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ESTIMATION OF PYRUVATE, PHOSPHATE DIKINASE ACTIVITY IN MAIZE LEAF TISSUE BY (PHOSPHOENOLPYRUVATE PLUS PYROPHOSPHATE)-DEPENDENT PHOSPHORYLATION OF AMP

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Key Word Index—*Zea mays*; Gramineae; maize; C_4 plants; pyruvate; phosphate dikinase; pyrophosphate metabolism; phosphorylation of AMP; phosphoenolpyruvate metabolism; pyruvate metabolism.

Abstract—Crude extracts of maize leaf tissue catalysed the phosphorylation of AMP by ^{32}PPi in the presence of phosphoenolpyruvate (PEP). The reaction was enhanced by F^- and NH_4^+. The optimum concentrations of AMP, PEP and PPi were 0.3, 10 and 1 mM, respectively. Under these conditions, *ca* 75% of the AMP phosphorylated by ^{32}PPi was present as ATP and *ca* 25% as ADP. The activity was reversibly cold labile. The specific activity of crude extracts in the presence of F^- was proportional to enzyme concentration only at protein concentrations < 25 μg/ml. Partially purified pyruvate, phosphate dikinase (PPD) from maize leaf quantitatively phosphorylated AMP to ATP in a (PEP plus PPi)-dependent reaction with the concomitant production of 0.9 mol of pyruvate per mol of AMP phosphorylated. It was concluded that (PEP plus PPi)-dependent phosphorylation of AMP provides a reliable method for estimating PPD activity in crude extracts of maize. Crude maize extracts also catalysed ^{32}Pi–ATP and ^{32}PPi–ATP exchange but these activities were not specific for PPD.

INTRODUCTION

Pyruvate, phosphate dikinase (PPD, EC 2.7.9.1) catalyses the synthesis of phosphoenolpyruvate (PEP) according to the reaction:

$$\text{Pyruvate} + \text{ATP} + \text{Pi} \rightleftharpoons \text{PEP} + \text{AMP} + \text{PPi} \quad (1)$$

The enzyme has been described in C_4 plants and micro-organisms [1, 2] and is present at low levels in CAM plants [3, 4]. PPD is reportedly absent in C_3 plants [2, 5, 6] although, more recently, it has been described in the immature grains and leaves of several C_3 plants [7–9]. The very low activity in the leaf tissue of C_3 relative to C_4 plants suggests that this property could be used to determine the mechanism of carbon dioxide assimilation in species in which this is unknown. Moreover, since PPD is the principal mechanism for the synthesis of PEP which is used in the primary carbon dioxide assimilation reaction in C_4 plants, this raises the possibility of a causal relationship between PPD activity in leaf tissue and the mechanism of carbon dioxide assimilation. Most criteria used commonly to ascertain the mechanism of carbon dioxide assimilation (e.g. leaf anatomy, structural dimorphism of chloroplasts, δ-^{13}C discrimination, carbon dioxide compensation point) are not causally related and, in some species, the mechanism of carbon dioxide assimilation is ambiguous or inconsistent with these criteria [10–13].

The methods available for measuring PPD activity in plant extracts are tedious, complex and subject to non-specific activity [1, 2], especially in crude extracts. These methods also require the prior purification of one or more other enzymes. Andrews and Hatch [14] postulated that the reaction catalysed by maize leaf PPD involves the following partial reactions:

$$\text{En} + \text{Pi}^0 + \text{ATP}^\Delta \rightleftharpoons \text{En–P} + \text{P}^0\text{P}^\Delta + \text{AMP} \quad (2)$$

$$\text{En–P} + \text{Pyruvate} \rightleftharpoons \text{En} + \text{PEP} \quad (3)$$

where En denotes the enzyme PPD and the superscripts ^{0} and $^\Delta$ denote phosphorus atoms derived from Pi and the γ-phosphorus of ATP, respectively. This paper reports a study of PEP-dependent phosphorylation of AMP by ^{32}PPi (equation 1, back reaction) catalysed by crude and partially purified extracts of maize. The properties of the reaction are consistent with those reported previously for PPD and we conclude that (PEP plus PPi)-dependent phosphorylation of AMP affords a simple, specific and reliable method for estimating PPD activity in crude leaf extracts. On the other hand, the ^{32}Pi–ATP and ^{32}PPi–ATP exchange reactions catalysed by crude extracts as predicted by equation 2 could not be specifically assigned to PPD activity.

RESULTS AND DISCUSSION

(PEP plus PPi)-dependent phosphorylation of AMP catalysed by crude extracts

Crude extracts, prepared from preilluminated maize leaf tissue by method 1, supported the synthesis of a ^{32}P-labelled product when incubated with ^{32}PPi, PEP, AMP and F^-. The product, which was adsorbed to charcoal and tentatively assumed to be ATP and/or ADP, was not formed in significant amounts in the absence of AMP or

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Table 1. Effect of preillumination and incubation conditions on (PEP plus PPi)-dependent phosphorylation of AMP* by crude extracts† of maize leaf tissue

Pretreatment of leaf tissue	AMP phosphorylated (nmol/ml · min)			
	Complete	Without AMP	Without PEP	Complete with pyruvate‡
Light (2 hr)	3.28	0.15	0.06	2.49
Dark (2 hr)	0.64	0.12	0.05	0.58

*Assayed by method A.
†Prepared by method 1.
‡Pyruvate supplied at 5 mM.

PEP (Table 1). The reaction was inhibited by Pi (27% and 43% inhibition at 1 and 5 mM, respectively) and pyruvate (Table 1). These characteristics, particularly the requirement for PEP and AMP, are consistent with the back reaction of equation 1. Extracts prepared from tissue placed in the dark for 2 hr exhibited *ca* 20% of the activity of extracts from preilluminated tissue. The latter result is consistent with the reported light activation of PPD [15]. Extracts prepared by method 1 lost 40% of their activity in 30 min when stored in a stoppered flask at room temperature. Crude extracts prepared by method 2 exhibited similar activity to fresh extracts made by method 1 but were relatively more stable. Accordingly, the source material was routinely illuminated for 2 hr before extracting the enzyme by method 2.

(PEP plus PPi)-dependent phosphorylation of AMP by partially purified PPD

Further evidence for assigning the PPi incorporation activity of crude extracts to PPD was sought. Crude extracts prepared by method 2 were treated according to a modified procedure of Sugiyama [16] for the partial purification of PPD. This treatment enhanced the specific activity of (PEP plus PPi)-dependent phosphorylation *ca* 17-fold (Table 2). The PPi incorporation activity of purified enzyme was completely dependent on PEP and AMP. The apparent K_m values of the enzyme for PPi and PEP were 40 and 140 μM, respectively; concentrations > 0.4 mM PPi and > 10 mM PEP were slightly inhibitory (results not shown). The enzyme exhibited very high affinity for AMP. The K_m value for this substrate could not be ascertained but 5 μM AMP supported rates in excess of 0.5 V_{max} in the presence of 1.12 mM ^{32}PPi and 10 mM PEP. The data in Fig. 1 are in good agreement

Table 2. Purification of the activity catalysing (PEP plus AMP)-dependent phosphorylation of AMP* from maize-leaf tissue

Treatment	Protein (mg)	Specific activity (μmol/mg protein · min)
Crude extract†	492	0.068
$(NH_4)_2SO_4$	125	0.221
DEAE-cellulose	19.3	1.15

*Assayed by method B.
†Prepared by method 2.

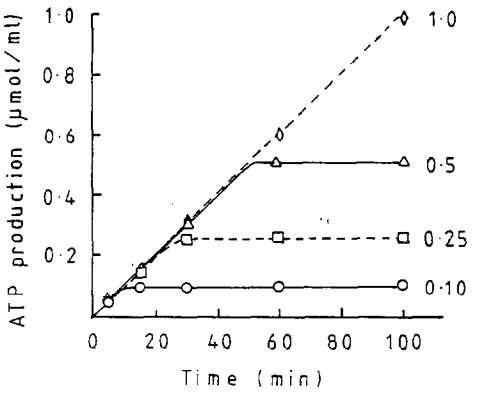

Fig. 1. Time course of the effect of AMP concentration on (PEP plus PPi)-dependent phosphorylation of AMP catalysed by partially purified PPD from maize leaf tissue. PPD activity was determined by method B except that AMP was adjusted to 0.1 (○), 0.25 (□), 0.5 (△) and 1 mM (◇). All incubations contained 20 μg partially purified protein/ml.

with the incorporation of 1 mol PPi/mol AMP consumed as predicted by equation 2. The abrupt cessation of incorporation at the time AMP became rate limiting provides further evidence for the very high affinity of the enzyme for AMP. Ammonium chloride (50 mM) enhanced the rate of PPi incorporation *ca* 3–4-fold; the apparent activation constant (K_a) was 5.4 mM. Pretreatment of the enzyme at 0° prior to determining activity at 30° inactivated the enzyme; the half-time of inactivation was 12 min. The half-time for reactivation of cold-inactivated enzyme at 30° was 5 min. Collectively, these results are in close agreement with those reported previously for the back reaction catalysed by PPD [2, 14, 16]. Accordingly, (PEP plus PPi)-dependent phosphorylation of AMP was assigned to PPD activity.

Equations 1–3 predict the synthesis of 1 mol pyruvate/mol AMP phosphorylated. The synthesis of pyruvate under conditions which support PPi incorporation was monitored by spectrophotometric oxidation of NADH at 340 nm in the presence of lactate dehydrogenase (LDH). Oxidation of NADH was completely dependent on PPD and PEP but proceeded at slow rates (22–30% of the complete system) in the absence of PPi, AMP and LDH implying that the purified PPD preparation contained an additional activity which oxidized NADH in the presence of PEP. Since PEP was free from

pyruvate as determined by assay with LDH and purified PPD was free from LDH and malate dehydrogenase, this rules out the possibility that pyruvate kinase, PEP carboxykinase and PEP carboxylase were involved. Nevertheless, the rate of production of pyruvate by the partially purified enzyme, as determined by LDH-dependent oxidation of NADH, was invariably 86–93% of the rate of (PEP plus PPi)-dependent phosphorylation of AMP over a range of concentrations of protein (10–40 μg/ml) and ammonium chloride (5–75 mM).

The nature of the ^{32}P-labelled product(s) which was adsorbed onto charcoal after terminating PPD assays was examined for incubations containing partially purified enzyme and 0.1–10 mM AMP. Most of the ^{32}P-label (83%) was eluted from the charcoal with 0.1 M ammonia in 50% ethanol. This material was subject to PC in solvents I–III. In assays containing < 0.5 mM AMP more than 90% of the ^{32}P-label was associated with ATP; the amount of ^{32}P-label associated with ADP was negligible. However, at higher AMP concentrations the proportion of ^{32}P-label associated with ATP decreased and the proportion associated with ADP increased (Table 3). This suggests that the partially purified PPD contains adenylate kinase activity. However, the latter enzyme does not interfere significantly with the accumulation of [^{32}P]ATP at the AMP concentration (0.5 mM) used in standard assays for determining PPD activity by PPi incorporation. Even at high AMP concentrations, any [^{32}P]ADP formed from [^{32}P]ATP by adenylate kinase activity would not result in underestimation of PPD activity since both compounds are adsorbed to charcoal.

Table 3. Effect of concentration of AMP on the distribution of ^{32}P-label between ATP and ADP under standard conditions* for the determination of (PEP plus PPi)-dependent phosphorylation of AMP by partially purified PPD and crude extract†

Concn of AMP (mM)	Purified enzyme		Crude extract	
	ATP (%)‡	ADP(%)‡	ATP(%)‡	ADP(%)‡
0.1	95	0	89	0
0.25	93	0	81	5
0.5	97	0	70	24
1	84	6	49	37
5	68	23	23	62
10	58	35	25	58

*Assayed by method B at the concentrations of AMP shown.
†Prepared by method 2.
‡The ^{32}P-labelled products were adsorbed onto charcoal as described for method 2 assays, eluted with 0.1 M ammonia in 50% ethanol and subjected to PC in solvent I. Results are expressed as a percentage of the ^{32}P-label eluted from the charcoal. The effect of AMP on the absolute level of ^{32}P-label incorporated into (ATP plus ADP) by crude enzyme is shown in Fig. 4.

The results with purified enzyme show that PEP-dependent synthesis of [^{32}P]ATP from AMP and ^{32}PPi affords a simple method for estimating PPD activity. For partially purified preparations, the method is not subject to error from the activity which supports the PEP-dependent oxidation of NADH in the absence of AMP and PPi. It was, therefore, concluded that the PPi incorporation method is more specific for estimating PPD activity in crude and partially purified extracts than methods based on the determination of pyruvate.

Conditions for determining PPD activity in crude extracts by (PEP plus PPi)-dependent phosphorylation of AMP

Extraction and stability. Crude extracts were routinely prepared by method 2 involving blending tissue under nitrogen in medium 2 containing 5 mM dithiothreitol (DTT) (see Experimental). The specific activity of extracts prepared in this way was *ca* three-fold greater than extracts prepared with a pestle and mortar in air (Table 4) implying that PPD activity was not associated with the additional protein extracted by the latter procedure. Extracts prepared by blending in air were relatively inactive (Table 4). Both the absolute and specific activities of extracts prepared by blending under nitrogen were highly reproducible.

The PPD activity of extracts prepared in medium 2 (containing 5 mM DTT) was unstable. The loss of activity was consistent with first-order kinetics and was more rapid at low temperatures; the half-times for loss of activity at 22° and 4° were *ca* 18 and 12 hr, respectively. In addition, crude extracts also exhibited short-term cold

Table 4. Effect of various procedures on the extraction of PPD activity from maize leaf tissue

Experiment	Procedure*			Protein extracted† (mg/ml)	PPD activity†‡ (nmol/mg protein · min)
	Equipment	Gas phase	Time (min)		
I	Mortar and pestle	Air	4	1.51	13.1
	Blender	N_2	0.5	0.54	38.5
	Blender	N_2	1	0.60	41.5
	Blender	N_2	2	0.65	40.4
II	Blender	N_2	1	0.44	42.3
	Blender	Air	1	0.40	5.2

*Medium 2 (preflushed with nitrogen) was used for all extractions.
†Means of duplicate extractions.
‡PPD activity was determined by method B in the presence of 50 mM ammonium chloride. For all treatments, the activity in the absence of either one of AMP or PEP was < 2% of the activity shown.

lability and reactivation at higher temperatures. The half-time for inactivation when an extract was transferred from 30° to 0° was 12 min and the half-time for reactivation of cold-inactivated enzyme (i.e. extracts transferred from 0° to 30°) was *ca* 5 min (Fig. 2). These characteristics of crude extracts are consistent with the properties of purified PPD reported previously [2, 14, 16].

Fluoride ion and protein concentration. PPD activity of crude extracts as determined by (PEP plus PPi)-dependent phosphorylation of AMP was highly dependent on the concentration of the extract, the duration of the incubation and the presence of F^- (Fig. 3), an inhibitor of Mg^{2+}-dependent pyrophosphatase [17]. At 125 mg protein/ml, ^{32}PPi incorporation in the presence of 10–20 mM F^- ceased after 10–20 min. Thereafter, the amount of ^{32}P-label associated with the nucleotide fraction decreased. Relatively little ^{32}PPi was incorporated in the absence of F^-. Under the latter conditions, PPi was quantitatively hydrolysed to Pi (4.5 μmol/ml) after 10 min but production of Pi in the presence of 10–20 mM F^- was relatively insignificant even after 60 min (< 0.14 μmol/ml). At a lower protein concentration (25 μg/ml) in the absence of F^-, PPi incorporation ceased at 10 min, after which the amount of ^{32}P-label associated with the nucleotide fraction decreased. These conditions were again associated with the production of large amounts of Pi (4.5 μmol/ml after 20 min). The results demonstrate that, in the absence of F^-, PPi is subjected to extensive hydrolysis by an endogenous pyrophosphatase which interferes with the determination of PPD activity. The loss of ^{32}P-label from ATP in prolonged incubations at high concentrations of protein in the presence of F^- implies that ATP is also subject to metabolism, presumably by ATPase activity. However, at low protein concentrations (< 25 μg/ml, equivalent to the protein from *ca* 8 mg fr. wt tissue/ml of incubation mixture) in the presence of 10–20 mM F^-, the rate of PPi incorporation was constant for *ca* 15–20 min and the highest specific activity was observed under these conditions. Accordingly, routine determinations of PPD activity in crude extracts were always performed within these limits.

Substrates, products and activators. The rate of the reaction catalysed by crude extracts increased with AMP concentrations up to 0.3 mM; higher concentrations were inhibitory (Fig. 4). However, 0.5 mM AMP was used in standard 15 min incubations since the reaction frequently proceeded at rates up to 20 nmol/ml·min. The time courses of PPi incorporation at various concentrations of AMP were similar to those shown for purified enzyme (see Fig. 1); for incubations containing crude extract and 0.1, 0.2 and 0.3 mM AMP, PPi incorporation ceased abruptly after *ca* 13, 25 and 37 min, respectively. At these times the ratios of Pi incorporated–AMP supplied for 0.1, 0.2 and 0.3 mM AMP were 0.89, 0.89 and 0.87, respectively. These values are slightly less than those for partially purified PPD (Fig. 1). Crude extracts also show a lower apparent affinity for AMP (Fig. 4) suggesting that AMP is subject to alternative forms of metabolism in crude extracts.

The nature of the ^{32}P-labelled product(s) synthesized by crude extracts in standard incubations was examined by the procedures used to investigate the purified enzyme. At 0.1 mM AMP almost all of the label was associated with ATP but, as the concentration of AMP increased, the proportion of label in ATP decreased and labelling of ADP increased (Table 3). For equimolar concentrations

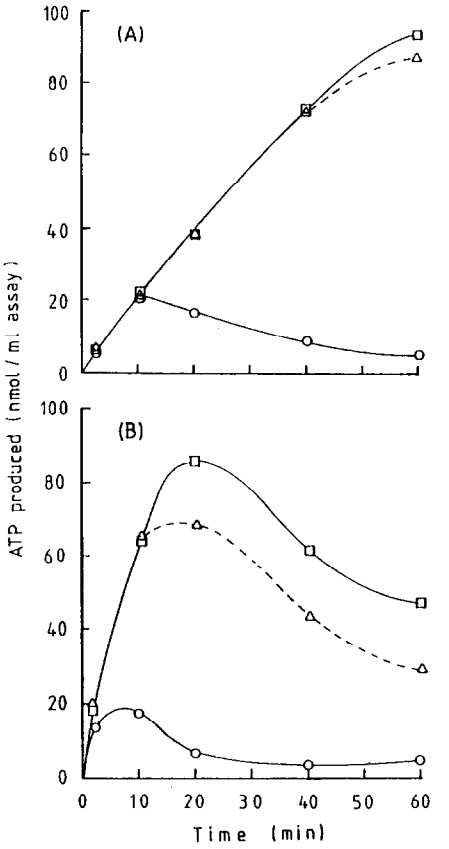

Fig. 3. Time course of (PEP plus PPi)-dependent phosphorylation of AMP by crude maize extracts at final protein concentrations of 25 μg/ml (A) and 125 μg/ml (B) in the presence of 20 (□), 10 (△) and 0 mM potassium fluoride (○). Incubations were as described for method B assays except that AMP and ^{32}PPi were supplied at 0.1 and 2.25 mM, respectively. Crude extracts were prepared by method 2. The amount of protein used in (A) and (B) represents the protein equivalent of 7.75 and 38.7 mg fr. wt of tissue/ml of incubation mixture, respectively.

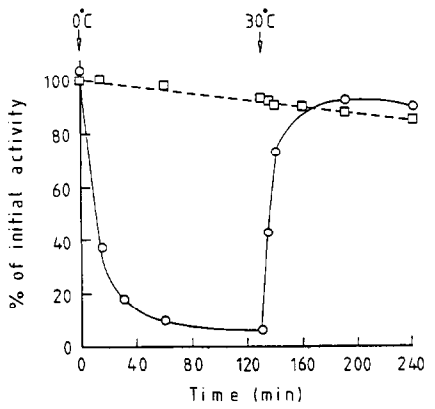

Fig. 2. Effect of preincubating crude maize extract at 30° and 0° on the activity catalysing (PEP plus PPi)-dependent phosphorylation of AMP. Crude extract, prepared by method 2, was preincubated at 30° (□) or initially at 0° and subsequently at 30° (○), prior to determining enzyme activity at 30° by method B.

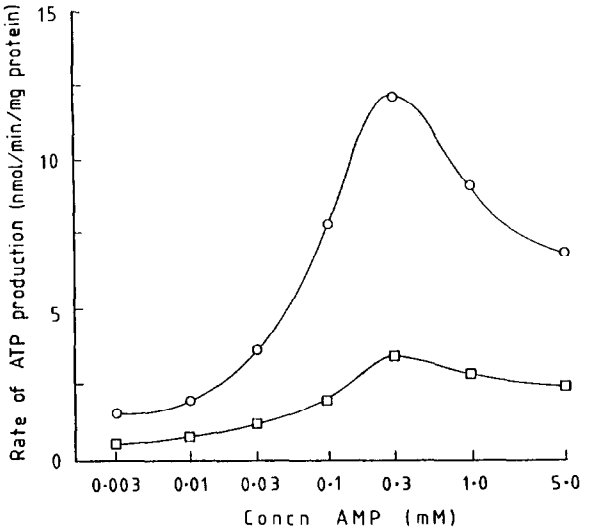

Fig. 4. Effect of concentration of AMP on (PEP plus PPi)-dependent phosphorylation of AMP in crude extracts prepared from maize plants pretreated in the light (○) for 2 hr at 300 $\mu E/m^2 \cdot sec$ and from plants pretreated in the dark (□) for 2 hr prior to extraction. Extracts were prepared by method 2 and activity determined by method B. The rates of ATP production in the absence of AMP by extracts from plants pretreated in the light and dark were 1.3 and 0.3 nmol/mg protein · min, respectively. The rate of ATP production was less than 1 % of maximum activity if PEP was omitted from the assay mixture.

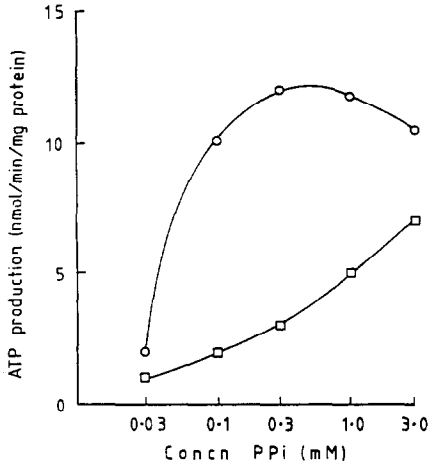

Fig. 5. Effect of concentration of PPi on (PEP plus PPi)-dependent phosphorylation of AMP by crude maize extracts in the presence (○) and absence (□) of 20 mM potassium fluoride. Incubation conditions were as described for method B assays except that AMP was supplied at 0.1 mM. The rate of ATP production was less than 1 % of maximum activity if either AMP or PEP was omitted from the incubation mixture.

of AMP, a greater proportion of label was associated with ADP in crude extracts relative to partially purified enzyme, presumably due to greater activity of adenylate kinase relative to PPD in crude extracts. However, as noted previously, adenylate kinase activity does not affect the quantitative estimation of PPD activity by (PEP plus PPi)-dependent phosphorylation of AMP.

Phosphorylation of AMP by ^{32}PPi in the absence of PEP was negligible. The rate of phosphorylation increased with the concentration of PEP up to 10 mM but higher concentrations were inhibitory (results not shown). In the presence of 20 mM F^- the optimum concentration of PPi was 0.3–1 mM; higher concentrations were slightly inhibitory (Fig. 5). In the absence of F^- the activity of crude extracts was much reduced, especially at low concentrations of PPi, and the rate of PPi incorporation increased with the concentration of PPi up to 3 mM again suggesting that under these conditions endogenous pyrophosphatase interferes with the estimation of PPD activity.

The PPD activity of crude extracts prepared from dark pretreated plants was *ca* 20% of extracts from pre-illuminated plants. Similar results were found over a wide range of concentrations of AMP (Fig. 4), PEP, ammonium chloride, PPi and crude protein implying that the (PEP plus PPi)-dependent activity from dark-treated tissue exhibited the same qualitative characteristics as the activity from illuminated tissue.

Ammonium chloride (50 mM) enhanced the PPi incorporation activity of crude extracts *ca* three-fold; the enhanced activity was dependent on both PEP and AMP. The activation constant (K_a) for ammonium chloride as determined from double-reciprocal plots was 6.2 mM. This is in general agreement with the value obtained for the purified enzyme by the same assay procedure and for independent determinations using different procedures [16].

^{32}Pi–ATP exchange activity of crude extracts

At protein concentrations < 50 μg/ml, crude extracts, prepared by method 1, catalysed ^{32}Pi–ATP exchange in the presence of 0.05 mM AMP, 0.5 mM PPi and 10 mM F^- at rates of *ca* 40 nmol/mg protein · min for up to 30 min. At higher protein concentrations, equivalent to *ca* 40 mg fr. wt/ml, the exchange rate decreased after 5 min. Under these conditions, net accumulation of ^{32}P-label in ATP ceased after 15 min and, thereafter, the ^{32}P-label associated with ATP gradually decreased (83% loss after 60 min) suggesting that ATP was subject to extensive metabolism. Omission of F^- decreased activity by *ca* 80%.

AMP was without significant effect on ^{32}Pi–ATP exchange at low concentrations (< 0.5 mM) but higher concentrations were inhibitory (*ca* 80% inhibition at 5 mM). This is consistent with a previous report [14] which attributed the lack of AMP stimulation to the very high affinity of PPD for AMP together with the presence of AMP as a contaminant in the ATP and/or endogenous ATPase activity. Low concentrations of PPi (< 0.5 mM) did not increase ^{32}Pi–ATP exchange significantly as predicted by equation 2 and high concentrations were inhibitory (*ca* 75% inhibition at 5 mM). This result is at variance with that reported for the purified maize enzyme [14], presumably due to the presence of low endogenous concentrations of PPi and/or very active pyrophosphatase activity in crude extracts. ^{32}Pi–ATP exchange was inhibited by PEP (50% inhibition at 2.5 mM) and pyruvate (20% inhibition at 15 mM) as predicted from equations 2 and 3. EDTA (13 mM) completely abolished activity. The ^{32}Pi–ATP exchange activity was unstable; at

room temperature activity decreased by 75% in 3 hr and extracts prepared with a blender instead of a pestle and mortar were completely inactive. Although the features of ^{32}Pi–ATP exchange are consistent with the reported properties of PPD, no single property of the exchange reaction is sufficiently specific to assign the activity to PPD.

^{32}PPi–ATP exchange activity of crude extracts

Crude extracts, prepared by method 1, catalysed ^{32}PPi–ATP exchange in the presence of 0.05 mM AMP, 0.5 mM Pi and 10 mM F^-. At a protein concentration of 25 μg/ml the rate was constant for up to 15 min. The properties of the reaction were similar to those for ^{32}Pi–ATP exchange with respect to the effect of F^- and protein concentration. ^{32}PPi–ATP exchange was enhanced slightly by Pi (10% stimulation at 0.5 mM) though this effect did not occur in the absence of F^-. Pyruvate (0.5 mM) inhibited exchange by 19% but PEP was without effect. Collectively, these results are consistent with the properties of the ^{32}PPi–ATP exchange reaction catalysed by purified PPD [14] but no single property of the exchange reaction was sufficiently specific to assign the whole or any part of the activity in crude extracts to PPD.

EXPERIMENTAL

Preparation of extracts. Field grown maize (*Zea mays* L. cv Flat Red) was the best source of extractable PPD activity. Optimum rates were obtained from fully expanded leaves at the onset of flowering. During winter, when field grown plants were not available, plants were raised in a growth cabinet on a 14 hr, 30°/10 hr, 20° day/night regime. The plants were grown under fluorescent tubes supplying 300 $\mu E/m^2 \cdot sec$ (at 400–700 nm) and harvested *ca* 8–9 weeks after sowing. The PPD activity of plants raised in cabinets was considerably less than field grown plants. Whole plants or detached leaves were illuminated (either in sunlight or under fluorescent tubes) for at least 2 hr immediately prior to preparing the extracts. The midribs were removed and the lamina tissue sliced finely with a razor blade. Extraction method 1 was used for studies of ^{32}Pi–ATP and ^{32}PPi–ATP exchange. It was also used for some preliminary investigations of (PEP plus PPi)-dependent phosphorylation of AMP in crude extracts. Sliced leaf material was extracted in a chilled pestle and mortar using 20 ml medium 1 (100 mM Tris–HCl buffer, pH 8.3, containing 5 mM mercaptoethanol and 6.5 mM $MgCl_2$) per g fr. wt. The extract was squeezed through cheese-cloth and the supernatant soln recovered by centrifuging at 15 000 *g* for 10 min. The extract was diluted five-fold in medium 1 prior to use (i.e. equivalent to 10 mg fr. wt/ml). Extraction method 2 (standard method) was used for most studies of (PEP plus PPi)-dependent phosphorylation of AMP. Sliced leaf tissue was extracted in medium 2 (20 ml/g fr. wt) under N_2 using a top drive blender for four periods of 15 sec; medium 2 (100 mM Tris–HCl buffer, pH 7, containing 6.5 mM $MgCl_2$ and 5 mM DTT) was flushed with N_2 for 30 min immediately prior to adding DTT. After centrifugation (as for method 1) the extract was diluted five-fold in medium 2. All operations were performed at room temp. (21–24°).

Purification of PPD. A modification of the method of ref. [16] was employed. Undiluted crude extract, prepared by method 2, was treated with $(NH_4)_2SO_4$ (243 g/l.) and the supernatant soln treated with additional $(NH_4)_2SO_4$ (63 g/l.). The ppt (containing the PPD activity) was suspended in 80% satd $(NH_4)_2SO_4$ and stored under N_2 at −10°; no loss of activity occurred under these conditions during 6 months. When required, the protein was recovered by centrifugation, dissolved in medium 2 and residual $(NH_4)_2SO_4$ removed by gel filtration through Sephadex G-25 equilibrated with medium 2. The extract was applied to a DEAE–cellulose column (bed vol. 54 ml) equilibrated with medium 2. The column was successively washed with medium 2 (150 ml) and 0.09 M KCl in medium 2 (150 ml) before eluting PPD with a linear gradient of KCl (from 0.09 to 0.3 M) in medium 2 (500 ml). Active fractions were stored in 80% $(NH_4)_2SO_4$ under N_2 at −10°. When required, PPD was dissolved in medium 2 and residual $(NH_4)_2SO_4$ removed as described above.

Enzyme assays. (PEP plus PPi)-dependent phosphorylation of AMP (PPD activity) was determined by two methods. For method A, activity was determined at 30° in incubation mixtures containing 15 mM PEP, 5 mM AMP, 2.55 mM ^{32}PPi (*ca* 9 GBq/mol), 6.5 mM $MgCl_2$, 10 mM KF, 5 mM mercaptoethanol, 100 mM Tris–HCl (pH 8.3) and 1.1 ml diluted crude extract (method 1) in a vol. of 1.42 ml. Reactions were terminated after 15 min by the addition of 2 ml TCA (7.5% w/v). [^{32}P]ATP was adsorbed onto charcoal and radioactivity determined with a gas-flow planchet counter [18]. ^{32}PPi incorporation was adjusted for controls lacking extract and corrected by a factor of 2 for the probability of ^{32}P-labelling of ATP implicit in the back reactions for equations 1 and 3. Activity is expressed as nmol ATP synthesized/mg protein · min. For method B, incubation mixtures contained 10 mM PEP, 0.5 mM AMP, 1.12 mM ^{32}PPi (*ca* 9 GBq/mol), 5 mM $MgCl_2$, 20 mM KF, 3 mM DTT, 100 mM Tris–HCl (pH 7) and 1.1 ml diluted crude extract (method 2) or purified PPD; all other details were as described for method A. The synthesis of pyruvate in method B assays was determined by replacing ^{32}PPi with 1.12 mM PPi and monitoring the oxidation of 0.14 mM NADH at 340 nm at 30° in the presence of LDH (1 unit/ml).

^{32}Pi–ATP exchange assays were conducted at 30° for 30 min in reaction mixtures containing 2.5 mM ATP, 1 mM ^{32}Pi (*ca* 9 GBq/mol), 0.05 mM AMP, 0.5 mM PPi, 5 mM mercaptoethanol, 6.5 mM $MgCl_2$, 10 mM KF, 100 mM Tris–HCl (pH 8.3) and 1.1 ml diluted crude extract (method 1) in a vol. of 1.42 ml. ^{32}P–ATP was adsorbed onto charcoal and radioactivity determined as described above. ^{32}Pi–ATP exchange, corrected for controls lacking extract, is expressed in nmol/mg protein · min.

^{32}PPi–ATP exchange was determined as described for ^{32}Pi–ATP exchange except that ^{32}Pi was replaced with 0.5 mM Pi and PPi replaced with 0.56 mM ^{32}PPi.

Elution of ^{32}P-labelled nucleotides from charcoal and PC. The ^{32}P-labelled products from method B assays which were adsorbed to charcoal were eluted with 0.1 M NH_3 in 50% EtOH. The extract was dried by rotary evaporation, dissolved in H_2O and subjected to PC in the following solvents: I, *n*-PrOAc–HCO_2H–H_2O (11:5:3); II, MeOH–HCO_2H–H_2O (80:15:5); III, *iso*-BuOH–NH_3–H_2O (66:1:3). ^{32}P-label was detected with a gas flow radiochromatogram scanner.

Other methods. Protein in crude extracts and $(NH_4)_2SO_4$ fractions was determined as in ref. [19]. Protein eluted from the DEAE–cellulose column during purification of PPD was determined as in ref. [20].

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