

Site-directed mutagenesis of maize recombinant C₄-pyruvate,orthophosphate dikinase at the phosphorylatable target threonine residue

Chris J. Chastain^{a,*}, Mary E. Lee^a, Molly A. Moorman^a, Patil Shameekumar^b, Raymond Chollet^b

^aDepartment of Biology, Moorhead State University, Moorhead, MN 56563, USA

^bDepartment of Biochemistry, University of Nebraska-Lincoln, G.W. Beadle Center, Lincoln, NE 68588-0664, USA

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Abstract A key regulatory enzyme of the C₄-photosynthetic pathway is stromal pyruvate,orthophosphate dikinase (PPDK, EC 2.7.9.1). As a pivotal enzyme in the C₄ pathway, it undergoes diurnal light–dark regulation of activity which is mediated by a single bifunctional regulatory protein (RP). RP specifically inactivates PPDK in the dark by an ADP-dependent phosphorylation of an active-site Thr residue (Thr-456 in maize). Conversely, RP activates inactive PPDK in the light by phosphorolytic dephosphorylation of this target Thr-P residue. We have employed a His-tagged maize recombinant C₄ PPDK for directed mutagenesis of this active-site regulatory Thr. Three such mutants (T456V, T456S, T456D) were analyzed with respect to overall catalysis and regulation by exogenous maize RP. Substitution with Val and Ser at this position does not affect overall catalysis, whereas Asp abolishes enzyme activity. With respect to regulation by RP, it was found that Ser can effectively substitute for the wild-type Thr residue in that mutant enzyme is phosphorylated and inactivated by RP. The T456V mutant, however, could not be phosphorylated and was, thus, resistant to ADP-dependent inactivation by RP.

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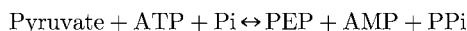
Key words: Pyruvate; Orthophosphate dikinase; Regulatory protein; C₄ plant; C₄ photosynthesis; Maize; *Zea mays*

1. Introduction

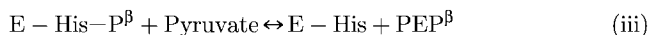
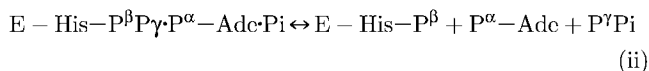
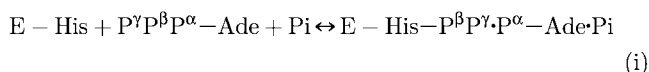
The C₄ photosynthetic pathway in higher plants is a unique form of photosynthetic carbon assimilation that eliminates the energetically wasteful process of photorespiration. This allows species with C₄ photosynthesis to be inherently more productive in terms of biomass production and grain yields [1,2]. Major crop species with C₄ photosynthesis include maize, sorghum, and sugarcane.

A key regulatory enzyme of the C₄-photosynthetic pathway is pyruvate,orthophosphate dikinase (PPDK) [1,3,4]. In C₄ leaves, PPDK is localized predominantly in the chloroplast stroma of mesophyll cells. In higher plants, the enzyme is active as a homotetramer of ≈95 kDa subunits. It has a cardinal role in the C₄ metabolic pathway as it catalyzes the

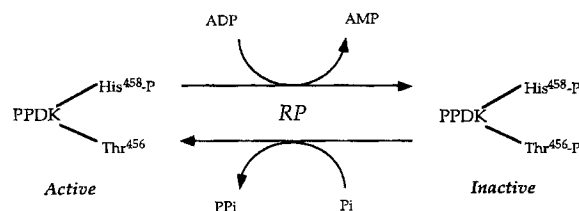
regeneration of the primary CO₂ acceptor, phosphoenolpyruvate (PEP), from pyruvate and ATP/Pi:



The reversible, three-step catalytic mechanism is complex and sequentially involves the (i) pyrophosphorylation of an active-site His residue (His-458 in maize PPDK) with the β- and γ-phosphates of ATP, forming PPDK-HisP^{βγ} and AMP, (ii) transfer of the γ-phosphate to Pi, yielding PPDK-HisP^β and PPi, and finally (iii) phosphorylation of pyruvate by PPDK-HisP^β, forming PEP and free enzyme [5,6]:



In addition to catalysis, PPDK, as a pivotal enzyme in the C₄ pathway, undergoes diurnal light-dark regulation of activity. The His-P form of the enzyme is specifically inactivated in the dark by an ADP-dependent phosphorylation of a nearby active-site Thr residue (Thr-456 in maize) [1,3,4,7]. Conversely, the free His-458 form of this inactive PPDK is preferentially activated in the light by a phosphorolytic dephosphorylation of the target Thr-P group. This light/dark regulation is mediated by the bifunctional stromal regulatory protein (RP):



PPDK RP represents a most unusual regulatory protein in three important respects. First, it is bifunctional, catalyzing both PPDK activation/dephosphorylation and inactivation/phosphorylation. Second, it uses ADP instead of ATP as the phosphoryl donor. Third, it employs a Pi-dependent, PPi-forming dephosphorylation mechanism, as opposed to simple hydrolysis as with most protein phosphatases. Its properties, however, are only partially characterized. This is largely

*Corresponding author. Fax: (1) (218) 236-2168.
E-mail: chastain@mhd1.moorhead.msus.edu

Abbreviations: DTT, dithiothreitol; IPTG, isopropyl-β-thiogalactoside; PEP, phosphoenolpyruvate; PMSF, phenylmethylsulfonyl fluoride; PPDK, pyruvate,orthophosphate dikinase; RP, regulatory protein

due to its extreme instability *in vitro* [4,8–10], and its low abundance in C_4 leaves ($\leq 0.04\%$ of soluble leaf protein [8]). Only a single early study has demonstrated purification to electrophoretic homogeneity as indicated by one dimensional SDS-PAGE [9]. More recent studies have failed to repeat this level of purity, even with improved purification protocols (e.g. FPLC, HPLC, ADP-affinity chromatography) [8,10]. It is this inability to adequately purify RP which has precluded efforts to develop an effective antibody or determine a partial peptide sequence. The latter two are obviously necessary for cloning the gene from a C_4 leaf cDNA library.

One approach for further illuminating PPDK regulation by RP is by selective mutagenesis of the active-site regulatory Thr-456 residue. To facilitate this approach, we have employed a histidine-tagged maize recombinant C_4 PPDK for site-directed mutagenesis. In this report, we describe several substitutions at this position that reveal insights into RP properties as well as the mechanism of regulatory inactivation of the target enzyme.

2. Materials and methods

2.1. Construction of a maize PPDK *E. coli* His-tag expression vector

The C_4 *pdk* ORF corresponding to the mature maize polypeptide was excised from our previously described pET-11a/*pdk* construct as a 2.6-kb *Bam*HI/*Nde*I fragment and inserted into *Nde*I/*Bam*HI cut pET-28a [11]. This construct is identical to pET-11a/*pdk* [11] except that sequences encoding a 20 amino acid/6X histidine peptide are fused to the N-terminal coding region of the maize gene.

2.2. Site-directed mutagenesis procedures

Site-directed mutagenesis was performed using a double-stranded plasmid mutagenesis procedure obtained in kit form ('Chameleon' site-directed mutagenesis kit) from Stratagene, Inc. Mutations were verified by two separate DNA sequencing runs of the same strand.

2.3. Purification of maize recombinant His-tag PPDK by Ni^{2+} -NTA affinity chromatography

This procedure is identical to our previous recombinant PPDK purification protocol [11] but deviates from it after $(NH_4)_2SO_4$ fractionation of the *E. coli* lysate. In this protocol, the 50% saturation $(NH_4)_2SO_4$ precipitate is resuspended in 8 ml of column binding-buffer (10 mM imidazole/50 mM KPi, pH 8.0, 300 mM KCl, 5 mM $MgSO_4$, 5 mM 2-mercaptoethanol, 1 mM PMSF). This and all subsequent steps were performed at room temperature. After a brief, clarifying centrifugation, the supernatant fluid was applied to a 1.5 ml column of Ni^{2+} -NTA agarose (Ni^{2+} -NTA 'Superflow', Qiagen, Inc.). To enhance recovery of enzyme, the flow-through was re-applied over the same column an additional seven times. After this binding step, the column was washed sequentially with 40 ml of wash buffer I (40 mM imidazole/50 mM KPi, pH 8.0, 300 mM KCl, 5 mM $MgSO_4$, 5 mM 2-mercaptoethanol, 1% (v/v) Tween 20) and 20 ml of wash buffer II (wash buffer I without Tween 20). PPDK was then eluted from the column with 10 ml 150 mM imidazole/50 mM KPi, pH 8.0, 300 mM KCl, 5 mM $MgSO_4$, 5 mM 2-mercaptoethanol. This eluate was then subjected to an overnight, 70% saturation $(NH_4)_2SO_4$ precipitation step at 0–4°C. Precipitated PPDK was then collected by centrifugation at $13,000 \times g$. The well-drained pellets were stored under N_2 in sealed tubes at $-80^\circ C$. Recombinant C_4 PPDK stored in this manner is stable for at least 4 months. The yield of His-tagged PPDK from this procedure is approximately 5 mg of high-purity enzyme per liter of IPTG-induced *E. coli* cells.

Prior to use in subsequent experiments, the enzyme pellet was resuspended in 100 mM Tris-HCl, pH 8.0, 10 mM $MgSO_4$, 0.5 mM EDTA, 5 mM DTT, and desalted by buffer exchange using a Centri-con 30 micro-concentrator (Amicon, Inc.).

2.4. PPDK and protein assays

PPDK activity was assayed spectrophotometrically at $30^\circ C$ in the PEP-forming direction [4,12]. Prior to assay, the enzyme was preincubated at $30^\circ C$ for 10 min to ensure full heat-reactivation [4]. PPDK

immunoblots were performed using standard methods with rabbit polyclonal antibodies raised against the maize-leaf PPDK monomer [13]. Protein was quantified by a sensitive dye-binding method with crystalline bovine serum albumin as standard [14].

2.5. RP-catalyzed inactivation/ ^{32}P -phosphorylation of PPDK

The source of RP for these experiments was a 'rapid' maize-leaf extract prepared as previously described from dark-adapted tissue [12]. Inactivation of active (dephospho) recombinant PPDK by RP was accomplished by incubating purified PPDK with RP and ADP (plus ATP/Pi) as previously described [10,12]. RP-catalyzed ^{32}P -phosphorylation of PPDK was carried out in a two-stage, single-tube procedure as previously outlined [12]. Briefly, $[\beta\text{-}^{32}P]\text{ADP}$ was generated from $[\gamma\text{-}^{32}P]\text{ATP}$, AMP and exogenous adenylate kinase in the first stage. This was followed by the immediate addition of purified PPDK and the RP-containing desalted leaf extract to the same reaction tube. The phosphorylation reaction was then incubated for 1 h at $30^\circ C$ and terminated by addition of SDS-PAGE sample buffer and heating to $100^\circ C$ for 2 min. Aliquots of the denatured reaction mixture were electrophoresed on 12% SDS-PAGE gels. The ^{32}P -radio-label associated with the $\approx 95\text{-kDa}$ PPDK polypeptide was detected on dried gels by conventional autoradiography at room temperature.

3. Results

3.1. Characterization of wild-type, His-tagged recombinant maize C_4 PPDK

We had previously developed a maize recombinant PPDK expression vector for synthesis of C_4 PPDK in *E. coli* cells [11]. For the present study, we inserted the maize *pdk* ORF from this previous construct into the His-tag expression vector, pET-28a. Hence, this recombinant enzyme is identical to our previous recombinant PPDK except that a 6X histidine 'tag', plus an additional 14 amino acids, is fused to the N-terminus of the mature *pdk* ORF. This has allowed us to greatly simplify the purification of wild-type and mutant enzymes (cf. [4,6,10,11]) by using rapid one-step Ni^{2+} -chelate affinity chromatography. Enzyme purified in this manner shows a high level of electrophoretic purity with few contaminating polypeptides (Fig. 1). Moreover, the specific activity of this recombinant wild-type construct is modestly increased by about 30% over our previous untagged recombinant enzyme (Table 1 and [11]). We also observed no differences in the His-tagged version with respect to regulatory phosphorylation as indicated by RP-inactivation assays (Fig. 2) and RP catalyzed ^{32}P -phosphorylation (Fig. 1). Moreover, no differences in specific activity are observed when the His-tag is selectively removed by thrombin proteinase (data not shown). Hence, like our previously reported recombinant C_4 PPDK [11], N-terminal His-tagged PPDK appears to be equivalent to the native enzyme extracted from illuminated maize leaves.

3.2. Analysis of site-directed mutants of the regulatory Thr-456 residue

For this study, three Thr-456 mutants were analyzed with respect to overall catalysis and regulation by RP. These were

Table 1
Specific activities (PEP-forming direction) of wild-type and mutant recombinant His-tagged maize PPDK purified by Ni^{2+} -NTA chromatography

Enzyme-form	Specific activity ($\mu\text{mol/min per mg protein}$)
Wild type	4.49
T456V	4.39
T456S	4.98
T456D	≈ 0

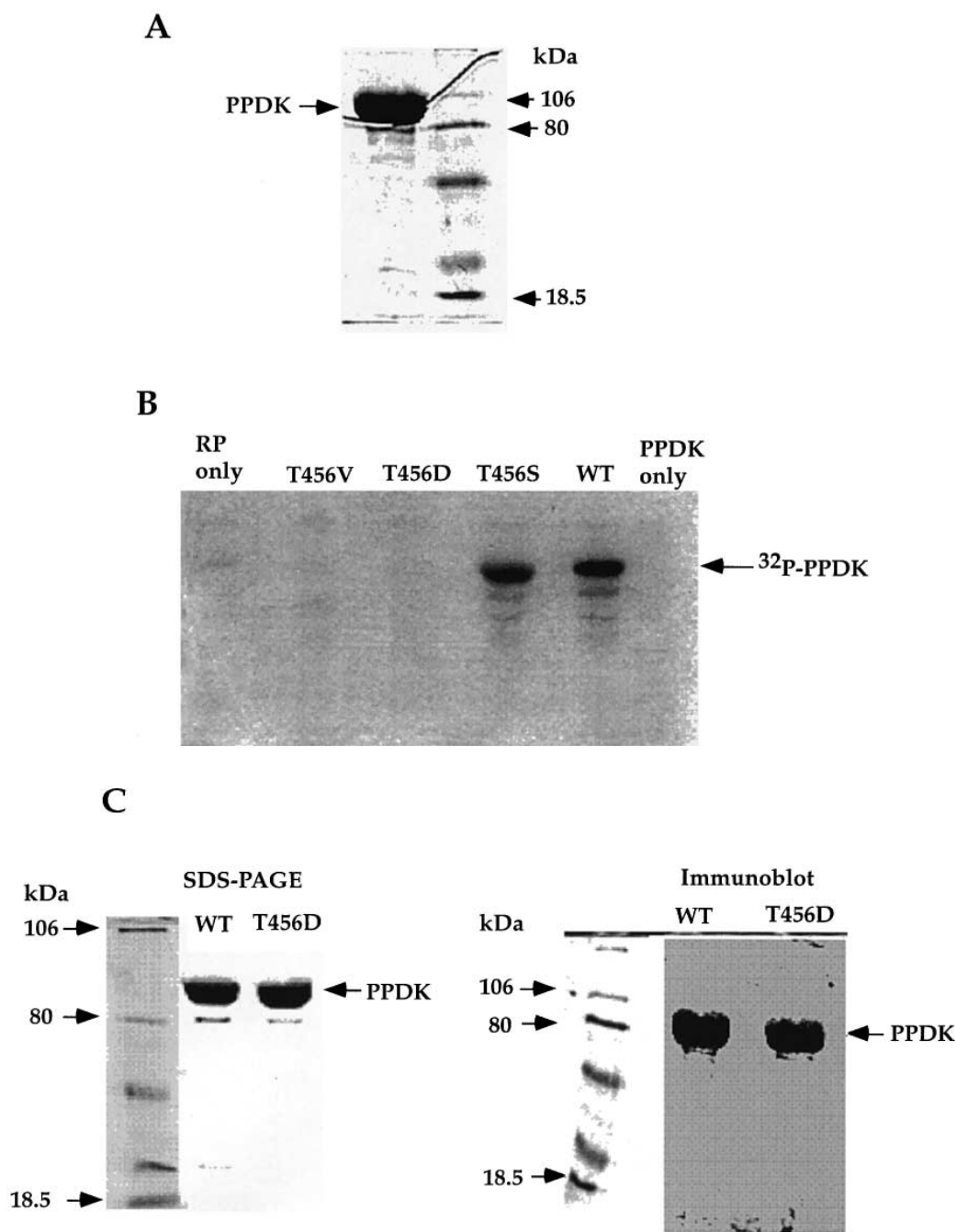


Fig. 1. (A) Coomassie blue-stained 12% SDS-PAGE mini-gel of wild-type His-tagged PPDK (15 µg protein) purified by Ni^{2+} -NTA affinity chromatography. Right lane depicts the molecular mass standards. (B) Autoradiograph of phosphorylation of wild-type and mutant maize recombinant PPDK by $[\beta\text{-}^{32}\text{P}]\text{ADP}$ and an RP-containing maize-leaf extract. Note the related RP-catalyzed inactivation data in Fig. 2. The arrow marks the position of the $\approx 95\text{-kDa}$ PPDK polypeptide as revealed by Coomassie blue-staining of the same gel. (C) SDS-PAGE and immunoblot analysis of His-tagged wild-type and T456D PPDK (10 µg protein) purified by Ni^{2+} -NTA affinity chromatography. Immunoblots were probed with maize-leaf PPDK antiserum. Unlabeled lanes are molecular mass standards.

T456S, T456V, and T456D. Serine was selected as one of the substitutions because of its frequency as a protein-Ser/Thr kinase target residue and because of its similar structure to the wild-type threonine. Alternatively, valine was inserted at this position because of its inability to accept phosphoryl groups as well as its electrical neutrality. Aspartate, a relatively rare protein kinase target [15], was selected in order to introduce negative charge into the active site via its carboxylic acid side chain, as does Thr-P^{2-} .

3.2.1. Catalysis

Overall catalysis was assayed with respect to the PEP-forming direction (Table 1). Specific activities of the T456S and T456V mutants were essentially equivalent to wild-type enzyme. However, substitution of an aspartate at this regulatory position resulted in PPDK being incompetent in overall catalysis, although enzyme purity and concentration were equivalent to wild-type PPDK as indicated by SDS-PAGE and immunoblot analysis, respectively (Fig. 1).

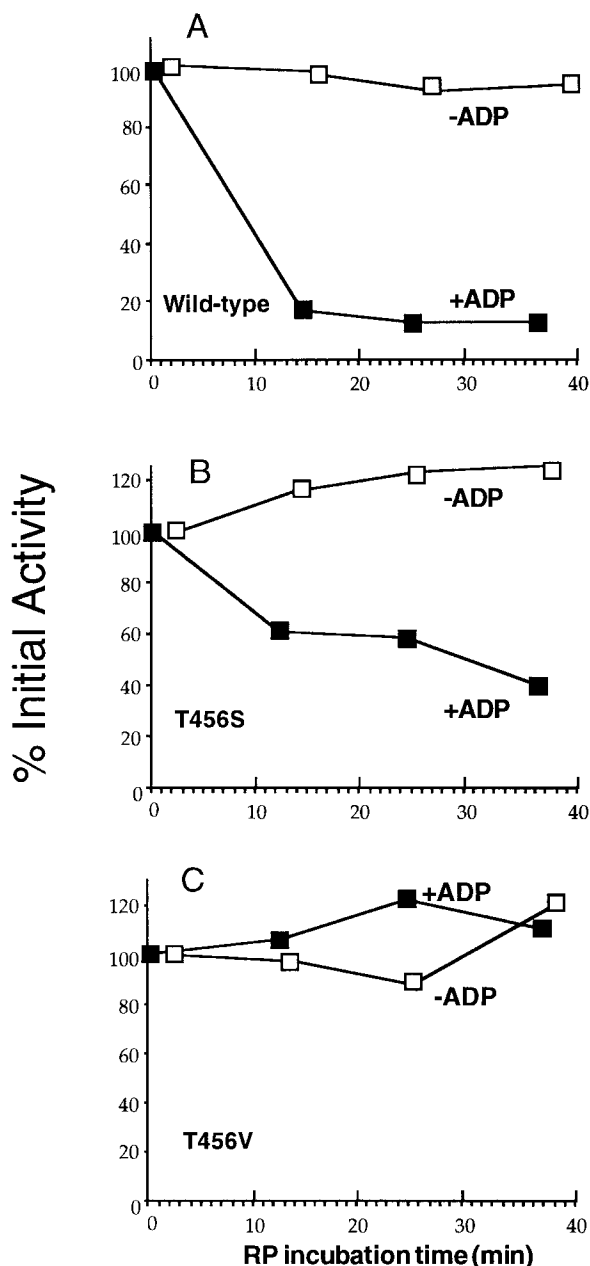


Fig. 2. PPDK/RP inactivation (phosphorylation) assays. Plot of PPDK activity versus incubation time with an RP-containing desalted leaf extract. PPDK activity is displayed as percent of initial activity (determined by immediate assay after addition of the desalted extract to the reaction tube). +/-ADP=addition or deletion of the RP phosphoryl donor to the incubation mixture. Due to technical limitations, the initial activity of the minus ADP series of assays ($\square$) was based on PPDK activity after a 2 min incubation with the RP-containing extract. Actual values of initial specific activities of +ADP and -ADP incubations were essentially identical (data not shown).

3.2.2. RP-catalyzed inactivation and ^{32}P -phosphorylation

When mutant and wild-type enzymes were subjected to RP-catalyzed, [^{32}P]ADP-phosphorylation *in vitro*, a strong and specific radiolabeling of wild-type PPDK was observed (Fig. 1). Of the three mutant enzymes, only T456S was similarly radiolabeled. No ^{32}P was detected in the T456D or T456V proteins, even upon prolonged exposure of the autoradiograph (data not shown). Concomitant with the phosphorylation of

Thr-456 is the inactivation of the target enzyme. This was demonstrated for wild-type enzyme using a rapid, crude maize-leaf extract as the source of exogenous RP. When wild-type, fully active recombinant PPDK is incubated with the RP-containing leaf extract, a rapid and marked inactivation of enzyme activity is observed ($>80\%$ inactivation after 14 min, Fig. 2). Although the desalted crude leaf extract contained a heterogeneous mixture of soluble proteins, inactivation was due specifically to RP because a control incubation minus ADP showed essentially no effect on enzyme activity (Fig. 2). Consistent with the ^{32}P -labeling data (Fig. 1) were the findings that the serine mutant was partially inactivated by RP in an ADP-dependent manner while the valine mutant was resistant to inactivation (Fig. 2 and C). However, the extent of inactivation of the serine mutant differed from the wild type in that 38% of the initial PPDK activity remained after the approximately 37 min incubation period, versus 13% for wild type.

4. Discussion

By directed mutagenesis of the regulatory Thr-456 residue in maize PPDK, we have gained specific insights into the nature of RP, as well as about the mechanism of inactivation by regulatory phosphorylation at this target residue. Of these is the *first* molecular-genetic demonstration that the sole RP phosphorylation residue is Thr-456. Previous biochemical studies had implicated this residue by phosphopeptide isolation, microsequencing, and comparison to the deduced sequence of maize C_4 PPDK [5,7,16]). Another significant insight is that Ser-456 can serve as a target for phosphorylation by maize RP. This indicates that the unusual RP likely belongs to the Ser/Thr family of protein kinases [15]. Alternatively, RP may also phosphorylate Tyr at this position, and hence potentially belong to the 'dual-specificity' family of kinases. The T456Y substitution is currently under development for future investigation. Although Ser-456 can serve as an alternative target for RP, it appears to be less efficiently phosphorylated as the magnitude of inactivation is reduced with respect to wild-type PPDK (62% versus 87% inactivation, respectively). This relative inefficiency may be due to the modest structural difference between the closely related Thr and Ser residues, with the former possessing an additional carbon on its side chain. Another possibility is that Ser-456 is phosphorylated to the same extent as the wild-type Thr (see Fig. 1), but its effect in the inactivation mechanism is attenuated for some unknown reason.

It is possible that RP inactivates overall catalysis in C_4 PPDK by introducing a the dianionic (2^-) phosphoryl group into the active-site domain ([17] and references therein). Recent evidence from detailed structural analysis of bacterial PPDK shows that the enzyme is organized into three distinct functional domains [18]. These are an N-terminal nucleotide binding domain, a central catalytic His-containing domain, and a C-terminal pyruvate binding domain. The remote N-terminal and C-terminal binding domains communicate during overall catalysis via swiveling of the central catalytic His to and from the flanking substrate binding domains. Thus, a dianionic phosphoryl group (PThr-456) positioned proximal to the central catalytic histidine (His-458) in maize PPDK may prevent interaction with bound substrate in the two remote domains, thus negating overall catalysis. This mechanism is strongly supported by the finding that substitut-

ing Asp for the regulatory Thr results in a non-phosphorylatable but catalytically incompetent enzyme. Although sterically far less bulky than a phosphoryl group, the Asp carboxyl group contributes a monoanionic (1^-) side chain into the active-site domain. Thus, overall catalysis is presumably prevented in the same manner as by regulatory phosphorylation, i.e. by altering the net charge about the central catalytic His-458. In more specific terms, it is the pyruvate to PEP partial reaction (partial reaction iii in Section 1: Introduction) that is negated. This assumption is based on prior studies showing that the PPDK Thr-P enzyme could catalyze partial reactions (i) and (ii) [19].

It should be noted that elimination of the RP-inactivation site altogether, as with the T456V mutant, results in a phosphorylation-incompetent, constitutively active maize enzyme. This may have useful consequences for genetic engineering of improved photosynthetic performance in maize leaves by providing a means of elevating the endogenous activity of this key C_4 enzyme [20].

References

- [1] Hatch, M.D. (1987) *Biochim. Biophys. Acta* 895, 81–106.
- [2] Hatch, M.D. (1992) *Plant Cell Physiol.* 33, 333–342.
- [3] Burnell, J.N. and Hatch, M.D. (1986) *Arch. Biochem. Biophys.* 245, 297–304.
- [4] Ashton, A.R., Burnell, J.N., Furbank, R.T., Jenkins, C.L.D. and Hatch, M.D. (1990) in: *Methods in Plant Biochemistry* (Lea, P.J., Ed.) Vol. 3, pp. 39–72, Academic Press, San Diego, CA.
- [5] Pocalyko, D.J., Carroll, L.J., Martin, B.M., Babbitt, P.C. and Dunaway-Mariano, D. (1990) *Biochemistry* 29, 10757–10765.
- [6] Carroll, L.J., Dunaway-Mariano, D., Smith, C.M. and Chollet, R. (1990) *FEBS Lett.* 274, 178–180.
- [7] Roeske, C.A., Kutny, R.M., Budde, R.J.A. and Chollet, R. (1988) *J. Biol. Chem.* 263, 6683–6687.
- [8] Smith, C.M., Duff, S.M.G. and Chollet, R. (1994) *Arch. Biochem. Biophys.* 308, 200–206.
- [9] Burnell, J.N. and Hatch, M.D. (1985) *Arch. Biochem. Biophys.* 237, 490–503.
- [10] Roeske, C.A. and Chollet, R. (1987) *J. Biol. Chem.* 262, 12575–12582.
- [11] Chastain, C.J., Thompson, B.J. and Chollet, R. (1996) *Photosynth. Res.* 49, 83–89.
- [12] Smith, C.M., Sarath, G. and Chollet, R. (1994) *Photosynth. Res.* 40, 295–301.
- [13] Budde, R.J.A. and Chollet, R. (1986) *Plant Physiol.* 82, 1107–1114.
- [14] Bradford, M.M. (1976) *Anal. Biochem.* 72, 248–254.
- [15] Hunter, T. (1991) *Methods Enzymol.* 200, 3–37.
- [16] Matsuoka, M., Ozeki, Y., Yamamoto, N., Hirano, H., Kano-Murakami, Y. and Tanaka, Y. (1988) *J. Biol. Chem.* 263, 11080–11083.
- [17] Wang, Y.-H., Duff, S.M.G., Lepiniec, L., Cretin, C., Sarath, G., Condon, S.A., Vidal, J., Gadal, P. and Chollet, R. (1992) *J. Biol. Chem.* 267, 16759–16762.
- [18] Herzberg, O., Chen, C.C.H., Kapadia, G., McGuire, M., Carroll, L.J., Noh, S.J. and Dunaway-Mariano, D. (1996) *Proc. Natl. Acad. Sci. USA* 93, 2652–2657.
- [19] Burnell, J.N. (1984) *Biochem. Biophys. Res. Commun.* 120, 559–565.
- [20] Furbank, R.T. and Taylor, W.C. (1995) *Plant Cell* 7, 797–807.