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Determination of the catalytic pathway of C_4-leaf pyruvate, orthophosphate dikinase from maize

Lawrence J. Carroll[1], Debra Dunaway-Mariano[1], Christopher M. Smith[2] and Raymond Chollet[2]

[1]*Department of Chemistry and Biochemistry, University of Maryland, College Park, MD 20742, USA* and [2]*Department of Biochemistry, University of Nebraska at Lincoln, Lincoln, NE 68583-0718, USA*

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The mechanism of the maize pyruvate, phosphate dikinase-catalyzed conversion of adenosine 5′-triphosphate, orthophosphate and pyruvate to adenosine 5′-monophosphate, inorganic pyrophosphate and phosphoenolpyruvate, respectively, was determined by using transient kinetic techniques. The data obtained demonstrate that catalysis in the maize pyruvate, phosphate dikinase active site involves initial transfer of the $^{\gamma}P^{\beta}P$-unit from adenosine 5′- triphosphate to the enzyme to form a pyrophosphorylenzyme intermediate, followed by sequential phosphoryl group transfer to orthophosphate (to form pyrophosphate and a phosphoenzyme intermediate) and pyruvate (to form phosphoenolpyruvate and free enzyme).

Pyruvate; Phosphate dikinase; *Zea mays* L.; Catalysis; Transient kinetics; Enzyme intermediate; Phosphoryl transfer

1. INTRODUCTION

Pyruvate, phosphate dikinase, an enzyme found in certain microorganisms and plants, catalyzes the reversible phosphorylation of orthophosphate and pyruvate with the γ- and β-phosphoryl groups, respectively, of ATP [1];

$$AdePP^{\beta}P^{\gamma} + P_i + Pyruvate \rightleftharpoons AdeP + PP_i^{\gamma} + PEP^{\beta}$$

The microorganisms which produce PPDK do so either to replace the action of pyruvate kinase in ATP synthesis or to replace the combined actions of pyruvate carboxylase and PEP carboxykinase in PEP synthesis [1]. The PPDK-catalyzed formation of PEP in C_4 and Crassulacean acid metabolism plants is essential to the initial fixation of atmospheric CO_2 by PEP carboxylase that takes place during photosynthesis [2].

Previous studies of the structure and catalytic functioning of PPDK have focused largely on the dikinases isolated from the bacteria *Propionibacterium shermanii* and *Bacteroides symbiosus* and from the C_4 plants, sugarcane and maize. The results from these investigations have suggested that the eucaryotic PPDK differs significantly from the procaryotic PPDK in quaternary structure [3,4], stability [3–6] and regulation [2,7]. In addition, earlier studies of the mechanism of action of the plant and bacterial dikinases showed that while both types of dikinase utilize an active site histidine to mediate phosphoryl transfer during catalytic turnover [7–9] they appear to differ in their overall mode of catalysis. As a case in point, the very extensive studies carried out by Wood and his coworkers on the bacterial dikinases led to the proposal of a three-step mechanism (shown in the ATP → PEP direction in Scheme I) involving the formation of first, a pyrophosphohistidine enzyme intermediate and then second, a phosphohistidine enzyme intermediate [10,11]. In contrast, studies of the sugarcane and maize dikinase, carried out by other researchers, point to a two-step mechanism for the plant dikinases involving the intermediacy of the singular phosphohistidine enzyme intermediate (see Scheme I) [4,12,13].

The relative instability of the plant dikinase [4,6] coupled with the primary focus placed on its mode of regulation by a complex phosphorylation (inactivation)/dephosphorylation (activation) cycle [2,7,9,14] delayed further studies of its mechanism of action and left open the question of how the phosphorylenzyme intermediate is formed in the first partial reaction (see Scheme I). In Scheme II we show two plausible chemical pathways for the overall reaction catalyzed by the plant dikinase that are both consistent with a Bi Bi Uni Uni kinetic mechanism [4,12,13] but which differ by virtue of the first reaction intermediate formed. Pathway I involves the initial formation of a pyrophosphohistidine enzyme intermediate (analogous to the reaction pathway of the bacterial dikinase (see Scheme I and [10,11])) which is in some way triggered by the P_i bound in the active site of the enzyme. Pathway II, on the other hand, requires phosphorylation of P_i with ATP to generate ADP as the first intermediate. The

Correspondence address: D. Dunaway-Mariano, Department of Chemistry and Biochemistry, University of Maryland, College Park, MD 20742, USA

Abbreviations: PPDK, pyruvate, phosphate dikinase; PEP, phosphoenolpyruvate; P_i, orthophosphate; PP_i, pyrophosphate; K^+ Hepes, potassium salt of *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; EPP, pyrophosphoryl PPDK; EP, phosphoryl PPDK

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Bacterial PPDK

(1) $E_{His} + ATP \rightleftharpoons E_{HisP}{}^{\beta}{}_{P}{}^{\gamma} + AMP$

(2) $E_{HisP^{\beta}P^{\gamma}} + P_i \rightleftharpoons E_{HisP}{}^{\beta} + P^{\gamma}P_i$

(3) $E_{HisP^{\beta}} + Pyruvate \rightleftharpoons E_{His} + PEP^{\beta}$

Plant PPDK

(1) $E_{His} + ATP + P_i \rightleftharpoons E_{HisP^{\beta}} + AMP + P^{\gamma}P_i$

(2) $E_{HisP^{\beta}} + Pyruvate \rightleftharpoons E_{His} + PEP^{\beta}$

Scheme I. The mechanisms proposed for the bacterial [10,11] and plant [4,12,13] PPDKs where E_{His} represents the free enzyme, E_{HisPP} the pyrophosphoenzyme intermediate and E_{HisP} the phosphoenzyme Intermediate.

ADP then phosphorylates the active site histidine to form the phosphorylenzyme.

In this paper we report the results of transient kinetic studies of the PPDK purified from maize leaves which unambiguously identify Pathway I (see Scheme II) as that followed during turnover in the active site of the plant dikinase.

2. MATERIALS AND METHODS

Maize PPDK was purified from preilluminated (~4 h) leaf tissue according to the methods of Roeske and Chollet [14] except for the following modifications. The desalted 40–50% saturation $(NH_4)_2SO_4$ fraction was applied directly to the hydroxylapatite (Bio-Gel HTP) column in the presence of 10 mM K^+-phosphate (in 50 mM Bis-Tris-propane, pH 7.0, 14 mM 2-mercaptoethanol, 5 mM $MgSO_4$, 0.5 mM EDTA, 5% v/v glycerol) and the dikinase eluted with 25 mM K^+-phosphate in the same buffer. The PPDK-enriched fractions were precipitated by 70% saturation $(NH_4)_2SO_4$, desalted, and further purified by FPLC-based anion-exchange chromatography on Mono-Q at pH 7.3 (50 mM Mops, 14 mM 2-mercaptoethanol, 5 mM $MgSO_4$, 0.1 mM EDTA, 5% v/v glycerol) and by dye-ligand chromatography on Blue A and Red A columns [14]. The enzyme used in these experiments had a specific activity of 4 μmol of PEP formed/min per mg of protein when assayed in a solution containing 15 mM NH_4Cl, 10 mM $MgCl_2$, 4 mM pyruvate, 1.5 mM ATP, 3.7 mM K_2HPO_4 and 100 mM Tris-HCl (pH 8.0) at 30°C. [γ-^{32}P]ATP was purchased from Amersham and purified prior to use on a 1-ml DEAE-Sephadex column with a 0.1–0.6 M linear gradient of NH_4HCO_3 serving as eluant. [β-^{32}P]ATP was prepared from [γ-^{32}P]ATP as described under footnote 9 in [15].

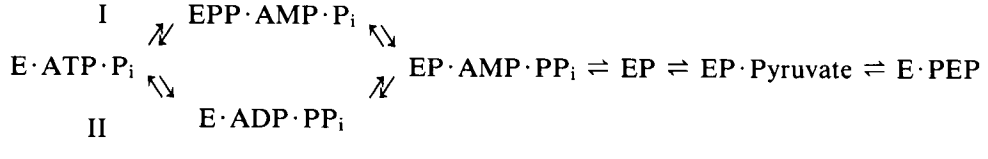
The time course for a single turnover in the PPDK active site [15] was measured by reacting radiolabelled substrate [γ-^{32}P]ATP or [β-^{32}P]ATP (40 μl) with excess enzyme (40 μl) at 30°C in a rapid quench apparatus designed and constructed by Dr Kenneth Johnson of the Pennsylvania State University (USA). The reactions were quenched with 0.6 N HCl (160 μl). The protein in the quenched sample was precipitated with CCl_4 (100 μl), separated by centrifugation, dissolved in boiling 10 N H_2SO_4 and assayed for ^{32}P content by liquid scintillation counting techniques. The radiolabelled reactants and products were separated by HPLC (Beckman Ultrasphere C^{18} analytical column, 25 mM K^+-phosphate, 2.5% triethylamine and 5% methanol (pH 6.5) isocratic elution) and quantitated by liquid scintillation techniques.

3. RESULTS AND DISCUSSION

In an earlier communication we reported [15] the successful application of rapid quench techniques to monitor the formation and reaction of the pyrophosphohistidine enzyme and phosphohistidine enzyme intermediates in the active site of the *Bacteroides symbiosus* PPDK during catalytic turnover. In the present study we utilized this transient kinetic approach to test for the formation of these same intermediates in the active site of the labile plant dikinase. By using enzyme in molar excess of substrates and a rapid quench apparatus for mixing and quenching in the millisecond time frame we were able to quantitate the substrates, intermediates and products formed during a single turnover in the enzyme active site as well as the unconsumed substrate.

The time course for the forward direction of the PPDK reaction was measured by using [γ-^{32}P]ATP to monitor EPP and PP_i formation and [β-^{32}P]ATP to monitor EPP + EP, PEP and ADP formation. The [γ-^{32}P]ATP and [β-^{32}P]ATP reactions were run sequentially and the amount of EP formed was calculated by subtracting the amount of radiolabelled enzyme formed in the [γ-^{32}P]ATP reaction from that formed in the [β-^{32}P]ATP reaction (see Scheme I). The results obtained with the Mn^{2+}/NH_4^+-activated maize PPDK are shown in Fig. 1. The formation of the EPP enzyme-intermediate and the concomitant loss of ATP in the PPDK active site are readily apparent from these data. Formation of the alternate intermediate complex, $E \cdot ADP \cdot PP_i$, on the other hand, did *not* occur at a detectable level. These results demonstrate that catalysis by the maize PPDK follows Pathway I of Scheme II and not Pathway II.

The time course shown in Fig. 1 also shows that the final product, PEP forms at a rate which is not significantly slower than that of PP_i formation and that there is no obvious burst of EP formation. These results



Scheme II. Possible chemical pathways of the plant PPDK catalyzed reaction.

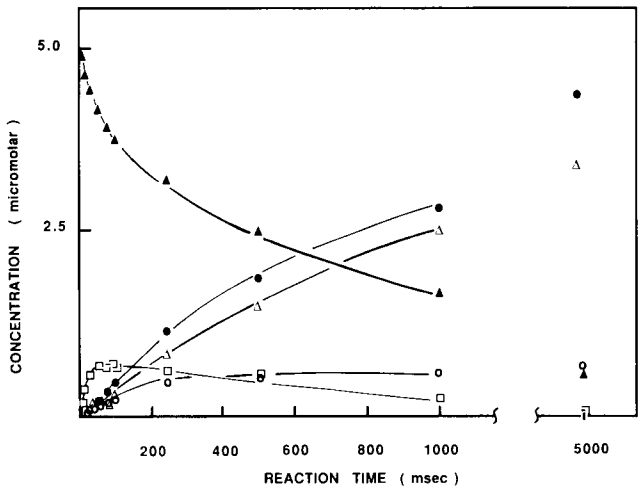


Fig. 1. Time course for a single turnover of ATP, P_i and pyruvate in the active site of Mn^{2+}/NH_4^+-activated maize PPDK at 30°C. The reaction mixture contained 5 μM [γ-^{32}P]ATP or [β-^{32}P]ATP, 15 μM PPDK active sites (94-kDa protomer), 10 mM P_i, 1 mM pyruvate, 2 mM $MnCl_2$, 10 mM NH_4Cl and 100 mM K^+Hepes (pH 8.5): (□) EPP, (○) EP, (△) ATP, (•) PP_i and (△) PEP.

suggest that under the selected reaction conditions (viz. Mn^{2+}/NH_4^+ cofactors; pH 8.5) the rate of formation of EPP is fast relative to the rate at which it reacts with P_i to form EP and PP_i and that the pyruvate reacts with EP at least as fast as the latter is formed. The results from studies (data not shown) carried out with Co^{2+}/NH_4^+ or Mg^{2+}/NH_4^+ serving as cofactors suggest that the relative rates of the three reaction steps are affected by the metal ion cofactors. It was observed that Mn^{2+} activated PPDK provided for the highest level of EPP accumulation during catalytic turnover while Mg^{2+} activated PPDK provided the lowest level (and it was for this reason that Mn^{2+} was chosen as the activating metal ion in the single turnover experiment reported in Fig. 1). This same trend was observed for the PPDK isolated from *B. symbiosus* [15].

In summary, contrary to previous evidence [1,4,12, 13], the catalytic pathways of the bacterial and plant pyruvate, phosphate dikinases are one in the same. The results reported in this paper for the plant PPDK, and in an earlier communication for the *B. symbiosus* dikinase [15], demonstrate that during turnover the $^{\gamma}PP^{\beta}$-unit of ATP is transferred to the enzyme and then sequential phosphoryl group transfer occurs to P_i and then pyruvate. Thus, while the bacterial and plant dikinases have diverged in terms of quaternary structure (α_2 and α_4, respectively) and regulation of catalysis, the chemistry itself and the active -site domain [7,8] have been conserved.

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