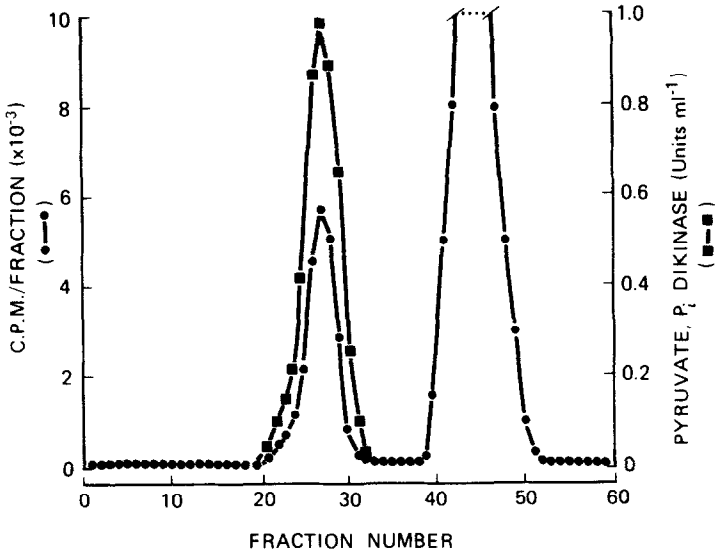

Fig. 4. Isolation of [^{32}P]phosphoryl enzyme by chromatography on Sephacryl S-300.
Pyruvate,P_i dikinase (4.8 units) was incubated for 20 min at 25°C with 10 mM Tris-HCl, pH 7.8, 5 mM $MgCl_2$, 5 mM dithiothreitol and 1 mM [^{32}P]phosphoenolpyruvate (3.3 x 10^5 cpm $nmol^{-1}$) in a total volume of 0.5 ml. The incubation mixture was then applied to a column (3.5 x 1.0 cm) of Sephacryl S-300 equilibrated with 50 mM Tris-HCl, pH 7.8, 5 mM $MgCl_2$ and 2 mM dithiothreitol. One ml fractions were collected.

prerequisite for inactivation then the partial effect of pretreatment with ATP plus P_i or phosphoenolpyruvate may reflect the proportion of enzyme recovered in its catalytically phosphorylated (E-P) form.

To further test this hypothesis experiments were conducted to determine if the plant enzyme can be recovered in a catalytically phosphorylated form. Such a phosphorylated form of the enzyme has been demonstrated for bacterial pyruvate,P_i dikinase (17). When purified pyruvate,P_i dikinase was incubated with [^{32}P]phosphoenolpyruvate and run through a column of Sephacryl S-300 the pyruvate,P_i dikinase was radioactively labelled (Fig. 4). Following SDS-polyacrylamide gel electrophoresis of this labelled protein (modification of previously described procedure, see METHODS) all the radioactivity appeared with the 100 kDa band previously shown to be the subunit of pyruvate,P_i dikinase (8). Pyruvate,P_i dikinase was also radioactively labelled in the presence of [β-^{32}P]ATP plus P_i, however, both the rate and extent of ^{32}P-labelling by [β-^{32}P]ATP plus P_i were lower than

with [^{32}P]phosphoenolpyruvate. There was a marked correlation between both the extent of catalytic phosphorylation and the rate and extent of inactivation of catalytically phosphorylated enzyme. Approximately 40% of the enzyme was phosphorylated (μmoles ^{32}P incorporated per μmol of active enzyme subunit - see (9)) by ATP plus P_i and approximately 40% of the enzyme was subsequently inactivated on the addition of ADP compared with approximately 60% catalytic phosphorylation and about 60% inactivation with phosphoenolpyruvate. The incomplete inactivation of pretreated pyruvate,P_i dikinase may be due to either incomplete catalytic phosphorylation of the enzyme during the pretreatment, or dephosphorylation of the enzyme during subsequent isolation. The fact that untreated pyruvate,P_i dikinase was inactivated to a small extent in the presence of partially purified PDRP, ADP, glucose and hexokinase (Fig. 3) was probably due to a small proportion of the pyruvate,P_i dikinase which remained catalytically phosphorylated during purification. Notably this inactivation was prevented by pretreatment with pyruvate (Fig. 1 & 3). Pyruvate pretreatment of pyruvate, P_i dikinase does not interfere with ADP plus ATP-dependent inactivation as long as the pyruvate is removed prior to inactivation.

Incubation of the ^{32}P-labelled enzyme with pyruvate removed the ^{32}P label supporting the view that the Zea mays pyruvate,P_i dikinase forms a catalytic-site phosphoryl-enzyme (E-P). Failure to detect catalytically-derived ^{32}P-labelling of purified pyruvate,Pi dikinase incubated with [β,γ-^{32}P]ATP by Ashton et al (9) was probably due to hydrolysis of the phosphate group during SDS-dissociation of the enzyme in acidic conditions prior to gel electrophoresis. Data presented in Table 1 supports this hypothesis. This view is also supported by the fact that acid hydrolysis, but not alkaline hydrolysis, quantitatively released the ^{32}P-label from the enzyme as [^{32}P]P_i. This phosphoryl-enzyme derivative is almost certainly the same as the acid-labile phosphoryl-enzyme intermediate formed by bacterial pyruvate,P_i dikinase in which the phosphate is bound to a histidine (18).

TABLE 1. Effect of pH and temperature during enzyme dissociation on the loss of ^{32}P-label from catalytically phosphorylated pyruvate P_i dikinase.

Treatment conditions during enzyme dissociation pH	(°C)	Radioactivity in the 100 kDa protein band after electrophoresis (cpm x 10^{-3})
6.8	100	3.3
6.8	60	8.8
7.8	100	19.8
7.8	60	22.6

Pyruvate,P_i dikinase was catalytically phosphorylated with [^{32}P]-phosphoenolpyruvate (see METHODS) and separated from [^{32}P]-phosphoenolpyruvate by Sephacryl S-300 chromatography (see Fig. 4). The most highly labelled fraction was divided into four equal volumes, the protein precipitated by adding acetone (80% v/v) and the protein incubated at the pH and temperature indicated in the table for 3 min in Laemmli's (13) modified "final sample buffer" (see METHODS). After the dissociated proteins were separated by SDS-polyacrylamide gel electrophoresis, and protein was located by briefly soaking the gel in 1M KCl, the gel was cut into 1 cm sections and the radioactivity counted in a scintillation counter.

CONCLUDING REMARKS

Previous studies have established that the in vitro inactivation of pyruvate,P_i dikinase is caused by phosphorylation of a threonine residue of the enzyme and that P_i-dependent reactivation is accompanied by dephosphorylation of the phosphothreonine. The present study demonstrates that the substrate for inactivation is the catalytic E-P intermediate form of the enzyme. For the bacterial pyruvate,P_i dikinase, the amino acid sequence of the peptide containing the histidine which is catalytically phosphorylated has been determined; a threonine residue is located next but one to the histidine residue involved in catalysis (19). If the amino acid sequence of the peptide at the active site of maize leaf pyruvate,P_i dikinase is similar to that described for the bacterial enzyme, steric interference by the ADP-dependent phosphorylation of the threonine residue in close proximity to a catalytically phosphorylated histidine residue may provide a mechanism for regulation. This hypothesis is currently under investigation.

REFERENCES

1. Hatch, M.D. and Slack, C.R. (1968) Biochem. J. 106, 141-146.
2. Hatch, M.D. (1981) in Photosynthesis iv. Regulation of Carbon Metabolism (Ed. G. Akoyunoglou) pp. 227-236. Balaban Int. Science Services, Philadelphia, Pennsylvania.

3. Hatch, M.D. and Osmond, C.B. (1976) in Encyclopedia of Plant Physiology (New Series) Vol. 3, Transport in Plants III, (Eds. C.R. Stocking and U. Heber), pp 144-184.
4. Sugiyama, T. and Hatch, M.D. (1981) Plant and Cell Physiol. 22, 115-126.
5. Hatch, M.D. and Slack, C.R. (1969) Biochem. J. 112, 549-558.
6. Chapman, K.S.R. and Hatch, M.D. (1981) Arch. Biochem. Biophys. 210, 82-89.
7. Burnell, J.N. and Hatch, M.D. (1983) Biochem. Biophys. Res. Commun. 111, 288-293.
8. Ashton, A.R. and Hatch, M.D. (1983) Biochem. Biophys. Res. Commun. 115, 53-60.
9. Ashton, A.R., Burnell, J.N. and Hatch, M.D. (1983) Arch. Biochem. Biophys.
10. Hatch, M.D. and Mau, S. (1977) Aust. J. Plant Physiol. 4, 207-216.
11. Hurlbert, R.B. (1957) Methods in Enzymol. 3, 785-805.
12. Hatch, M.D. (1979) Aust. J. Plant Physiol. 6, 607-619.
13. Laemmli, U.K. (1970) Nature 227, 680-685.
14. Feldhaus, P., Frolich, T., Goody, T., Isakov, M. and Schirmer, R.H. (1975) Eur. J. Biochem. 57, 197-205.
15. Andrews, T.J. and Hatch, M.D. (1969) Biochem. J. 114, 117-125.
16. Goss, N.H. and Wood H.G. (1982) Meth. Enzymol. 87, 51-66.
17. Milner, Y., Michaels, G. and Wood, H.G. (1978) J. Biol. Chem. 253, 878-883.
18. Spronk, A.M., Yoshida, H. and Wood, H.G. (1976) Proc. Natl. Acad. Sci. U.S.A. 73, 4415-4419.
19. Goss, N.H., Evans, C.T. and Wood, H.G. (1980) Biochemistry, 19, 5805-5809.