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REGULATION OF C_4 PHOTOSYNTHESIS CATALYTIC DEPHOSPHORYLATION AND P_i-MEDIATED ACTIVATION OF PYRUVATE P_i DIKINASE

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In experiments designed to test the reversibility of ADP-dependent inactivation and P_i-dependent activation of pyruvate,P_i dikinase, it was found that the preferred substrate for P_i dependent activation is the catalytically non-phosphorylated form of pyruvate,P_i dikinase. Only the second of the two partial reactions catalysed by pyruvate,P_i dikinase is inhibited when pyruvate,P_i dikinase is inactivated by ADP-dependent phosphorylation. Neither ADP-dependent inactivation nor P_i-dependent activation reactions were found to be reversible.

Pyruvate,P_i dikinase (EC 2.7.9.1), which catalyses the conversion of pyruvate to phosphoenolpyruvate in C_4 plants, is under light/dark-mediated control (1,2). This control can be simulated in vitro by an ADP-dependent inactivation and a P_i-dependent activation (3,4); both of these reactions are catalysed by the same protein (5). The level of pyruvate,P_i dikinase activity in maize leaves is controlled, to some extent, by the adenylate charge in the mesophyll chloroplasts (6).

Earlier work reported from this laboratory demonstrated that the inactivation of pyruvate,P_i dikinase is caused by phosphorylation of a threonine residue of the pyruvate,P_i dikinase (7) and that the substrate for inactivation is the catalytically-phosphorylated form of the enzyme where the enzyme is phosphorylated on a histidine residue (8,9) (see Figure 1).

Andrews and Hatch (10) reported that pyruvate,P_i dikinase catalyses two partial reactions.

$$E + ATP + P_i \rightleftharpoons E\text{-}P + AMP + PP_i$$

$$E\text{-}P + \text{Pyruvate} \rightleftharpoons E + \text{Phosphoenolpyruvate}$$

This paper reports that ADP-dependent inactivation inhibits only the second partial reaction. It also reports that the preferred substrate for P_i-dependent activation is the non-catalytically phosphorylated form of pyruvate,P_i dikinase. In addition it reports that AMP is the nucleotide

Abbreviations: PEP, phosphoenolpyruvate; PDRP, pyruvate,P_i dikinase regulatory protein.

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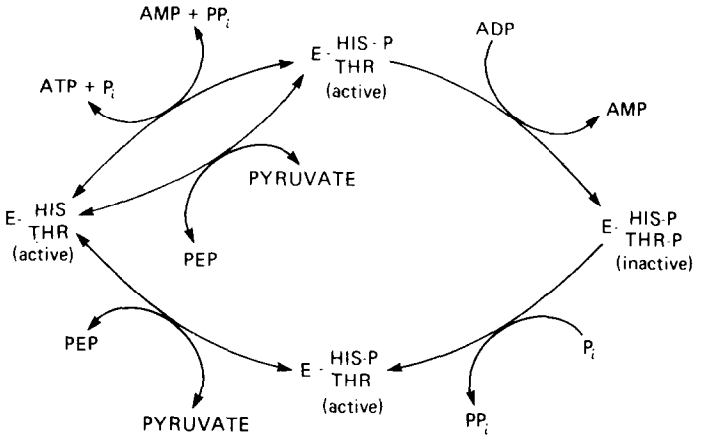

Fig. 1. Scheme describing the mechanism of inactivation and activation of pyruvate,P$_i$ dikinase.

end-product of ADP-dependent pyruvate,P$_i$ dikinase inactivation and that neither AMP-dependent activation nor PP$_i$-dependent inactivation could be demonstrated.

MATERIALS AND METHODS

The source of maize leaves (Zea mays var. Dekalb XL81), biochemicals, radiochemicals and reagent enzymes was as described previously (7), [β-^{32}P]ATP was synthesised and isolated as described previously (8), inactivation and activation of pyruvate,P$_i$ dikinase were assayed as described previously (11) and active pyruvate,P$_i$ dikinase was purified from maize leaves as described by Hatch (12). Dowex ion exchange chromatography of inactivation and activation incubation mixtures was conducted as described previously (8).

Incubation mixtures for testing the reversibility of ADP-dependent inactivation of pyruvate,P$_i$ dikinase contained 50 mM Tris-HCl, pH 7.2-8.3, 5 mM $MgCl_2$, 2 mM dithiothreitol, 0.6 mg inactive pyruvate,P$_i$ dikinase, 1.2 units PDRP, 2 mg BSA, and varying concentrations of AMP. Incubation mixtures for testing the reversibility of P$_i$-dependent activation of pyruvate,P$_i$ dikinase contained 50 mM Tris-HCl, pH 7.2-8.3, 5 mM $MgCl_2$, 2 mM dithiothreitol, 0.8 mg pyruvate,P$_i$ dikinase, 1.2 units PDRP, 2 mg BSA and varying concentrations of PP$_i$.

[^{14}C]ATP-AMP and [^{14}C]pyruvate-PEP exchange reactions were conducted as described by Andrews and Hatch (10) except that reactions were terminated by the addition of 20 µl 2 M trifluoroacetic acid, and nucleotides, PEP and pyruvate were separated by thin layer chromatography on PEI-cellulose in 1 M LiCl/0.5 M acetic acid.

ATP and AMP spots were located by UV fluorescence quenching. Pyruvate was located using 2,4-dinitrophenylhydrazine (13) and PEP was located as described previously (14). Those sections of the TLC plates corresponding to either ATP, AMP, pyruvate or PEP were excised and the radioactivity determined using a scintillation counter.

Pyruvate,P$_i$ dikinase regulatory protein (PDRP) was prepared from mesophyll chloroplasts. Chloroplast extracts were prepared as described previously (15) except that phosphate (2 mM) was added to the freeze-thaw mixture. The chloroplast extract was treated with solid $(NH_4)_2SO_4$ and the protein precipitating between 20 and 45% saturation was dissolved in buffer (50 mM Tris-HCl, pH 8.3, 5 mM $MgCl_2$, 2 mM dithiothreitol and 2 mM P$_i$) and applied to a column (1.5 x 35 cm) of Sepharose 6B. The active fractions

were pooled, the protein precipitated with 65% saturated $(NH_4)_2SO_4$, dissolved in a minimal volume of buffer and mixed with Blue dextran (5 mg/ml) prior to running through a column (1.5 x 35 cm) of Sephacryl S-300. The regulatory protein activity coeluted with the Blue dextran and was stored at 4°C until required. The purified PDRP was free of inorganic pyrophosphatase, phosphatase, adenylate kinase, ATPase and pyruvate, P_i dikinase activities.

RESULTS

The scheme for the regulation of pyruvate,P_i dikinase outlined in a recent report from this laboratory (9) and summarized in the INTRODUCTION, raised the question as to the identity of the nucleotide end-product of inactivation. To identify the end-product of inactivation, [U-^{14}C]ADP was included in inactivation incubation mixtures and the end-products of the reaction were subjected to PEI-cellulose thin layer chromatography. The only ^{14}C-labelled compound formed during inactivation of pyruvate,P_i dikinase was shown to be AMP. Control experiments were run to confirm the PDRP-dependent production of AMP during inactivation (see Table 1). Knowing the specific activity of the [U-^{14}C]ADP (3.6 x 10^3 cpm. nmole^{-1}) and assuming a specific activity of 10 units/mg of protein for pyruvate,P_i dikinase (see ref. 11) about 1 µmol of [^{14}C]AMP was released from [U-^{14}C]ADP per µmol of pyruvate,P_i dikinase inactivated.

Experiments were then conducted to determine the reversibility of P_i-dependent activation and ADP-dependent inactivation reactions. These reactions were not reversible in a purified system in which the pyruvate,P_i dikinase was catalytically phosphorylated. However, the question remained as to the phosphorylation status of pyruvate,P_i dikinase required for the reverse reactions. In an attempt to solve this problem the effect of ADP-dependent

TABLE I

IDENTIFICATION OF THE END-PRODUCT OF ADP-DEPENDENT INACTIVATION OF PYRUVATE,P_i DIKINASE

TREATMENT	RADIOACTIVITY IN AMP cpm	UNITS PPDK INACTIVATED
Complete reaction	1475	0.57
" " minus PDRP	30	<0.1
" " minus BSA	0	<0.1
" " minus PPDK	35	<0.1

*Specific activity of [^{14}C]ADP was 3.6 x 10^3 cpm. nmole^{-1}.

Reaction mixtures contained 0.6 units purified pyruvate,P_i dikinase, 0.22 µmol *[U-^{14}C]ADP, 0.3 units purified regulatory protein, 0.1 µmol ATP, 0.1 µmol AMP (added to prevent removal of [^{14}C]AMP once it was formed), 2 mg BSA, 0.9 µmol $MgCl_2$ and 9 µmol Tris-HCl, pH 8.3, in a total volume of 0.18 ml. Following inactivation of pyruvate,P_i dikinase (95% inactivation) at 25°C 40 µl aliquots of each incubation mixture was spotted onto a polyethylene-imine cellulose T.L.C. plate, developed with 1M$LiCl_2$/0.5 M acetic acid, dried, and the radioactivity determined by scintillation counting.

TABLE II

EFFECT OF ADP-MEDIATED INACTIVATION ON THE [^{14}C]ATP-AMP AND THE [^{14}C]PYRUVATE-PEP EXCHANGE REACTIONS

TREATMENT	EXCHANGE RATES (μmoles.min^{-1}) x 10^{-2}	
	[^{14}C]ATP-AMP	[^{14}C]Pyruvate-PEP
PPDK	11.3	17.3
PPDK plus 2 mM ADP	11.4	17.1
PDRP	<0.01	<0.01
PDRP plus 2 mM ADP	<0.01	<0.01
PPDK + PDRP + 2 mM ADP (30 min)	11.2	0.11

[^{14}C]ATP-AMP and [^{14}C]pyruvate-PEP exchange reactions were conducted as described in the METHODS. Additions to exchange assay mixtures were made as described in the table ADP-dependent inactivation of pyruvate,P_i-dikinase was run for 30 min at which time 98% of the activity was inactivated.

inactivation upon the two partial reactions catalysed by pyruvate,P_i dikinase was investigated and the results shown in Table II. ADP-mediated inactivation of pyruvate,P_i dikinase almost completely inhibited [^{14}C]pyruvate-PEP exchange but did not affect the [^{14}C]ATP-AMP exchange.

Since the first partial reaction catalysed by pyruvate,P_i dikinase is not affected by ADP-mediated inactivation, a means of examining the catalytic phosphorylation status of pyruvate,P_i dikinase required for inactivation is available. It should be possible to remove the catalytic phosphate of ADP-dependent inactivated pyruvate,P_i dikinase by incubating with AMP and PP_i, the activation of this catalytically-dephosphorylated enzyme can then be followed and the catalytic phosphorylation status of pyruvate,P_i dikinase required for activation determined.

An experiment was conducted to determine whether or not the catalytic phosphate could be removed from ADP-inactivated pyruvate,P_i dikinase. Experimental details and the results of this experiment are given in Table III. AMP plus PP_i treatment, but not pyruvate treatment, removed ^{32}P-label from catalytically-phosphorylated pyruvate,P_i dikinase. With this knowledge it was possible to determine whether or not the pyruvate,P_i dikinase had to be catalytically dephosphorylated for it to act as a substrate for activation. The results (Table IV) show that AMP plus PP_i-treated ADP-inactivated pyruvate,P_i dikinase activated at a significantly greater rate than enzyme treated with either AMP or PP_i alone or pyruvate. However, the final level of enzyme activation was not affected by pretreatment with AMP, PP_i or pyruvate. Taken together the results in Table III and IV indicate that the preferred substrate for P_i-dependent activation is pyruvate,P_i dikinase which

TABLE III

REMOVAL OF THE CATALYTIC PHOSPHATE FROM ADP-INACTIVATED PYRUVATE,P_i DIKINASE

TREATMENT	RADIOACTIVITY OF THE ADP-INACTIVATED PYRUVATE,P_i-DIKINASE cpm
H_2O (Control)	8770
Pyruvate (2 mM)	7340
AMP (2 mM) plus PP_i (2 mM)	1210

Purified pyruvate,P_i dikinase was inactivated in the presence of [β-^{32}P]ATP, ADP and purified regulatory protein and the nucleotides removed by Dowex-ion exchange chromatography (see 8). The column eluate was divided into three equal aliquots, treated with either H_2O, pyruvate or AMP plus PP_i for 10 min and again run through a Dowex column to remove any unbound ^{32}P-labelled compounds. The radioactivity of the column eluates was determined by Cerenkov radiation counting in a scintillation counter.

is not catalytically phosphorylated. Although non-catalytically phosphorylated pyruvate,P_i dikinase is the preferred substrate for P_i-mediated activation, the catalytically phosphorylated form of the enzyme is activated albeit at a slower rate.

Using this information a re-investigation of the reversibility of the reactions involved in regulation of pyruvate,P_i dikinase was conducted. Both catalytically-phosphorylated and dephosphorylated pyruvate,P_i dikinase were

TABLE IV

EFFECT OF PYRUVATE,PP_i AND AMP TREATMENT UPON THE RATE OF P_i-DEPENDENT ACTIVATION OF PYRUVATE,P_i DIKINASE

TREATMENT (Addition)	INITIAL RATE OF ACTIVATION (Units min^{-1}) x 10^{-3}	FINAL PPDK ACTIVITY AFTER ACTIVATION (units)
H_2O (Control)	3.27	1.62
AMP	4.55	1.58
PP_i	3.66	1.63
AMP plus PP_i	9.1	1.62
Pyruvate	3.8	1.60

Purified pyruvate,P_i dikinase was inactivated by incubation with purified PDRP, ADP and ATP. When 95% of the enzyme had been inactivated the incubation mixture was run through a column of Dowex AG2 x 8 to remove the nucleotides (see 8). The filtrate was divided into five equal aliquots and incubated with either pyruvate, AMP and/or PP_i - each 2 mM final concentration. After incubating for 20 min the incubation mixtures were run through Dowex columns to remove the pyruvate, AMP and PP_i. Orthophosphate (2 mM) was added to the filtrates and activation of pyruvate,P_i dikinase monitored.

tested as substrates for the reverse reactions. No AMP-dependent activation was detected in the presence of high AMP concentrations (5 mM) and high concentrations of inactive pyruvate,P_i dikinase (0.6 mg) and purified PDRP (1.2 units). Similarly, no PP_i-dependent inactivation of pyruvate,P_i dikinase was detected in the presence of 0.1-5 mM PP_i and high concentrations of both pyruvate,P_i dikinase (0.72 mg) and PDRP (1.2 units). Both reactions were tested at pH 7.2, 7.8 and 8.3 in the presence or absence of Mg^{2+}. The effect of possible metabolic effectors was not studied.

DISCUSSION

As recently discussed (11) the reversal of P_i-dependent activation of pyruvate,P_i dikinase should not offer any energetic problem. However in a system free of pyrophosphatase, no PP_i-dependent inactivation was detected. The finding that the P_i-dependent activation of pyruvate,P_i dikinase was not reversible may be due to steric interference of PP_i binding to the catalytic site of pyruvate,P_i dikinase and preventing pyruvate,P_i dikinase binding to the active site of the regulatory protein. Similarly, the failure to demonstrate AMP-dependent activation may be due to AMP binding to the catalytic site of pyruvate,P_i dikinase and so inhibiting (or preventing) pyruvate,P_i dikinase binding to the active site of the regulatory protein. Steric interference could occur if the histidine residue (involved in catalytic phosphorylation) is located close to a threonine residue (involved in inactivation-dependent phosphorylation) in plant pyruvate,P_i dikinase as suggested previously (9).

The above findings are consistent with the hypothesis that the preferred substrate for P_i-dependent activation is the non-catalytically phosphorylated form of pyruvate,P_i dikinase. The finding that ADP-dependent inactivation inhibits only the second partial reaction catalysed by pyruvate,P_i dikinase provides a possible explanation of why pyruvate-treatment of ADP-inactivated pyruvate,P_i dikinase does not remove the phosphate catalytically bound to the enzyme and required for inactivation (9).

Like ATP glutamine synthetase adenyltransferase (EC 2.7.7.42) [ATase- the enzyme which adenylates and deadenylates *E. coli* glutamine synthetase (16)] PDRP catalyses both the activation and inactivation of pyruvate,P_i dikinase. However, unlike ATase, the PDRP catalyses two essentially different reactions rather than a single reversible reaction.

The results presented in this paper indicate that activation and inactivation of pyruvate,P_i dikinase are catalysed by a single enzyme involving two functionally different active sites. In this respect the PDRP resembles the isocitrate dehydrogenase kinase/phosphatase activities reported for the regulation of isocitrate dehydrogenase in *E. coli* (17).

CONCLUDING REMARKS

The results presented in this paper indicate that both the inactivation and activation of pyruvate,P_i dikinase consist of two partial reactions; inactivation requires phosphorylation of a catalytically important histidine residue followed by an ADP-dependent phosphorylation of a threonine residue, whilst activation involves dephosphorylation of the histidine residue prior to the P_i-dependent dephosphorylation of the threonine residue.

The proximity of the catalytically important histidine residue and the threonine residue important in the regulation of pyruvate,P_i dikinase is currently being determined.

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