Substrate specificity and regulation of the maize (*Zea mays*) leaf ADP:protein phosphotransferase catalysing phosphorylation/inactivation of pyruvate,orthophosphate dikinase

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The protein substrate specificity of the maize (*Zea mays*) leaf ADP:protein phosphotransferase (regulatory protein, RP) was studied in terms of its relative ability to inactivate/phosphorylate pyruvate,orthophosphate dikinase from *Zea mays* and the non-sulphur purple photosynthetic bacterium *Rhodospirillum rubrum*. The dimeric bacterial dikinase was inactivated by the maize leaf RP via phosphorylation, with a stoichiometry of ~ 1 mol of phosphate incorporated/mol of 92.7-kDa protomer. Inactivation required both ADP and ATP, with ADP being the specific donor for regulatory phosphorylation. The requirements for inactivation/phosphorylation in this heterologous system were identical with those previously established for the tetrameric maize leaf dikinase. The ADP-dependent maize leaf RP did not phosphorylate alternative protein substrates such as casein or phosvitin, and its activity was not affected by cyclic nucleotides, Ca^{2+} or calmodulin. The regulation of the maize leaf ADP:protein phosphotransferase was studied in terms of changes in adenylate energy charge and pyruvate concentration. The change in adenylate energy charge necessary to substantially inhibit phosphorylation of maize leaf dikinase was not suggestive of it being a physiological modulator of phosphotransferase activity. Pyruvate was a potent competitive inhibitor of regulatory phosphorylation ($K_i = 80 \mu\text{M}$), consistent with its interaction with the catalytic phosphorylated intermediate of dikinase, the true protein substrate for ADP-dependent phosphorylation/inactivation.

INTRODUCTION

Phosphorylation of both soluble and membranous proteins has been established as an important mechanism in metabolic regulation (Ingebritsen & Cohen, 1983). Numerous polypeptides are phosphorylated, yet neither the specific protein phosphotransferase catalysing phosphorylation nor the protein substrate has been identified in most cases. Very little is known concerning the number, substrate specificity, or regulation of protein phosphotransferases in plants. In 'C₄' mesophyll chloroplasts the most intensely phosphorylated soluble protein is the 94 kDa protomer of pyruvate,orthophosphate dikinase (PPDK, EC 2.7.9.1) (Ashton & Hatch, 1983). In marked contrast with all other known examples of protein phosphorylation, the PPDK system is unique in that the specific phosphate donor is ADP (Ashton & Hatch, 1983; Budde *et al.*, 1985) and not ATP or PEP.

PPDK reversibly catalyses the conversion of pyruvate

into PEP. The reaction mechanism has been elucidated and involves three partial reactions for the non-photosynthetic bacterial enzyme (Goss & Wood, 1982) and only two partial reactions for the C₄ leaf enzyme (Jenkins & Hatch, 1985). The mechanism that the enzyme from photosynthetic bacteria uses is not known. Common to both catalytic mechanisms is a covalent phospho-enzyme intermediate complex (E-P), which is an acid-labile phosphohistidine (Burnell, 1984a; Goss & Wood, 1982). Conversely, maize leaf PPDK is inactivated via regulatory phosphorylation to form an acid-stable phosphothreonine residue from the β -phosphate of ADP (Budde *et al.*, 1985; Edwards *et al.*, 1985). There is an absolute co-requirement for either ATP (and presumably P_i) or PEP during regulatory phosphorylation which is due to the E-P catalytic intermediate being the true protein substrate for the ADP:protein phosphotransferase (Burnell & Hatch, 1984a). Thus the catalytic and regulatory mechanisms share a common phosphorylated intermediate complex (E-His-P):

Abbreviations used: C₄ plant, one in which the first stable product of photosynthetic CO₂ fixation is a four-carbon dicarboxylic acid; PPDK, pyruvate,P_i dikinase; PEP, phosphoenolpyruvate; RP, regulatory protein; PEI, poly(ethyleneimine)-cellulose; Ap₅A, P₁,P₅-di(adenosine-5') pentaphosphate; BSA, bovine serum albumin; C₃ plant, one in which the first stable product of photosynthetic CO₂ fixation is 3-phosphoglyceric acid.

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Reactivation of PPDK (i.e. dephosphorylation of the threonine residue) has been shown to require the same regulatory protein (RP) and P_i , with PP_i being the product (Edwards *et al.*, 1985).

We have examined the protein substrate specificity of the maize leaf regulatory protein in a heterologous system using PPDK from the non-sulphur purple photosynthetic bacterium *Rhodospirillum rubrum*. Our results indicate that the maize leaf RP will inactivate/phosphorylate the bacterial dikinase with ADP only if the enzyme is in the catalytic E-His-P intermediate form. This absolute specificity for the catalytic intermediate as the protein substrate precludes phosphorylation of casein or phosvitin, two common substrates for protein phosphotransferases. Our data also indicate that the maize leaf ADP:protein phosphotransferase activity is not influenced by cyclic nucleotides, Ca^{2+} or Ca^{2+} plus calmodulin, unlike many ATP-dependent protein kinases. The extent of inhibition of RP-dependent phosphorylation of maize leaf dikinase by adenylate energy charge and increasing pyruvate concentration indicates that adenylate energy charge is not itself a likely modulator of PPDK inactivation *in vivo*, but pyruvate (at concentrations comparable with those occurring *in vivo*) is a potent inhibitor of regulatory phosphorylation.

EXPERIMENTAL

PPDK was purified from maize (*Zea mays* L., cv. Golden Cross Bantam) leaves as previously described (Budde *et al.*, 1985). Maize leaf RP was partially purified through Blue Sepharose CL-6B (Pharmacia) by the method of Burnell & Hatch (1983). PPDK was purified ~80-fold to an apparent electrophoretic purity of ~60% from *Rhodospirillum rubrum*, strain S-1, grown photoheterotrophically on DL-lactate plus HCO_3^- (Ernst *et al.*, 1986). Spectrophotometric assays for dikinase activity (PEP formation) were performed at pH 8.0 and 30 °C by coupling to exogenous PEP carboxylase (Boehringer Mannheim) and NADH:malate dehydrogenase (Hatch & Slack, 1975). Protein was determined by the method of Bradford (1976), with crystalline BSA as the standard.

$[\gamma\text{-}^{32}\text{P}]\text{ADP}$ was synthesized from $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (Amersham) and purified as described by Budde *et al.* (1985). The integrity of the ^{32}P -labelled ADP, in terms of conversion into ATP and AMP by traces of contaminating adenylate kinase during inactivation/phosphorylation assays, was determined by PEI-t.l.c. analysis as described by Budde *et al.* (1985). All reactions contained Ap_5A , a potent inhibitor of adenylate kinase activity (Feldhaus *et al.*, 1975).

Protein samples for autoradiography were treated initially in an acidic denaturing medium to hydrolyse any acid-labile phosphohistidine formed during catalysis, and then neutralized with NaOH before SDS/polyacrylamide-gel electrophoresis on gradient gels (Budde *et al.*, 1985). Laemmli (1970) gel and electrode buffers were used for electrophoresis.

For the protein-substrate-specificity studies, phosphorylation/inactivation reactions contained purified maize leaf PPDK or *R. rubrum* PPDK, partially purified maize leaf regulatory protein, 1 mM-ADP, 0.2 mM-ATP, 0.4 mM- Ap_5A and BSA (0.1 mg/ml) in a buffer containing 50 mM-Tris/HCl, pH 8.3, 5 mM-MgCl₂, 0.1 mM-Na₂ EDTA and 10 mM- β -mercaptoethanol. Reactions

were performed at 30 °C and initiated with Blue Dextran-stabilized RP (Burnell, 1984b). Samples were removed at the specified times and assayed spectrophotometrically for PPDK activity or for protein phosphorylation by the filter-paper binding assay (Markwell *et al.*, 1982), in which case either $[\beta\text{-}^{32}\text{P}]\text{ADP}$ or $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ was used. The level of ^{32}P incorporation was determined by Čerenkov counting, and radioactivity (d.p.m.) was calculated on the basis of a 50% counting efficiency. Reaction mixtures minus PPDK were used as background controls.

For studies on the regulation of ADP:protein phosphotransferase activity, a specific adenylate energy charge was established by incubating various concentrations of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and AMP, BSA (0.25 mg/ml) and 2 units of adenylate kinase in 50 μl of buffer consisting of 50 mM-Tris/HCl, pH 8.0, 5 mM-MgCl₂ and 7 mM- β -mercaptoethanol for ~30 min (30 °C) before addition of purified maize leaf PPDK, RP, pyruvate (0–0.5 mM), and 2 mM- P_i . The concentration of total adenylates used was 0.5 mM, which is thought to be similar to the pool size *in vivo* (Nakamoto & Edwards, 1983).

RESULTS AND DISCUSSION

When purified maize leaf PPDK is incubated with ADP, ATP and the maize leaf RP, it is inactivated in a time- and RP-dependent fashion (Fig. 1; Ashton *et al.*, 1984; Budde *et al.*, 1985). If exogenous ATP is omitted and an ATP-scavenging system is included, neither inactivation (Fig. 1) nor phosphorylation occurs to any significant extent. Likewise, inactivation/phosphorylation of the *R. rubrum* PPDK is dependent upon the presence of ADP, ATP and the maize leaf RP (Figs. 1 and 2). Maize PPDK is inactivated more rapidly than the bacterial enzyme. Four different attempts at inactivating *R. rubrum* PPDK resulted in losses of 20–60% of the initial activity after 2 h at 30 °C. Inactivation is not complete, owing to the instability of the RP, as evidenced by further inactivation/phosphorylation upon addition of fresh RP. The relative initial rates of phosphorylation were estimated by a filter-paper binding assay and indicated a 5–6-fold greater rate of phosphorylation for maize leaf PPDK (Table 1).

Recently, we (Budde *et al.*, 1985) and Ashton & Hatch (1983) have firmly established that ADP is the specific donor for the regulatory phosphorylation of maize leaf PPDK by RP. Likewise, ADP is the specific donor for the phosphorylation-dependent inactivation of *R. rubrum* PPDK in this hybrid system (Fig. 1; Fig. 2, lanes 3 versus 5). Thus the bacterial PPDK/maize RP heterologous system displays the same ADP-plus-ATP requirement for inactivation/phosphorylation as does the homologous C₄ leaf system, with ADP being the specific phosphate donor. It is likely that the ATP requirement in this heterologous system is identical with that in the plant system, i.e. the maize leaf regulatory protein only recognizes the E-His-P catalytic intermediate of PPDK (see eqn. 1 and Burnell & Hatch, 1984a). The inability of the RP to phosphorylate either the trypsin-treated (trypsin activity being quenched with a 10-fold molar excess of leupeptin) or urea-denatured (urea being removed by extensive dialysis) maize leaf enzyme (Table 1) is presumably also due (at least) to the lack of E-His-P.

It has been shown both *in vitro* (Ashton *et al.*, 1984; Budde *et al.*, 1985) and *in vivo* (Budde *et al.*, 1985) that

Fig. 1. Regulatory protein- and ATP-dependent inactivation of PPDK activity

Maize PPDK (ZmDK) or *R. rubrum* PPDK (RrDK), plus or minus ~ 700 μ g of maize leaf RP, was incubated at pH 8.3 and 30 °C with 1 mM-ADP, 0.2 mM-ATP, BSA (0.1 mg/ml) and 0.4 mM- Ap_5A except where noted [–ATP], in which case exogenous ATP was omitted and replaced with an ATP-scavenging system comprising 2 mM-D-glucose and 4 units of hexokinase. At the times indicated, samples were withdrawn for measurement of PPDK activity. The initial (100%) specific activity was 3.0 μ mol of PEP formed/min per mg of protein for the bacterial enzyme and 4.5 units $\cdot$ mg of protein $^{-1}$ for maize PPDK. The RP preparation contained no spectrophotometrically detectable PPDK activity.

Fig. 2. ADP-dependent phosphorylation of *R. rubrum* PPDK (RrDK) by the maize leaf regulatory protein (RP)

Proteins were separated by SDS/polyacrylamide-gel electrophoresis and subjected to autoradiography. Lane 1, M_r markers (including phosphorylase *b*, BSA, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor and lysozyme) stained with Coomassie Blue R-250; lane 2, maize PPDK (ZmDK) inactivated *in vitro* with [β - ^{32}P]ADP (4×10^7 d.p.m./ μ mol of ADP), ATP and RP; lane 3, bacterial PPDK inactivated *in vitro* with [β - ^{32}P]ADP (4×10^7 d.p.m./ μ mol of ADP), ATP and RP; lane 4, control sample lacking exogenous ATP and containing an ATP-scavenging system; lane 5, bacterial PPDK inactivated *in vitro* with [γ - ^{32}P]ATP (6×10^7 d.p.m./ μ mol of ATP), ADP and RP; lanes 6 and 7, control samples lacking RP and bacterial PPDK respectively.

Table 1. Maize leaf RP specificity and inhibition of PPDK phosphorylation

Unless noted otherwise, assays contained ~ 10 μ g of protein substrate, 0.4 mM- Ap_5A , partially purified RP, γ -labelled ATP (0.2 mM) plus ADP (1 mM) or ATP (0.2 mM) plus β -labelled ADP (1 mM) and BSA (0.1 mg/ml) at pH 8.3 in a final volume of 0.1 ml. When casein or phosphovitin was used as substrate, only the indicated labelled nucleotide was present. Reactions were quenched after 30 min at 30 °C.

Protein substrate	^{32}P -labelled substrate	^{32}P incorporation (%)
Maize PPDK	β -ADP	100
	γ -ATP	1
	+ Trypsin	~0
	+ Urea	7
<i>R. rubrum</i> PPDK	+ 1 mM- Ap_5A	73
	β -ADP	17
	γ -ATP	~0
Phosvitin	β -ADP	5
	γ -ATP	80
Casein	β -ADP	5
	γ -ATP	60

the stoichiometry for complete inactivation of maize leaf PPDK is 1 mol of phosphate incorporated/mol of 94 kDa protomer. The corresponding stoichiometry for the bacterial PPDK phosphorylation/inactivation heterologous system was estimated by desalting a portion of the reaction mixture through a Sephadex G-25 column

containing 0.2% SDS. Activity losses of 21 and 40% yielded stoichiometries of 0.22 and 0.41 mol of phosphate/92.7-kDa protomer of *R. rubrum* PPDK respectively. Our preliminary attempts to identify the amino acid modified in the bacterial PPDK hybrid system by h.p.l.c.-based phospho amino acid analysis (Budde *et al.*, 1985) suggested a phosphothreonine residue, but an insufficiency of ^{32}P -labelled material precluded a definitive answer.

Burnell (1984a) has reported that PPDK from the non-photosynthetic bacterium *Bacteroides symbiosus* could not serve as a substrate for the maize leaf regulatory protein as measured by inactivation/phosphorylation assays. Considering the nearly identical active-site sequence homology between *B. symbiosus* and *Z. mays* PPDK (Burnell, 1984a; Goss *et al.*, 1980), Burnell (1984a) suggested that differences in quaternary structure may explain the inability of the bacterial enzyme to serve as an effective substrate. Whereas PPDK from *B. symbiosus* is a 170 kDa homodimer (Goss *et al.*, 1980), the enzyme from *Z. mays* is a 387 kDa homotetramer (Sugiyama, 1973); both oligomers have 94 kDa subunits (Goss *et al.*, 1980; Sugiyama, 1973). However, like the enzyme from *B. symbiosus*, PPDK from *R. rubrum* is also probably a homodimer of 92.7 kDa subunits (Ernst *et al.*, 1986). Taken collectively, these findings indicate that more subtle properties than gross quaternary structure determine whether a given PPDK protein can serve as an effective substrate for the maize leaf regulatory protein.

The maize leaf RP preparations were only partially purified and were found to contain both ADP- and ATP-dependent protein phosphotransferase activities (Table 1). These two activities could be partially separated by casein-Sepharose affinity chromatography (results not shown). ATP was not an effective substrate for phosphorylating either maize leaf or *R. rubrum* PPDK (Table 1 and Fig. 2), and ADP was ineffective in phosphorylating phosvitin or casein (Table 1), two substrates commonly used in protein phosphorylation assays. The phosphorylation of casein and phosvitin by ATP was inhibited by ADP by 34 and 40% respectively. Although all phosphorylation assays contained 0.4 mM- Ap_5A to inhibit traces of contaminating adenylate kinase, this activity could not be completely eliminated. PEI-t.l.c. analysis indicated that ~6% of the ^{32}P ADP was converted into ^{32}P ATP during the course of the phosphorylation reaction. Therefore low ^{32}P incorporation rates of 5% or less for the alternative nucleotide (i.e. ADP versus ATP) are likely to be overestimates. The use of di(adenosine-5') oligophosphates such as Ap_5A has

been shown by Shoyab (1985) to inhibit protein kinase activity, as was also observed with the maize leaf RP (Table 1).

Protein phosphorylation in several plant systems has been shown to be stimulated by Ca^{2+} and calmodulin (Ranjewa *et al.*, 1983) and inhibited by cyclic nucleotides (Baker *et al.*, 1982). Our studies indicate that cyclic AMP or cyclic GMP has little or no effect on the phosphorylation of maize leaf PPDK by ADP or the phosphorylation of casein or phosvitin by ATP or ADP (Table 2). Likewise, Ca^{2+} plus or minus calmodulin has essentially no effect on the ADP-dependent phosphorylation of maize PPDK, whereas Ca^{2+} appears to inhibit phosphorylation of both casein and phosvitin (Table 2, plus EGTA versus Ca^{2+}). Calmodulin and Ca^{2+} have been shown to stimulate the ATP-dependent phosphorylation of both soluble and membranous proteins in etiolated maize coleoptiles (Veluthambi & Poovaiah, 1984).

The maize leaf RP appears to have a bivalent-cation requirement for the maintenance of activity; desalting the partially purified RP preparation on a Sephadex G-25 column in the absence of Mg^{2+} causes a total loss of any ADP:protein phosphotransferase activity when added to a reaction mixture containing Mg^{2+} , ADP, ATP and PPDK. This procedure did not inactivate the ATP:protein phosphotransferase activity with phosvitin or casein.

The inability of cyclic nucleotides, Ca^{2+} and/or calmodulin to modulate the ADP:protein phosphotransferase activity (Table 2) leaves unresolved the basis for light/dark regulation of this enzyme *in vivo*. Preliminary studies in our laboratory indicate that crude leaf extracts prepared from light- and dark-adapted maize plants possess both PPDK-inactivating and re-activating activities. This observation seemingly excludes the involvement of a bicyclic cascade regulatory system in which the ADP:protein phosphotransferase activity is modulated via covalent modification. Similarly, the requirement for Mg^{2+} and broad overlapping pH optima (pH 7.8–8.4) for inactivation/re-activation (Burnell & Hatch, 1985) seem to preclude the dark/light changes in pH and Mg^{2+} concentration which occur within the chloroplast stroma as factors involved in the regulation of the ADP:protein phosphotransferase.

Suggestions have been made that chloroplast adenylate energy charge along with pyruvate (Nakamoto & Edwards, 1983), or the ADP concentration itself (Burnell & Hatch, 1985) may be responsible for controlling inactivation/re-activation of the dikinase. Although it is not known how much the adenylate energy charge changes upon dark–light transitions in C_4 mesophyll

Table 2. Effect of various ligands on protein phosphorylation catalysed by partially purified maize leaf regulatory protein(s)

Ligand concentrations used were: 1 mM-cyclic AMP; 1 mM-cyclic GMP; 0.25 mM-EGTA; 2 mM- CaCl_2 . See Table 1 for reaction conditions. The values in parentheses are for 2 mM- CaCl_2 plus 1.6 μM spinach calmodulin.

Protein substrate	^{32}P -labelled substrate	^{32}P incorporation (% of control)			
		Cyclic AMP	Cyclic GMP	EGTA	Ca^{2+}
Maize PPDK	β -ADP	101	83	78	91 (93)
	β -ADP	103	102	102	65
	γ -ATP	106	96	120	40 (62)
Casein	β -ADP	91	102	89	54
	γ -ATP	95	103	105	35 (57)

Fig. 3. Inhibition of maize leaf PPDK regulatory phosphorylation by (a) adenylate energy charge and (b) pyruvate at two different adenylate-energy-charge (AEC) values

The adenylate energy charge was established by incubating (pH 8.0, 30 °C, 30 min) $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (3×10^8 d.p.m./ μmol) with AMP, 2 units of adenylate kinase and BSA (0.25 mg/ml). Pyruvate was added as indicated. Regulatory phosphorylation was initiated by addition of PPDK, RP and 2 mM- P_i in a final volume of 100 μl . After 20 min the reactions (in triplicate) were assayed in duplicate for ${}^{32}\text{P}$ incorporation into protein by a filter-paper binding assay. Reaction mixtures minus PPDK were used as background controls for each adenylate-energy-charge value. In (b), $1/v$ represents $10^3 \times$ the reciprocal net d.p.m. incorporated.

chloroplasts, recent studies using rapid subcellular fractionation techniques with 'C₃' leaf protoplasts found the stromal adenylate energy charge to be 0.72 ± 0.12 in the light and 0.61 ± 0.07 in the dark (Stitt *et al.*, 1982).

The effect of adenylate energy charge on RP-catalysed phosphorylation (inactivation) of maize leaf PPDK was studied by measuring ${}^{32}\text{P}$ incorporation from ADP; this indicated that maximum phosphorylation occurred at adenylate energy charge values of 0.5–0.8 (Fig. 3a). Only at adenylate energy charge levels less than 0.3 and greater than 0.95 was phosphorylation less than 50% of the maximum level.

The concentrations of AMP, ADP and ATP at any adenylate energy charge can be calculated (Atkinson &

Chapman, 1979) if one knows the adenylate pool size and equilibrium constant for adenylate kinase; the latter value is dependent upon pH and Mg^{2+} concentration (Noda, 1973). An equilibrium constant of 0.75 (in the direction of ATP formation) was determined experimentally for our reaction conditions. The equilibrium constant *in vivo* is thought to be ~ 0.5 (Stitt *et al.*, 1982). Such calculations indicate that the chloroplast ADP concentration would be saturating for the maize leaf RP ($K_m = 52 \mu\text{M}$; Burnell & Hatch, 1985), except at very high (greater than 0.88) and very low (less than 0.12) adenylate-energy-charge values.

These observations suggest that adenylate energy charge itself *cannot* yield the 10–15-fold difference in PPDK activity seen in dark- versus-light-adapted C₄ plants (Budde *et al.*, 1985; Edwards *et al.*, 1985). Pyruvate would seem to be a more likely effector candidate, since it would markedly decrease the lifetime of the E-His-P catalytic intermediate, the true protein substrate for ADP-dependent phosphorylation/inactivation catalysed by the RP (see eqn. 1). To explore this possibility, phosphorylation (inactivation) of maize leaf PPDK was studied as a function of pyruvate concentration at the two physiological levels of adenylate energy charge (Stitt *et al.*, 1982). A Dixon-type plot of the data (Fig. 3b) indicated that pyruvate was a competitive inhibitor of regulatory phosphorylation, which is consistent with the proposed mechanism for pyruvate inhibition of inactivation by removal of the protein substrate during catalysis (see eqn. 1 and Burnell & Hatch, 1984b). The K_i (app.) for inhibition was 80 μM , which is essentially identical with the K_m , pyruvate value for catalysis (Jenkins & Hatch, 1985). The plots were non-linear at pyruvate concentrations greater than 250 μM (Fig. 3b), indicating that pyruvate also inhibits in an additional manner (i.e., two different binding sites). Pyruvate concentrations greater than 1 mM completely inhibited phosphorylation. Although the concentration of pyruvate *in vivo* within a C₄ mesophyll chloroplast is not known precisely, it has been shown that this metabolite is taken up by maize chloroplasts via light-dependent active transport and the total concentration *in vivo* in the C₄ mesophyll cell in the light is 6 mM (Flügge *et al.*, 1985). Considering that the K_m , pyruvate values are ~ 1 mM for the carrier on the C₄ chloroplast envelope (Huber & Edwards, 1977) and 82 μM for catalysis by PPDK (Jenkins & Hatch, 1985), it is quite conceivable that pyruvate concentrations within the C₄ chloroplast stroma in the light are sufficient to inhibit inactivation/phosphorylation of the dikinase. In comparison with other protein-phosphorylation systems regulated by various ligands, the absolute specificity of RP for the E-His-P catalytic intermediate of PPDK (whose lifetime is dependent on the level of the substrate, pyruvate) provides a novel means for regulating the phosphorylation/inactivation of this important C₄-photosynthesis enzyme.

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