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Technical communication

Maize recombinant C_4-pyruvate,orthophosphate dikinase: Expression in *Escherichia coli*, partial purification, and characterization of the phosphorylatable protein

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Abstract

The gene for C_4-pyruvate,orthophosphate dikinase (PPDK) from maize (*Zea mays*) was cloned into an *Escherichia coli* expression vector and recombinant PPDK produced in *E. coli* cells. Recombinant enzyme was found to be expressed in high amounts (5.3 U purified enzyme-activity liter^{-1} of induced cells) as a predominantly soluble and active protein. Biochemical analysis of partially purified recombinant PPDK showed this enzyme to be equivalent to enzyme extracted from illuminated maize leaves with respect to (i) molecular mass, (ii) specific activity, (iii) substrate requirements, and (iv) phosphorylation/inactivation by its bifunctional regulatory protein.

Abbreviations: DTT – dithiothreitol; FPLC – fast-protein liquid chromatography; HAP – hydroxyapatite; IPTG – isopropyl-β-thiogalactoside; MOPS – 3-(*N*-morpholino)propanesulfonic acid; PCR – polymerase chain reaction; PEP – phospho*enol*pyruvate; PMSF – phenylmethylsulfonyl fluoride; PPDK – pyruvate,orthophosphate dikinase; RP – regulatory protein

Introduction

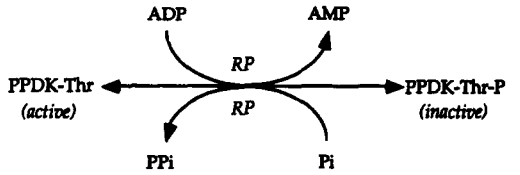
A key regulatory enzyme of the C_4-photosynthetic pathway of higher plants is pyruvate, orthophosphate dikinase (PPDK; EC 2.7.9.1) (Burnell and Hatch 1984; Hatch 1987; Ashton et al. 1990). In C_4 leaves, PPDK is localized predominantly in the chloroplast stroma of mesophyll cells. It has a cardinal role in the C_4 metabolic pathway as it catalyzes the rate-limiting regeneration of the primary CO_2 acceptor PEP from pyruvate and ATP/Pi:

$$\text{Pyruvate} + \text{ATP} + \text{Pi} \overset{\text{PPDK}}{\longleftrightarrow} \text{PEP} + \text{AMP} + \text{PPi}$$

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 from ATP, forming PPDK-His$P^{\beta}P^{\gamma}$ 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, forming PEP and free enzyme (Carroll et al. 1990; Pocalyko et al. 1990).

In addition to catalysis, PPDK, as a pivotal enzyme in the C_4 pathway, undergoes diurnal light-dark regulation of activity. The HisP 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) (Burnell 1984; Burnell and Hatch 1984; Hatch 1987; Roeske et al. 1988; Ashton et al. 1990). Conversely, the free His form of this inactive PPDK is preferentially activated in the light by a phosphorolytic dephosphorylation of the target Thr-P group. This *in vivo* light/dark regulation is mediated by a single bifunctional regulatory protein

(RP) which represents a most unusual form of protein kinase/phosphatase because it utilizes ADP in place of ATP as the phosphoryl donor and requires Pi for dephosphorylation (Burnell and Hatch 1984; Ashton et al. 1990):



The *pdk* gene from the nonphotosynthetic bacterium *Clostridium symbiosum* has previously been cloned into an *Escherichia coli* protein expression vector and active, recombinant PPDK enzyme produced in *E. coli* (Pocalyko et al. 1990). This has allowed site-directed and deletion mutagenesis of the gene for studies concerning specific details of the three-step catalytic reaction mechanism as related to protein structure (Xu et al. 1995a,b; Yankie et al. 1995). The maize dikinase shares a high degree of sequence homology with the *C. symbiosum* enzyme, including an identical active site (Pocalyko et al. 1990):

Maize	452 RGGMT^{456}SH^{458}AAVVAR 464
C. symbiosum	449 RGGMT^{453}SH^{455}AAVVAR 461

Interestingly, the bacterial PPDK, although possessing the same active-site Thr (Thr-453 in *C. symbiosum*), does not undergo regulatory phosphorylation at this residue (Pocalyko et al. 1990). Moreover, the bacterial enzyme is not inactivated/phosphorylated *in vitro* by exogenous maize RP (Burnell 1984; C.M. Smith and R. Chollet, unpublished). Another notable difference, despite the high degree of primary-structure homology, is that maize PPDK is active as a homotetramer while the bacterial enzyme is active as a homodimer of ≈ 95-kDa subunits.

Our knowledge concerning higher plant PPDK has relied solely on enzyme extracted from leaves of C_4 plants. A greater understanding of enzyme regulation and catalysis as related to protein structure requires that the plant dikinase be amenable to mutagenesis. This can only be accomplished by producing the active leaf enzyme as a recombinant form, e.g. in *E. coli.* Moreover, enzyme produced in *E. coli* would presumably consist wholly of the free Thr, active form (non-phosphorylated form) primarily due to the likely absence of RP in bacteria, a highly specific stromal protein kinase for the PPDK active-site Thr (Burnell 1984; C.M. Smith and R. Chollet, unpublished). On the other hand, enzyme extracted from leaves contains both Thr-P and free Thr forms of PPDK. The presence of both forms in leaves is presumably due to a varying activity-ratio of the bifunctional RP *in vivo*. A recent report has shown that PPDK from three plant species, maize, *Flaveria bidentis*, and *F. brownii*, can be produced in recombinant form in *E. coli* cells (Usami et al. 1995). However, analysis of this recombinant enzyme was limited to measurement of activity in crude cell lysates. No further characterization beyond interspecific comparisons of enzyme activity with respect to cold stability was performed. Therefore, in order to perform further in-depth biochemical analyses of the recombinant C_4 enzyme, including site-directed mutagenesis studies, we have cloned and expressed the C_4 form of maize PPDK in *E. coli*, subjected it to extensive purification, and compared its activity and regulation to the authentic leaf enzyme. We report here that this recombinant enzyme is equivalent in its activity and regulatory properties to PPDK isolated from illuminated maize leaves.

Materials and methods

Construction of a maize PPDK E. coli *expression vector*

The full length maize cDNA clone corresponding to the C_4 *pdk* gene was previously cloned and inserted into pUC 12 as a *Bam* HI fragment (Sheen 1991). The open reading frame corresponding to the mature, ≈95-kDa PPDK polypeptide was assembled using this cDNA clone by first installing an in-frame translation start site (ATG) just before the N-terminal Thr of the mature polypeptide (Figure 1) (Matsuoka 1990). This was accomplished using PCR mutagenesis; the 33-mer 5′-PCR mutagenic primer contained an in-frame ATG as an *Nde* I site at the N-terminus of the mature polypeptide (Matsuoka 1990):

(GCGCCGCATATGACG/ACC/AAA/AAG/AGG/GTG/TTC)
Nde I Thr Thr Lys Lys Arg Val Phe

The 31-mer 3′-PCR primer spanned the unique *Sst* I site 549 bp downstream from the first Thr codon of the mature coding sequence:

(C/AAA/GAG/CTC/GTG/GGT/CAG/TAC/AAG/GAG/GTC)
Sst I

The resulting 585 bp PCR fragment, generated using the maize cDNA clone as template, was cloned into a pNoTA/T7 cloning vector (5 Prime-3 Prime, Inc.) and subsequently released as a 547 bp *Nde*I -*Sst* I fragment. This fragment was ligated with a 2327 bp *Sst* I-*Bam* HI fragment, derived from the maize *pdk* cDNA clone, to form an open reading frame corresponding to the mature polypeptide. This latter fragment contained the adjoining 3′-*pdk* sequence, including the C-terminal end of the polypeptide. The resulting 2874 bp fragment was inserted into an *E. coli* expression vector as an *Nde* I/*Bam* HI fragment (pET 11a, Novagen, Inc.) and subsequently transformed into *E. coli* BL21 (DE3) cells. This vector was labeled as pET 11a/*pdk* (Figure 1).

Partial purification of maize recombinant and leaf PPDK

Four liters of LB medium containing 50 μg/ml ampicillin was inoculated with 0.5 ml of an overnight culture of *E. coli* BL21 (DE3) cells previously transformed with the pET 11a/*pdk* construct. The culture was grown at 37 °C on an orbital shaker until an A_{600nm} of 0.6 was attained. Induction of recombinant PPDK synthesis was then initiated by addition of 1 mM IPTG and growth continued for another 3 h. The cells were then collected by centrifugation, frozen in liquid N_2, and stored for up to 7 d at –80 °C. An extract was prepared by first thawing the cells in extraction buffer (100 mM Tris-HCl, pH 8.0, 10 mM $MgSO_4$, 1 mM EDTA, 2 mM K-Pi, 5 mM pyruvate, 14 mM 2-mercaptoethanol) containing the following proteinase inhibitors: 1 mM fresh PMSF, 1 mM benzamidine-HCl, 50 mg/ml soybean-trypsin inhibitor, 1 mM 1,10-phenanthroline. All subsequent steps, including FPLC, were performed at room temperature in order to preclude cold-inactivation/dissociation of the active homotetramer (Ashton et al. 1990; Usami et al. 1995). Cells were lysed via 3 passes through a French press. The cell lysate was clarified by ultracentrifugation (100000 *g*, 30 min) and immediately subjected to $(NH_4)_2SO_4$ fractionation, with the 40–50% saturation precipitate collected for further purification. This fraction was resuspended in HAP buffer (50 mM bis-tris-propane, pH 7.0, 5 mM $MgSO_4$, 0.5 mM EDTA, 1 mM pyruvate, 10 mM K-Pi, 14 mM 2-mercaptoethanol, 1 mM PMSF, 5% [v/v] glycerol) plus the other proteinase inhibitors indicated above, and desalted on a PD-10 column (Pharmacia) pre-equilibrated with this same buffer. The desalted extract was then purified using a combination of HAP and FPLC chromatography as described previously for the maize-leaf enzyme (Carroll et al. 1990) with the following modifications. Briefly, the desalted sample was applied to a 2.5 × 10 cm column of HAP [Bio-Gel HTP (Bio-Rad)]. The column was then washed with 80 ml of HAP buffer and the PPDK-containing fractions step-eluted with 50 mM K-Pi in this same buffer. Peak PPDK activity-fractions were pooled, diluted 2-fold with Mono-Q buffer (50 mM MOPS-KOH, pH 7.3, 5 mM $MgSO_4$, 0.1 mM EDTA, 14 mM 2-mercaptoethanol, 5% [v/v] glycerol) and loaded directly onto a prepacked Mono-Q FPLC anion-exchange column (Pharmacia). The 0.5 × 5 cm column was washed with this same buffer and the PPDK fractions eluted with a linear gradient of 1 M KCl in Mono-Q buffer. Peak PPDK activity-fractions were pooled, diluted 3-fold in Mono-Q buffer, and directly re-chromatographed on the same column. The peak PPDK fractions from this second separation were again pooled and protein precipitated by addition of $(NH_4)_2SO_4$ to 70% saturation. The precipitate was collected by centrifugation and the well-drained pellets stored at –80 °C under N_2 for up to 2 weeks. 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 microdialyzed for 1 h against this same buffer at room temperature.

Maize leaf PPDK was purified using the same protocol as above with the following modifications: 25 g of greenhouse-grown/high-light illuminated (7 h) maize leaves were harvested directly into liquid N_2. The frozen leaf tissue (minus the liquid N_2) was then placed in a Waring blender and homogenized in 125 ml extraction buffer (plus 2% [w/v] insoluble polyvinylpolypyrrolidone and 1 mM fresh PMSF) for 1 min. The homogenate was filtered through 2 layers of cheesecloth and clarified by high-speed centrifugation. The centrifiuged extract was then subjected to the same purification protocol as outlined above.

PPDK and protein assays

PPDK activity was assayed spectrophotometrically at 30 °C in the PEP-forming direction (Ashton et al. 1990; Smith et al. 1994b). Prior to assay, the dialyzed enzyme was preincubated at 30 °C for 10 min to ensure full heat-reactivation. PPDK immunoblots were performed using standard methods with rabbit polyclonal antibodies raised against the maize-leaf PPDK monomer (Budde and Chollet 1986). Protein was quantitated by

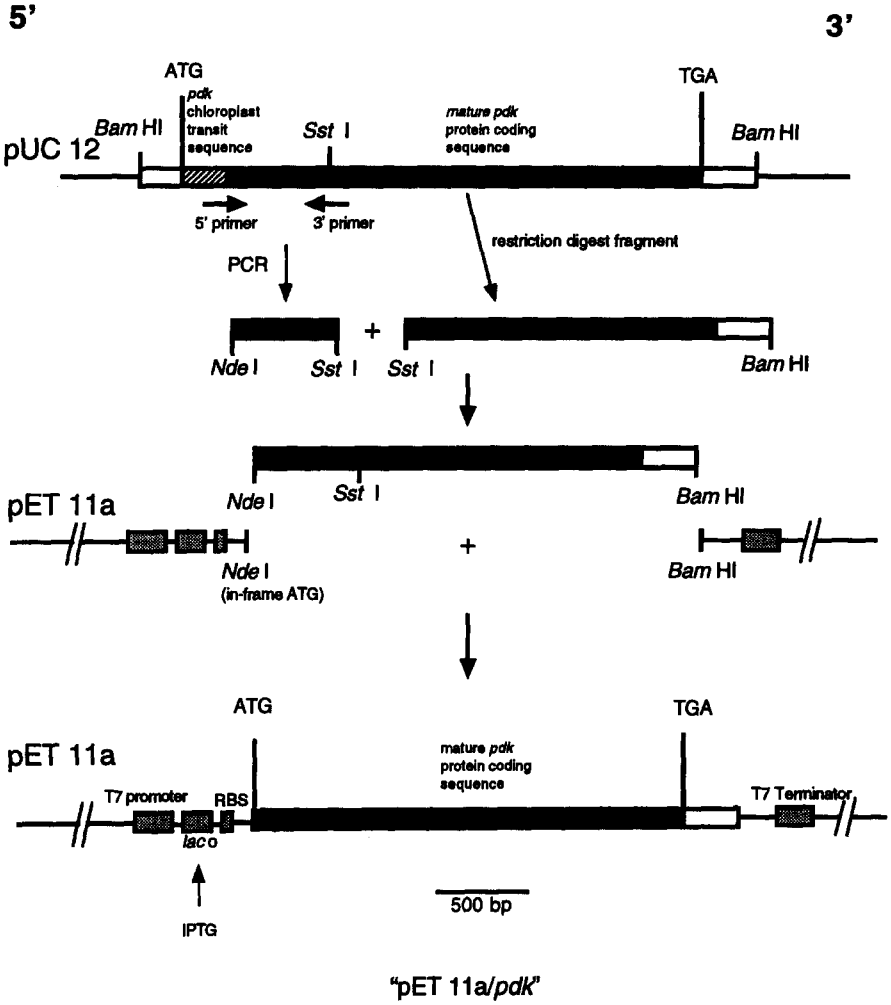


Figure 1. Subcloning of the mature C_4 *pdk* protein coding sequence from maize into the *E. coli* protein expression vector, pET 11a. An ATG translational start codon was introduced just before the N-terminal Thr of the mature polypeptide using PCR mutagenesis. Expression of *pdk* message from the bacteriophage T7 promoter was induced by removal of the *lac* repressor from the *lac* operator (*lac* o) by IPTG. TGA, native translational stop codon. RBS, *E. coli* ribosomal binding sequence.

a sensitive dye-binding method (Bradford 1976) with crystalline bovine serum albumin as standard.

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

RP was partially purified from maize leaves as described previously (Roeske and Chollet 1987; Smith et al. 1994a), but only through chromatography on Blue Sepharose CL-6B (Pharmacia). Inactivation of active PPDK by RP was accomplished by preincubating partially purified PPDK with RP and ADP (plus ATP/Pi) as previously described (Roeske and Chollet 1987; Smith et al. 1994b). RP-catalyzed ^{32}P-phosphorylation of PPDK was carried out in a two-stage, single-tube procedure as previously outlined (Smith et al. 1994b). Briefly, in the first stage, [β-^{32}P]ADP was generated from [γ-^{32}P]ATP, AMP and exogenous adenylate kinase. This was followed by immediate addition of partially purified PPDK and RP to the reaction tube. The phosphorylation reaction was then incubated for 1 h at 30 °C and terminated by addition of SDS-PAGE sample buffer and heating to 100 °C for 2 min. Aliquots of the quenched reaction mixture were electrophoresed on 12% SDS-PAGE gels and the gels transferred to filter paper and dried. The ^{32}P-radiolabel associated with the ≈95-kDa PPDK polypeptide was detected by phosphorimager analysis. When 'rapid' maize-leaf extracts

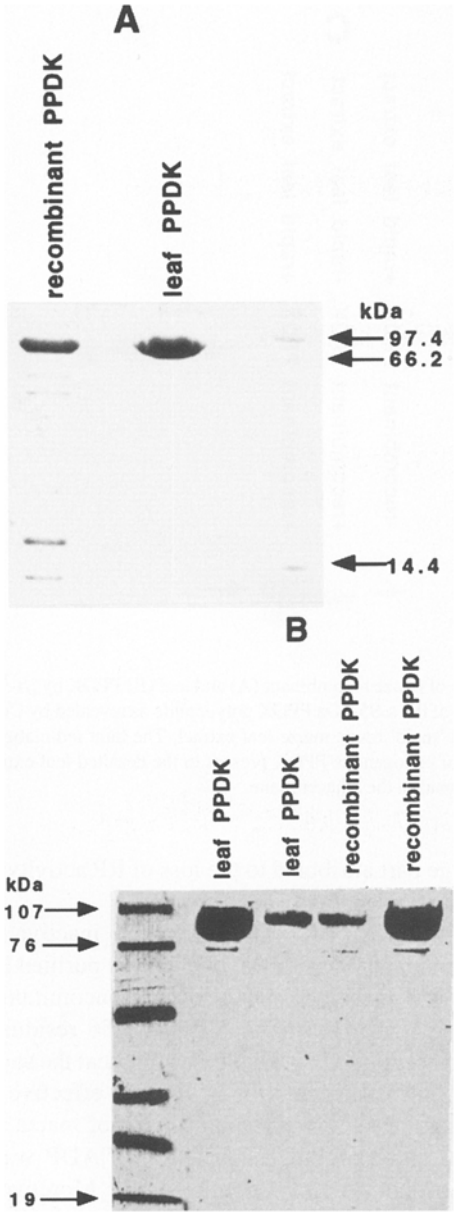


Figure 2. SDS-PAGE and immunoblot analysis of partially purified PPDK. (A) Coomassie blue-stained 12% SDS-PAGE gel of partially purified maize recombinant and leaf PPDK. The first two lanes represent 5 μg protein. (B) Immunoblot analysis of partially purified maize recombinant and leaf PPDK probed with maize-leaf PPDK antiserum. Lanes from left to right are 10 and 2 μg leaf protein and 3 and 15 μg recombinant protein, respectively. Unlabeled lanes are molecular-mass standards.

were used as the source of crude RP for the above phosphorylation assay, they were prepared as previously described from dark-adapted tissue (Smith et al. 1994b).

Results and discussion

Expression of recombinant maize PPDK in E. coli *and its partial purification and characterization*

Active PPDK produced in *E. coli* was subjected to a purification protocol modified from that developed previously for the maize leaf enzyme (Carroll et al. 1990). Recombinant maize PPDK purified in this manner produced a final yield of 5.3 units (μmol min^{-1}) of soluble PPDK activity per liter of induced cells. The protein was essentially non-degraded and of the same monomeric molecular mass (≈95 kDa) as purified leaf PPDK as evidenced by Coomassie blue-stained SDS-PAGE gels (Figure 2A) and Western blot analysis (Figure 2B). However, some contaminating *E. coli* polypeptides remained in the recombinant PPDK preparation (Figure 2A). Although the amount of contaminating protein was not quantitated, the modestly lower specific activity of the recombinant PPDK versus the leaf enzyme (3.1 and 5.3 μmol min^{-1} mg^{-1} protein, respectively) was likely attributable to this extraneous protein. Native C_4-leaf PPDK is fully active as a homotetramer (Hatch 1987; Ashton et al. 1990). Similarly, non-denaturing PAGE analysis suggested that the active recombinant enzyme was present largely as the tetramer based on its equal mobility relative to maize leaf PPDK in a 6% polyacrylamide gel (data not shown).

Overall catalysis by PPDK in the PEP-forming direction has a strict substrate requirement for pyruvate, Pi, and ATP (see 'Introduction'). This forward reaction is unable to proceed when any of these substrates are omitted from the reaction mixture (Hatch 1987; Ashton et al. 1990). The partially purified recombinant maize PPDK revealed the same strict substrate requirements in that no activity was detected when ATP or pyruvate was omitted from the assay medium. A very limited amount of activity was evident when exogenous Pi was deleted (about 6% of the complete assay). This is likely due to traces of Pi carried into the reaction medium as a contaminant of the ATP since the leaf enzyme also showed a similar trace of activity in the absence of exogenous Pi.

Regulatory phosphorylation of recombinant PPDK by maize-leaf RP

Maize-leaf PPDK is inactivated when Thr-456 is specifically phosphorylated by its bifunctional RP (Burnell 1984; Burnell and Hatch 1984; Hatch 1987;

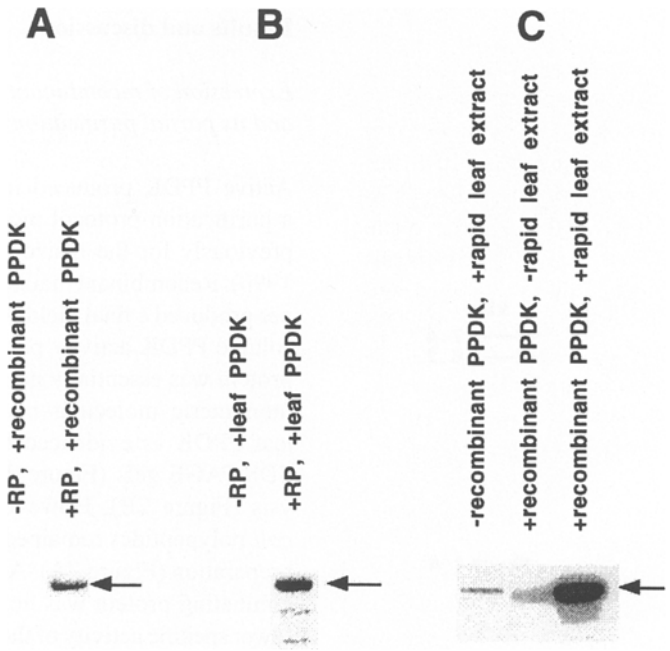


Figure 3. Phosphorimager analysis of RP-catalyzed phosphorylation (inactivation) of maize recombinant (A) and leaf (B) PPDK by [β-^{32}P]ADP and a partially purified preparation of maize-leaf RP. The arrows mark the position of the ≈95-kDa PPDK polypeptide as revealed by Coomassie blue-staining of the same gel. (C) Same as panel A except the source of RP was a 'rapid' crude maize-leaf extract. The faint radiolabeled band in the + rapid leaf extract/– recombinant PPDK lane represents phosphorylation of endogenous PPDK present in the desalted leaf extract. The middle lane in panel C shows a faint pseudo-band due to trailing of the intense signal in the adjacent lane.

Roeske et al. 1988; Ashton et al. 1990; Smith et al. 1994b). In *E. coli*, stromal RP is absent and therefore the recombinant protein would be expected to be present wholly in its active, non-phosphorylated form. To assess the extent of *in vitro* regulation of recombinant PPDK by leaf RP, the recombinant enzyme was compared to the active, predominantly dephosphorylated form of leaf PPDK with respect to the ability of RP to (i) inactivate the enzyme *in vitro*, and (ii) phosphorylate the enzyme *in vitro* using [β-^{32}P]ADP as substrate. When recombinant PPDK was incubated for 30 min at 30 °C with partially purified maize-leaf RP and ADP (plus ATP/Pi), activity was 62% of the minus-ADP control (control activity = 3.7 μmol min^{-1} mg^{-1} protein). Results were similar for the maize leaf enzyme, where activity was 68% of the corresponding control after 30 min of incubation with RP and ADP (control activity = 5.4 μmol min^{-1} mg^{-1} protein). It should be noted that experiments utilizing partially purified RP are complicated by its extreme instability during isolation and storage (Ashton et al. 1990; Smith et al. 1994a). Therefore, the incomplete inactivation of active PPDK in these *in vitro* experiments is in large part attributed to the loss of RP activity during purification, storage, and assay.

The observed ADP-dependent inactivation of recombinant maize PPDK by partially purified RP (see above) is presumably due to the concomitant phosphorylation of the regulatory Thr-456 residue. This view is supported by the observation that the same partially purified preparation of RP was effective in catalyzing the *in vitro* phosphorylation of maize recombinant and leaf PPDK when [β-^{32}P]ADP was used as phosphoryl donor (Figure 3A, B). Moreover, ^{32}P-incorporation into the recombinant PPDK monomer occurred to the same relative extent as with the leaf enzyme (dpm μg^{-1} protein; data not shown). The recombinant enzyme was also strongly and specifically radiolabeled *in vitro* with ^{32}P when the source of RP for the ADP-dependent phosphorylation reaction was a 'rapid' leaf extract (Figure 3C). Altogether, the above results indicate that the recombinant maize enzyme is in an active, soluble form and amenable to *in vitro* inactivation/phosphorylation by leaf RP in a similar manner as authentic leaf PPDK.

Concluding remarks

We have demonstrated the equivalency of recombinant maize PPDK to the homologous leaf enzyme. Producing an active, higher-plant recombinant C_4 PPDK has obvious advantages in terms of experimental manipulation. First, precise studies concerning active-site residues and structural domains involved in catalysis will now be possible by exploiting site-directed and deletion mutagenesis of the cloned gene (Xu et al. 1995 a,b; Yankie et al. 1995). Second, genetically engineering the protein for enhancing the photosynthetic properties of maize leaves should also be possible (Furbank and Taylor 1995); for example, expressing the protein in C_4 leaves without a regulatory Thr-phosphorylation site or decreasing the cold sensitivity of the enzyme *in vivo* (Usami et al. 1995). Finally, we believe the most significant advance a recombinant higher plant PPDK system will offer will be the use of recombinant mutant enzymes for obtaining a more complete understanding of how the unique bifunctional regulatory protein interacts with this target protein to so dramatically alter its catalytic activity.

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