Properties and Mechanism of Action of Pyruvate, Phosphate Dikinase from Leaves

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1. Sugar-cane leaf pyruvate, P_i dikinase was prepared free of enzymes that would interfere with studies on the stoichiometry and mechanism of the reaction it catalyses. The reaction was unequivocally shown to involve the conversion of equimolar amounts of pyruvate, ATP and P_i into phosphoenolpyruvate, AMP and PP_i . 2. The purified enzyme was stable at pH 8.3 only if stored at about 20° in the presence of Mg^{2+} and a thiol-reducing reagent, care being taken to prevent the oxidation of the thiol. 3. The apparent Michaelis constants for phosphoenolpyruvate and PP_i were 0.11 mM and 0.04 mM respectively and that for AMP was less than 4 μM . 4. At pH 8.3 the initial velocity of the reaction was about 6 times as fast in the direction towards phosphoenolpyruvate synthesis as in the reverse direction. 5. With the exception of ATP, all the products of the reaction in both directions were inhibitory. 6. The phosphate groups of PP_i were derived from P_i and from the terminal phosphate of ATP. 7. Isotope-exchange studies indicated that the reaction proceeds in the following steps:

Pyruvate, P_i dikinase, previously named PEP* synthase, was described originally from leaves (Hatch & Slack, 1967, 1968). The enzyme apparently catalyses the interconversion of pyruvate, ATP and P_i with PEP, AMP and PP_i and operates specifically in the C_4 -dicarboxylic acid pathway of photosynthesis (Hatch & Slack, 1966, 1968, 1969; Slack & Hatch, 1967; Johnson & Hatch, 1968). Recent studies have revealed that it may have an important regulatory role in photosynthesis (Slack, 1968; Hatch & Slack, 1969; Hatch, Slack & Bull, 1969). The enzyme has now been reported in protozoa (Reeves, 1968) and bacteria (Evans & Wood, 1968; Reeves, Menzies & Hsu, 1968).

The present paper establishes unequivocally the stoichiometry for the reaction catalysed by pyruvate, P_i dikinase and describes further studies on the kinetic properties and stability of the enzyme. Isotope-exchange studies provide information about the mechanism of the reaction.

* Abbreviation: PEP, phosphoenolpyruvate.

MATERIALS

Leaves were obtained from field-grown sugar cane (*Saccharum* hybrid sp. var. Pindar). The following chemicals and enzymes were obtained from Sigma Chemical Co. (St Louis, Mo., U.S.A.): sodium pyruvate, PEP, ATP, ADP, AMP, NADH, $NADP^+$, aspartate aminotransferase, hexokinase, glucose 6-phosphate dehydrogenase, lactate dehydrogenase, pyruvate kinase, 5'-adenylate deaminase and inorganic pyrophosphatase. Sources of other chemicals and enzymes were: dithiothreitol (Calbiochem, Los Angeles, Calif., U.S.A.); $Na_2^{14}CO_3$, sodium $[1-^{14}C]$ pyruvate, ammonium $[U-^{14}C]$ AMP, ammonium $[U-^{14}C]$ ATP, $[^{32}P]P_i$ and tetrasodium $[^{32}P]PP_i$ (The Radiochemical Centre, Amersham, Bucks.); Hypatite C (Clarkson Chemical Co., Williamsport, Pa., U.S.A.); malate dehydrogenase (Worthington Biochemical Corp., Freehold, N.J., U.S.A.). Partially purified PEP carboxylase, free of pyruvate, P_i dikinase, was prepared as described by Hatch & Slack (1968).

METHODS

Partial purification of pyruvate, P_i dikinase

The steps used for purifying pyruvate, P_i dikinase were essentially the same as described by Hatch & Slack (1968)

except for the following modifications. 1 mM-EDTA was included in all solutions and the concentrations of 2-mercaptoethanol and MgSO_4 were increased to 50 mM and 6 mM respectively. For the treatments on Sephadex 2-mercaptoethanol was replaced by 5 mM-dithiothreitol. The $(\text{NH}_4)_2\text{SO}_4$ fraction used was that precipitated between 43% and 50% saturation. For the first Sephadex G-200 treatment and the Hypatite C treatment the columns were 380 ml. (4 cm. $\times$ 30 cm.) and 64 ml. (2 cm. $\times$ 20 cm.) respectively, twice the volumes used previously. For the second Sephadex G-200 treatment superfine grade of gel was used and the flow rate was decreased to 0.2 ml./min. Enzyme was stored at 4° as a suspension in 66%-saturated $(\text{NH}_4)_2\text{SO}_4$ solution. As required samples of this suspension were centrifuged and the protein was dissolved in 50 mM-tris-HCl buffer, pH 8.3, containing 6 mM- MgSO_4 , 5 mM-dithiothreitol and 1 mM-EDTA.

Pyruvate, P_i dikinase assays

Assay 1. For some studies pyruvate, P_i dikinase was assayed in the forward direction, the direction proceeding towards PEP synthesis, by measuring the rate of fixation of ^{14}C bicarbonate into aspartate as described by Hatch & Slack (1968) except that the pyruvate concentration was 6 mM and 2-mercaptoethanol was replaced by dithiothreitol.

Assay 2. An alternative forward-direction assay involved measuring PEP formation spectrophotometrically in the presence of NaHCO_3 , PEP carboxylase, malate dehydrogenase and NADH. Reaction mixtures contained, in addition to enzyme, 50 mM-tris-HCl buffer, pH 8.3, 10 mM- MgSO_4 , 5 mM-dithiothreitol, 2.5 mM- NaHCO_3 , 0.25 mM-NADH, 1.25 mM-pyruvate, 1.25 mM-ATP, 2.5 mM- P_i , approx. 0.8 unit of malate dehydrogenase and approx. 0.2 unit of partially purified PEP carboxylase in a total volume of 0.8 ml. Reactions were started by the addition of ATP, and the rate of decrease in E_{340} was measured at 25°.

Assay 3. Pyruvate, P_i dikinase was assayed in the reverse direction by measuring the rate of ATP production spectrophotometrically in a reaction coupled with hexokinase and glucose 6-phosphate dehydrogenase. In addition to enzyme, reaction mixtures contained 50 mM-tris-HCl buffer, pH 8.3, 5 mM- MgSO_4 , 5 mM-dithiothreitol, 0.5 mM-NADP⁺, 2.5 mM-glucose, 1.25 mM-PEP, 0.5 mM-AMP, 0.5 mM- PP_i , approx. 1 unit of hexokinase and approx. 0.5 unit of glucose 6-phosphate dehydrogenase in a total volume of 0.8 ml. Reactions were started by the addition of PP_i and the rate of increase in E_{340} was measured at 25° or 30°.

Assay 4. Pyruvate, P_i dikinase was also assayed in the reverse direction spectrophotometrically by measuring pyruvate production in the presence of lactate dehydrogenase. In addition to enzyme, reaction mixtures contained 50 mM-tris-HCl buffer, pH 8.3, 6 mM- MgSO_4 , 10 mM-dithiothreitol, 0.15 mM-NADH, 1 mM-PEP, 1 mM-AMP, 1 mM- PP_i and approx. 6 units of lactate dehydrogenase in a total volume of 0.8 ml. The rate of decrease in E_{340} dependent on the addition of PP_i was measured at 30°.

With assays 1, 2 and 3 there was a decline in the rate after an initial linear phase, possibly due to the product-inhibition effects described in the Results section. For assays 2 and 3 the initial rate was determined by monitoring the first 0.1 unit change in extinction on the expanded scale of a Cary model 14 spectrophotometer. A unit of enzyme activity is defined as the amount of enzyme catalysing the

conversion of 1 μmole of substrate in 1 min. under the conditions of temperature and pH stated for individual experiments.

Assays of other enzymes

PEP carboxylase (Slack & Hatch, 1967), adenylate kinase and pyrophosphatase (Hatch *et al.* 1969) were assayed as described previously.

Isotope-exchange studies

When referring to isotope-exchange reactions, a prefix in square brackets is employed to indicate the type of isotope used, and which of the two components of the exchange was initially labelled. Thus the ^{14}C ATP-AMP exchange is the exchange between ATP and AMP, ATP being the component initially labelled. When it is not essential to specify which of the components was initially labelled, the prefix is omitted.

Isotope-exchange reactions were carried out at either 25° or 30°. When isotope-exchange rates were compared with the rate of the overall reaction both determinations were conducted at the same temperature. Unless otherwise stated, isotope-exchange-reaction mixtures contained the following components in addition to 25 mM-tris-HCl buffer, pH 8.3, and 5 mM-dithiothreitol, in a total volume of 0.2 ml.: for the ^{14}C pyruvate-PEP exchange, 6 mM- MgSO_4 , 2 mM-PEP and 1 mM-[1- ^{14}C]pyruvate (2.6 mc/m-mole); for the ^{32}P P_i - PP_i exchange, 5 mM- MgSO_4 , 0.5 mM- ^{32}P P_i (approx. 5 mc/m-mole), 1 mM- PP_i and 0.25–0.5 mM-ATP; for the ^{14}C ATP-AMP exchange, 8 mM- MgSO_4 , 0.5 mM-[U- ^{14}C]ATP (2.5 mc/m-mole), 2 mM-AMP and, when added, 1 mM- P_i and 0.04 mM- PP_i . For the ^{14}C AMP-ATP exchange, 0.5 mM-[U- ^{14}C]AMP (2.5 mc/m-mole) was used with 2 mM-ATP.

Reactions were started by the addition of enzyme and stopped after various times by the transfer of a sample of the reaction mixture into a tube containing an EDTA solution equivalent to twice the molar quantity of Mg^{2+} in the reaction sample. Tubes were immediately placed in a boiling-water bath for 2 min., then chilled rapidly, and samples were chromatographed.

Chromatography. Pyruvate and PEP were separated on Whatman no. 1 paper by descending chromatography with butan-1-ol-propionic acid-water (10:5:7, by vol.) as solvent. A development time of 16 hr. was required. ATP, ADP and AMP were separated by ascending chromatography on Whatman DEAE-cellulose paper in 4 hr. with 0.6 M-ammonium formate solution, pH 3.1, as solvent (Morrison & Cleland, 1966). P_i and PP_i were separated by descending chromatography on Whatman 3MM paper that had been washed with EDTA and acetic acid as described by Bielecki & Young (1963). The solvent was propan-1-ol-aq. NH_3 (sp.gr. 0.9)-water (6:3:1, by vol.) and a period of approx. 40 hr. was required to achieve separation. ATP, P_i and PP_i were separated on DEAE-cellulose paper with 0.15 M-ammonium formate solution, pH 8.5, containing 5 mM-EDTA as solvent. Ascending development for 6 hr. was employed, the chromatograms being over-run into an absorbent pad of filter paper attached to the top of the DEAE-cellulose paper.

When markers were used they were located either by the spraying technique of Bandurski & Axelrod (1951) for phosphate esters or by u.v. fluorescence for nucleotides.

Glucose 6-phosphate was located by spraying with aniline hydrogen phthalate (Block, Durrum & Zweig, 1955).

Measurement of radioactivity on chromatograms. After development and drying, chromatograms were cut into strips and passed through a Nuclear-Chicago chromatogram scanner to locate areas of radioactivity. The radioactivity in these areas was measured either by planimeter measurement of the areas of the peaks or by scintillation counting. The latter method was more accurate with exchanges of less than 10%. The sections of the chromatogram containing radioactivity were rolled, inserted vertically in small counting vials and dried at 80°. The vials were filled with scintillator fluid and counted in a Nuclear-Chicago scintillation counter, the channels-ratio method being used for correction of quenching. With both ^{14}C and ^{32}P the counting efficiency varied depending on the quantity and grade of the chromatography paper present in the counting vial.

Calculation of isotope-exchange rates. Except for the $^{32}\text{P}\text{P}_1\text{-ATP}$ exchange, all exchanges followed first-order kinetics. The exchange rates were calculated by using the equations derived by Friedlander, Kennedy & Miller (1964).

Determination of AMP and ADP contamination in ATP

ADP was assayed spectrophotometrically by measuring NADH oxidation at 340 nm. in a solution containing, in addition to ATP, 50 mM-tris-HCl buffer, pH 7.5, 5 mM-MgSO₄, 1 mM-dithiothreitol, 75 mM-KCl, 0.8 mM-PEP, 0.3 mM-NADH and lactate dehydrogenase. AMP was assayed after ADP by the addition of adenylate kinase. A correction for AMP occurring in the NADH preparation was applied.

Location of ^{32}P in products from $^{32}\text{P}\text{PP}_i$

Pyruvate, P_i dikinase was incubated at 30° in a reaction mixture containing 50 mM-tris-HCl buffer, pH 8.3, 4 mM-MgSO₄, 5 mM-dithiothreitol, 1 mM-PEP, 1 mM-AMP and 1 mM- $^{32}\text{P}\text{PP}_i$ (4.03 mc/m-mole) in a total volume of 0.2 ml. After 15 min. the reaction was stopped by the addition of EDTA as described for isotope-exchange reactions. A sample of the reaction mixture was chromatographed on DEAE-cellulose paper at pH 8.5 as described above and the proportions of the radioactivity in individual compounds were determined. Radioactive ATP was isolated from the other reactants by adsorption on activated charcoal and eluted with 0.1% (w/v) NH₃ in 50% (v/v) ethanol, after the column had been washed with water to remove other radioactive compounds. Its radiochemical purity was confirmed by chromatography on DEAE-cellulose at pH 3.1 as described above. The amount of ^{32}P in the γ -position was determined by incubating a sample of this ATP with 50 mM-tris-HCl buffer, pH 8.3, 10 mM-MgSO₄, 5 mM-glucose, 1 mM-ATP and approx. 1 unit of crystalline hexokinase in a total volume of 0.2 ml. After 60 min. 40 μl . of 0.1 M-EDTA was added and a sample of the mixture was chromatographed on DEAE-cellulose paper at pH 3.1 as described above. The radioactivity was determined in the glucose 6-phosphate isolated.

Other determinations

P_i was determined by the procedure of Mozersky, Pettinati & Kolman (1966). PP_i was assayed by measuring the

increase in P_i after treatment with inorganic pyrophosphatase. Protein was determined by the method of Warburg & Christian (1941). Where necessary, samples for protein determination were freed of u.v.-absorbing thiol compounds by treatment on a small column of Sephadex G-25.

RESULTS

Purification of pyruvate, P_i dikinase

The purification procedure used by Hatch & Slack (1968) freed pyruvate, P_i dikinase from several enzymes that would interfere with studies of the stoichiometry and mechanism of pyruvate, P_i dikinase, but appreciable quantities of an alkaline pyrophosphatase remained. With the modified procedure described in the Methods section interfering enzymes including pyrophosphatase were totally removed. Modification of the early steps in the purification gave additional removal of pyrophosphatase. However, the most important modification was the use of the superfine grade of Sephadex G-200 instead of medium grade. With the improved resolution pyrophosphatase moved as a separate peak immediately after pyruvate, P_i dikinase. With the information provided by recent studies on the inactivation of pyruvate, P_i dikinase in darkened leaves and its reactivation in extracts (Hatch & Slack, 1969) it would now be possible to commence purification with an enzyme preparation of much higher specific activity than that employed either previously (Hatch & Slack, 1968) or during the present study.

Enzyme stability

Partially purified pyruvate, P_i dikinase was rapidly inactivated after the enzyme was centrifuged from a suspension in ammonium sulphate solution and redissolved in buffer (Hatch & Slack, 1968). The rate of inactivation was decreased by adding Mg^{2+} , 2-mercaptoethanol and to a smaller extent pyruvate, but was higher at 0° than at 22°. During the present studies we showed that the rate of inactivation was related to the rate at which 2-mercaptoethanol was oxidized by oxygen. The rate of inactivation was markedly decreased by excluding oxygen or by prior treatment of the enzyme on Sephadex G-25 to remove small-molecular-weight materials, presumably including heavy-metal ions originating from the ammonium sulphate. Heavy-metal ions catalyse the oxidation of thiols by oxygen. The Sephadex-treated enzyme in buffer containing 5 mM-2-mercaptoethanol and 5 mM-Mg²⁺ was stable for at least 24 hr. under nitrogen. For the present studies dithiothreitol was substituted for 2-mercaptoethanol wherever possible, to take advantage of its lower rate of oxidation by oxygen.

Table 1. *Stoichiometry of the pyruvate, P_i dikinase reaction*

In addition to enzyme and substrates, the reaction mixtures contained 55 μ moles of tris-HCl buffer, pH 8.3, 4.6 μ moles of MgSO₄ and 0.2 μ mole of dithiothreitol in a total volume of 1 ml. After incubation at 30°, the reactions were chilled and 50 μ l. of 40% (w/v) HClO₄ was added. Each solution was quickly reneutralized with 0.16 ml. of 2M-KHCO₃ and maintained at 0° until precipitation of KClO₄ was complete. Pyruvate, PEP and ATP were determined as described by Hatch & Slack (1967) and P_i and PP_i as described in the Methods section. AMP was assayed by measuring the decrease in *E*₂₆₅ accompanying the conversion of AMP into IMP in a solution containing 50 mM-citrate buffer, pH 6.5, and 5'-adenylate deaminase.

Time (min.)	Amount (μ moles/reaction mixture)					
	PEP	AMP	PP _i	Pyruvate	ATP	P _i
0	0.98	0.94	0.89	0.01	0	0.01
50	0.67	0.62	0.60	0.30	0.32	0.30
Net change	...	-0.31	-0.32	+0.29	+0.32	+0.29

As the period of storage of the enzyme as a suspension in ammonium sulphate solution increased, the initial activity of the redissolved enzyme decreased. However, complete reactivation occurred after about 3 hr. at pH 8.3 and 22° in a solution containing 5 mM-dithiothreitol and 5 mM-Mg²⁺. Under these conditions no decrease in the final activity of the enzyme stored in 66%-saturated ammonium sulphate at 4° was observed over a period of several months.

Stoichiometry

Hatch & Slack (1968) demonstrated that, after correction for P_i produced by the contaminating pyrophosphatase, equimolar amounts of pyruvate, ATP and P_i were formed when pyruvate, P_i dikinase was incubated with PEP, AMP and PP_i. With the enzyme preparation free of pyrophosphatase, prepared as described in the Methods section, the consumption of PEP, AMP and PP_i was accompanied by the production of equimolar amounts of pyruvate, ATP and P_i (Table 1).

Apparent Michaelis constants

Hatch & Slack (1968) found the apparent Michaelis constants for pyruvate, ATP and P_i to be 0.11 mM, 0.09 mM and 0.5 mM respectively. By using the reverse-direction assay procedures described in the Methods section the apparent Michaelis constants for PEP and PP_i have now been determined. A linear Lineweaver-Burk plot was obtained when PEP concentration was varied, the apparent *K_m* being 0.11 mM. A similar plot of the data for PP_i gave an upward curve, but in a simple plot of velocity against PP_i concentration the velocity was maximal and essentially constant over the range of PP_i concentrations from 0.2 mM to 2.5 mM. If this velocity was taken as the maximum velocity, the PP_i concentration giving half-maximal velocity was 0.04 mM. The apparent *K_m*

for AMP was too low to be determined. No decrease in the maximum rate was observed at an AMP concentration of 4 μ M.

Ratio of activities in the forward and the reverse directions

The reaction proceeding towards PEP synthesis has been arbitrarily defined as the forward reaction. Pyruvate, P_i dikinase was assayed in the forward direction by using assay 2 and in the reverse direction by using assay 3 (see the Methods section). At pH 8.3 and 30° the initial velocity in the forward direction was 6 times that in the reverse direction.

Product inhibition

Forward reaction. All three products of the pyruvate, P_i dikinase reaction in the forward direction had some inhibitory effect on the reaction in that direction. With assay 2 0.5 mM-PP_i and 0.5 mM-AMP caused 54% and 52% inhibition respectively. Inhibition by AMP was related to the ATP concentration in a competitive manner. To investigate the effect of PEP on the forward reaction the enzyme was assayed, in the absence of a coupling system, by observing the conversion of [¹⁴C]ATP into AMP; arsenate was substituted for P_i so that PP_i was not formed. PEP caused only 38% inhibition at a concentration of 3 mM and was less inhibitory than the other products. Comparable inhibitory effects of these reactants on the reverse reaction were not observed during the determination of their Michaelis constants.

Reverse reaction. In the reverse direction pyruvate and to a smaller extent P_i were inhibitory. With assay 3 0.5 mM-pyruvate caused 56% inhibition and 1.6 mM-P_i caused 29% inhibition. Inhibition by pyruvate and P_i was competitive with respect to PEP and PP_i respectively. With assay 4 ATP had no significant effect on the reverse reaction at a concentration of 1 mM. At comparable concentra-

tions pyruvate and P_i did not affect the velocity of the forward reaction.

Fate of $[^{32}P]PP_i$ in the reverse reaction

When $[^{32}P]PP_i$ was provided with PEP and AMP in the reaction mixture described in the Methods section P_i and ATP each contained 22% of the total radioactivity after 15 min. at 30°. Since all the radioactivity appeared in glucose 6-phosphate when ATP was treated with glucose and hexokinase (as described in the Methods section) all the ^{32}P in ATP was located in the γ -position.

Isotope-exchange studies

$[^{14}C]$ Pyruvate-PEP exchange. When the enzyme was incubated with $[^{14}C]$ pyruvate and unlabelled PEP in the presence of Mg^{2+} radioactivity rapidly appeared in PEP. The rate of this $[^{14}C]$ pyruvate-PEP exchange was 3.4 times as fast as the reverse rate for the overall reaction. The concentrations of pyruvate and PEP used were sufficient to saturate the overall reaction. No labelled PEP was produced if EDTA was added in excess of the Mg^{2+} concentration, or if non-labelled PEP was omitted. At concentrations that would be optimum for the overall reaction ATP, AMP, PP_i and P_i had no effect on this exchange, either when added singly or when either ATP + AMP or P_i + PP_i were added together.

$[^{32}P]P_i$ - PP_i exchange. When the enzyme was incubated with $[^{32}P]P_i$, unlabelled PP_i and ATP in the presence of Mg^{2+} , radioactivity appeared in PP_i and to a smaller extent in ATP. No radioactive PP_i was formed when either PP_i or ATP was omitted or when PEP was substituted for ATP. When the labelled ATP was isolated and treated with hexokinase as described in the Methods section the label was found solely in the γ -position. No exchange was observed if EDTA was added in excess of the Mg^{2+} .

With the reaction mixture described in the Methods section for the $[^{32}P]P_i$ - PP_i exchange a requirement for AMP could not be demonstrated. However, AMP added at concentrations of 20 μM or higher inhibited the exchange, whereas lower concentrations had no effect. Freshly prepared solutions of ATP used in these experiments contained concentrations of ADP and AMP equivalent to 0.44% and 0.12% respectively of the ATP. Since the concentration of ATP usually included in these exchanges was 0.25 mM, any requirement for AMP must be satisfied by very low concentrations. The K_m for AMP in the overall reaction was, however, much less than 4 μM and could not be measured. Inhibition of the P_i - PP_i exchange by higher concentrations of AMP was competitive with both ATP and P_i (Table 2).

Table 2. *Inhibition of ATP-dependent $[^{32}P]P_i$ - PP_i exchange by AMP*

Reaction mixtures contained, in addition to enzyme and the components described below, 25 mM-tris-HCl buffer, pH 8.3, 4.6 mM- $MgSO_4$, 5 mM-dithiothreitol and 1 mM- PP_i in a total volume of 0.2 ml. P_i was labelled with ^{32}P (0.34 μC /reaction mixture). Other details are described in the Methods section. The values in parentheses represent the percentage inhibition with respect to the reaction containing no added AMP.

Concn. of AMP added (mM)	$10^3 \times$ Exchange rate (μ moles/min.)			
	Concn. of P_i (mM)	0.50	0.10	0.50
	Concn. of ATP (mM)	0.25	0.25	0.05
0		9.8	4.8	7.9
0.02		7.1 (28)	1.4 (71)	1.7 (78)
0.10		1.7 (83)	0.3 (94)	0.3 (96)

Fig. 1. Requirement of ATP for the $[^{32}P]P_i$ - PP_i exchange. Reaction mixtures contained, in addition to enzyme, 25 mM-tris-HCl buffer, pH 8.3, 8 mM- $MgSO_4$, 5 mM-dithiothreitol, 0.5 mM- $[^{32}P]P_i$ (5.29 mC/m-mole), 5 μM -AMP, 1 mM- PP_i and the concentrations of ATP indicated in a total volume of 0.2 ml. Other details are provided in the Methods section.

The dependence of the $[^{32}P]P_i$ - PP_i exchange on the ATP concentration is shown in Fig. 1. In this experiment 5 μM -AMP was included in the reactions to eliminate the possible effects of AMP contamination in ATP that would occur if this exchange does require AMP.

The observed rate of the ATP-dependent $[^{32}\text{P}]\text{P}_i\text{-PP}_i$ exchange was about 0.5 times the rate for the overall reaction in the reverse direction. The maximum exchange rate would be somewhat higher since a less than saturating concentration of P_i , namely 0.5 mM, was used. Technical difficulties concerned with maintaining adequate ratios of P_i to PP_i , and with the precipitation of the $\text{Mg}^{2+}\text{-PP}_i$ complex, prohibited the use of higher P_i concentrations. Conditions under which rates for this exchange are higher than the reverse rate for the overall reaction are discussed below.

ATP-AMP exchange. Pyruvate, P_i dikinase catalyses a slow exchange reaction between ATP and AMP in the presence of Mg^{2+} . The exchange was the same when either ATP or AMP was provided as the radioactive reactant. The rate of this exchange was stimulated as much as 15-fold by the addition of P_i . 0.5 mM- P_i was required for maximal stimulation and the exchange rate remained essentially constant at concentrations of P_i between 0.5 mM and 2.5 mM. PP_i was also required for this exchange, but the effect of its addition depended on the source of the P_i used. Different samples contained different quantities of PP_i . With some samples the addition of 40 μM - PP_i increased the exchange rate fivefold. Higher concentrations of PP_i caused strong inhibition of the exchange (Fig. 2). However, with apparently optimum concentrations of all reactants the exchange rate was only about 0.15

Fig. 2. Inhibition of $[^{14}\text{C}]\text{ATP-AMP}$ exchange by PP_i . In addition to enzyme, the reaction mixtures contained 30 mM-tris-HCl buffer, pH 8.3, 7.5 mM- MgSO_4 , 5 mM-dithiothreitol, 0.5 mM- $[\text{U-}^{14}\text{C}]\text{ATP}$ (2.5 mc/m-mole), 2 mM-AMP, 0.5 mM- P_i and PP_i in the concentrations indicated. Other details are provided in the Methods section.

times the reverse rate for the overall reaction. The residual low activity observed in the absence of added P_i and PP_i was accounted for by their presence as contaminants in the nucleotides. The concentrations of P_i and PP_i were 17 μM and 4 μM

Fig. 3. Effects of PP_i and AMP on the $[^{32}\text{P}]\text{P}_i\text{-ATP}$ exchange. In addition to enzyme, the reaction mixtures contained 25 mM-tris-HCl buffer, pH 8.3, 8.5 mM- MgSO_4 , 5 mM-dithiothreitol, 1 mM- $[^{32}\text{P}]\text{P}_i$ (1.87 mc/m-mole), 2.5 mM-ATP and PP_i and AMP at the concentrations indicated in a total volume of 0.2 ml. Other details are given in the Methods section. (a) +, 0.01 mM- PP_i ; $\circ$, 0.075 mM- PP_i ; $\triangle$, 0.2 mM- PP_i ; $\square$, 0.5 mM- PP_i ; (b) $\bullet$, 0.5 mM- PP_i + 0.01 mM-AMP; $\blacktriangle$, 0.5 mM- PP_i + 0.1 mM-AMP; $\blacksquare$, 0.5 mM- PP_i + 2.5 mM-AMP. The unbroken lines refer to ATP and the broken lines to PP_i .

respectively in reaction mixtures to which only [^{14}C]AMP and ATP were added.

Additional evidence for the PP_i requirement was obtained by adding pyrophosphatase to reaction mixtures. With 1.8 units of pyrophosphatase in a 0.2 ml. reaction mixture the exchange was inhibited by about 90%. Pyrophosphatase was ineffective when it was heated at 100° for 2 hr. before use.

Although AMP inhibits the P_i - PP_i exchange no comparable effect on the ATP-AMP exchange was observed. The ATP-AMP exchange rate was constant over the range 0.1–2 mM-AMP and declined below 0.1 mM.

[^{32}P] P_i -ATP exchange. When [^{32}P] P_i and unlabelled ATP were incubated with pyruvate, P_i dikinase in the presence of Mg^{2+} no exchange occurred unless PP_i was added. The P_i -ATP exchange rate increased with increasing PP_i concentration, as also did the degree of labelling of PP_i (Fig. 3). The kinetics of the P_i -ATP exchange were not first-order and from the data of Fig. 3(a) we concluded that the radioactivity must enter ATP via PP_i . Both exchanges were stimulated by low concentrations of AMP and inhibited by higher concentrations (Fig. 3b). Since the stimulation by AMP was maximal at 10 μM , the lowest concentration used, the exchange that occurs in the absence of added AMP is probably due to traces of AMP introduced with ATP.

[^{32}P] PP_i -ATP exchange. Pyruvate, P_i dikinase catalysed a slow [^{32}P] PP_i -ATP exchange in the presence of these two compounds alone, but the rate was greatly increased by adding P_i and AMP (Table 3). The kinetics of this exchange was first-order. Again it seems reasonable to assume that the exchange occurring in the absence of P_i or AMP

was due to the presence of these compounds as contaminants in the other reactants. When P_i was added there was, as expected, an exchange from [^{32}P] PP_i to P_i , which was also first-order. It is important to note that with the high ATP concentration used the [^{32}P] PP_i - P_i exchange is stimulated by the addition of a low concentration of AMP, and that the stimulated rate is twice that reported above.

Elimination of exchanges by inactivation of pyruvate, P_i dikinase. Pyruvate, P_i dikinase loses activity irreversibly in the absence of Mg^{2+} , thus providing a relatively specific means of inactivating the enzyme. The rates of exchanges were examined with enzyme previously incubated in 50 mM-tris-hydrochloric acid buffer, pH 8.3, containing 5 mM-dithiothreitol so that about 92% of its activity was lost compared with a sample similarly treated but to which 6 mM-magnesium sulphate was also added. The [^{14}C]AMP-ATP, [^{32}P] PP_i - P_i and [^{14}C]pyruvate-PEP exchange rates with the enzyme pre-incubated without Mg^{2+} were decreased comparably with the rates for the overall reaction. This result appears to eliminate any possibility that the exchange reactions may be catalysed by enzymes other than pyruvate, P_i dikinase.

DISCUSSION

It was inferred from previous studies (Hatch & Slack, 1968) that pyruvate, P_i dikinase catalyses the following reaction:

Stoichiometry data reported in the present studies provide unequivocal evidence for this reaction. Evidence is also provided that PP_i gives rise to P_i and the terminal phosphate of ATP. It follows that the β -phosphate of ATP must provide the phosphate of PEP. Similar conclusions were reached during studies on the bacterial enzyme (Reeves *et al.* 1968).

The maximum velocity for the enzyme in the direction of PEP synthesis was about 6 times the rate for the reverse direction. In contrast the equilibrium for the reaction was reported by Hatch & Slack (1968) to favour the reverse direction strongly. However, studies with the bacterial enzyme by Reeves *et al.* (1968) indicate that the equilibrium for the above reaction does not strongly favour the reverse direction at higher pH. From the present studies it becomes apparent that, at least with the leaf enzyme, inhibition by products may interfere with the determination of the equilibrium point. Other reasons for this discrepancy are not apparent.

In leaves with the C_4 -dicarboxylic acid pathway of photosynthesis pyruvate, P_i dikinase operates to

Table 3. *Effects of AMP and P_i on the [^{32}P] PP_i -ATP and [^{32}P] PP_i - P_i exchanges*

Reaction mixtures contained, in a total volume of 0.2 ml., 25 mM-tris-HCl buffer, pH 8.3, 6.5 mM- MgSO_4 , 5 mM-dithiothreitol, 2.5 mM-ATP, 0.5 mM-[^{32}P] PP_i (3.55 mc/m-mole), 0.012 unit of pyruvate, P_i dikinase (reverse direction) and the AMP and P_i concentrations shown below. Other details are given in the Methods section. Exchanges are expressed as the ratio of the exchange rate to the rate of the overall reaction in the reverse direction.

Addition(s)	Exchange rate ($\mu\text{moles/min.}$ /reverse-direction unit)	
	[^{32}P] PP_i -ATP exchange	[^{32}P] PP_i - P_i exchange
Nil	0.06	—
0.5 mM- P_i	0.29	0.41
0.05 mM-AMP	0.06	—
0.5 mM- P_i + 0.05 mM-AMP	0.81	1.06

regenerate PEP, the acceptor for photosynthetic carbon dioxide fixation (Hatch & Slack, 1968). It seems reasonable to assume that the product-inhibition effects described in the present studies may perform a regulatory function *in vivo*. In leaves pyruvate, P_1 dikinase activity is regulated by light-intensity (Slack, 1968; Hatch & Slack, 1969). However, this effect is not immediate and product inhibition may provide a control that is more rapid and more sensitive. Inhibition of the reaction proceeding towards PEP synthesis by AMP, PP_1 and PEP could contribute to maintaining a balance between PEP production and its utilization by carboxylation. This may prevent the conversion of PEP into 3-phosphoglycerate, which would result in imbalance of the distribution of carbon between the two cycles operating in the C_4 -dicarboxylic acid pathway (Hatch & Slack, 1966). Inhibition of the reverse reaction by pyruvate may contribute towards preventing reversal of the reaction, with accompanying depletion of photosynthetic intermediates by conversion into pyruvate, during periods of decreased light-intensity or in the dark.

Evans & Wood (1968) have briefly reported the results of isotope-exchange studies catalysed by pyruvate, P_1 dikinase from propionibacteria. These authors concluded that the reaction proceeds in the following manner:

The following evidence would appear to exclude this mechanism for the leaf enzyme: (a) the enzyme did not catalyse a PEP-dependent P_1 - PP_1 exchange; (b) the ATP-AMP exchange required both P_1 and PP_1 ; (c) in addition to ATP, the P_1 - PP_1 exchange apparently required AMP. The latter conclusion is supported by the observation that this exchange was stimulated by low concentrations of AMP when a high concentration of ATP was provided. The K_m for AMP in this exchange, as for the overall reaction, was extraordinarily low. Since the inhibitory effect of higher concentrations of AMP was competitive with respect to ATP, it is possible that increasing the concentration of ATP would not only alleviate inhibition but also increase the concentration of AMP required for maximum exchange activity. This would facilitate the observation of an AMP requirement.

Present evidence is consistent with the following reaction sequence:

Points (b) and (c) listed above are consistent with a 'sequential' mechanism for the addition of ATP and P_1 and for the release of AMP and PP_1 . On the other hand the rapid rate of the pyruvate-PEP exchange, and the fact that it was unaffected by any of the other components of the overall reaction, indicate that the addition of pyruvate and release of PEP must follow a 'ping-pong' sequence. The terms 'sequential' and 'ping-pong' are used in the sense ascribed to them by Cleland (1967).

Even at high concentrations there was little or no inhibition of the P_1 - PP_1 exchange by ATP or of the ATP-AMP exchange by P_1 , consistent with ATP and P_1 reacting with the enzyme in a random manner. No definite conclusions can be drawn from present results about the mode of release of AMP and PP_1 . However, the facts that the PP_1 -ATP and P_1 - PP_1 exchanges were considerably faster than the ATP-AMP exchange and that PP_1 strongly inhibited the ATP-AMP exchange favour an ordered mechanism with PP_1 leaving before AMP. Either the binding or the release of AMP would then be the rate-limiting step for the ATP-AMP exchange. However, this mechanism does not explain the inhibition of the P_1 - PP_1 exchange by AMP. The formation of a dead-end Enzyme-AMP- P_1 complex cannot be invoked because this would mean that AMP should also inhibit the ATP-AMP exchange, and this was not observed.

The ATP-AMP exchange was the only exchange that was slower than the overall reaction in the slower direction. Since it was slower than both the P_1 - PP_1 and PP_1 -ATP exchanges it appears that either the binding or the release of AMP must be the limiting process. It follows that this limiting process must be accelerated when all substrates for the overall reaction are present. If the release of AMP is the slower process it is possible that pyruvate is able, though not obliged, to bind to the enzyme at some point in the reaction sequence before it is required to participate in bond-breaking reactions. Under these circumstances the enzyme may be modified in such a way that the release of AMP is facilitated. If binding of AMP is the slower process, similar considerations, applied to the reverse reaction, would mean that pyruvate is not obliged to dissociate from the enzyme immediately it is formed. With the pyruvate thus bound to the enzyme the rate of AMP binding may be increased.

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