Regulatory phosphorylation of plant phospho*enol*pyruvate carboxylase: role of a conserved basic residue upstream of the phosphorylation site

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Abstract In order to mimic regulatory phosphorylation of the Ser-15 of maize C_4-form phospho*enol*pyruvate carboxylase (PEPC), we replaced Ser-15 and Lys-12 with Asp (S15D) and Asn (K12N), respectively, by site-directed mutagenesis. Although both mutant enzymes were catalytically as active as the wild-type PEPC, they showed much less sensitivity to malate, an allosteric inhibitor, similarly to the phosphorylated wild-type PEPC. A maize protein kinase of 30 kDa which is known to be specific to PEPC (PEPC-PK), phosphorylated K12N as well as the wild-type PEPC but not S15D. The phosphorylation of K12N further diminished the sensitivity to malate. Thus, a positive charge of the conserved Lys-12 is not required for the recognition by PEPC-PK but contributes to the intrinsic sensitivity to malate inhibition.

Key words: Phospho*enol*pyruvate carboxylase; Regulatory phosphorylation; Site-directed mutagenesis; Protein kinase; *Zea mays*

1. Introduction

In C_4 plants such as maize and sorghum, phospho*enol*pyruvate carboxylase (PEPC, EC 4.1.1.31) plays a key role in photosynthetic CO_2 fixation [1]. The enzyme fixes HCO_3^- on phospho*enol*pyruvate to form oxaloacetate and orthophosphate. The PEPCs of maize and sorghum consist of four identical subunits of 970 and 952 amino acid residues, respectively [2,3]. It is now well established that the sensitivity of PEPC to malate, decreases under light conditions through phosphorylation of Ser in the N-terminal domain [1,4,5]. This regulatory phosphorylation was mimicked by substitution of Ser-8 to Asp in sorghum PEPC [6,7] or by S-carboxymethylation of Cys introduced in place of Ser-8 [8]. The regulatory phosphorylation seems to occur ubiquitously in higher plant PEPCs [1], since the residues around the phosphorylation site (underlined) are well conserved as follows [9]; (Asp/Glu)-(Lys/Arg)-X-X-Ser-Ile-Asp-Ala-Gln-(Leu/Met)-Arg [1,9,10]. Among these residues the role of Lys or Arg at the 3rd position upstream of Ser is of particular interest, since many protein kinases (PKs) such as cyclic AMP-dependent PK (PK-A) [11] and calcium-dependent/calmodulin-independent PK (CDPK) [12] require the basic residue for recognition of Ser/Thr to be phosphorylated. It seems worthwhile to investigate whether this is also the case with PK for PEPC. Furthermore, it is interesting to examine whether a negative charge shift not only at the phosphorylatable Ser but also at nearby amino acids causes similar modification of enzyme properties. In the present study, we prepared recombinant mutant PEPCs of maize, in which Ser-15 and Lys-12 are individually replaced to Asp and Asn, respectively. We investigated their kinetic properties and the occurrence of phosphorylation by maize PK. For the plant PKs involved in the regulatory phosphorylation (PEPC-PKs), Wang and Chollet [13,14] reported that the major PK is calcium-independent and about 30 kDa in size. In addition we pointed out the possible involvement of a CDPK [15]. Since the former PK is much easier to detect and purify than the latter, we used the PK preparation of Wang and Chollet [13] in the present study.

2. Materials and methods

2.1. Preparation of recombinant wild-type and mutant PEPCs

The entire coding sequence of maize C_4-form PEPC was cut out of pKM10 [16] by digestion with *Nco*I (partial) and *Hin*dIII, and inserted into the pTV119N vector (Takara). The resulting expression plasmid was designated as pTM94. Site-directed mutagenesis was carried out according to the method of Deng and Nickoloff [17] using a Transformer mutagenesis kit (Clontech). The sequences of oligonucleotides for mutagenesis are given in Fig. 1. The selection primer to change the unique restriction site from *Hin*dIII to *Mlu*I had the following sequence, 5′-CAGGCATGCACGCGTGGCTATGCA-3′. After the confirmation of the desired mutations by DNA sequencing, the intact coding region of pTM94 downstream from *Nru*I (2.9 kb) was cut out with *Nru*I and *Xba*I, and inserted to each mutant plasmid from which the corresponding segment had been removed.

The cells of *Escherichia coli* F15, a *recA* variant of the *ppc* deletion mutant [18], which had been transformed with pTM94 or mutant plasmids encoding S15D and K12N, were grown with shaking at 37°C in Terrific broth supplemented with ampicillin. When OD_{660} of the cultures reached 0.6–0.8, isopropyl-1-thio-β-D-galactopyranoside was added to a final concentration of 1 mM and they were incubated at 30°C for further 6 h with shaking for the induction of recombinant PEPC. The harvested cells were disrupted with a French pressure cell in the presence of 20% glycerol, 0.1 M Tris-HCl (pH 7.4), 1 mM dithiothreitol (DTT) and 1 mM phenylmethylsulfonyl fluoride (PMSF). PEPCs were purified about 470-fold with yields of around 40%, essentially according to [19]. The purity of each preparation was about 90% as judged by SDS-PAGE [20]. Protein was determined by the dye-binding method [21] using bovine serum albumin as a standard.

2.2. Assay of PEPC activity

The enzyme activity was assayed at nearly physiological pH, pH 7.4, and low concentrations of bicarbonate, since the malate inhibition of maize PEPC is more obvious under these conditions than at the optimum pH, pH 8.0 [19,22,23]. The standard reaction mixture contained, in a total volume of 1.0 ml, 0.1 M HEPES-NaOH (pH 7.4), 1 mM $KHCO_3$, 10 mM $MgSO_4$, 2 mM phospho*enol*pyruvate, 0.2 mM

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Abbreviations: CDPK, calcium-dependent/calmodulin-independent protein kinase; DTT, dithiothreitol; $I_{0.5}$, the concentration of inhibitor required for 50% inhibition; PEPC, phospho*enol*pyruvate carboxylase; PK, protein kinase; PK-A, cyclic AMP-dependent protein kinase; PMSF, phenylmethylsulfonyl fluoride; K12N and S15D, mutant maize PEPCs in which Lys-12 and Ser-15 are replaced by Asn and Asp, respectively; WT, wild-type maize C_4-form PEPC

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    A   P   G   P   G   E   K   H   H   S15  I   D   A   Q
5'-GCA CCC GGC CCT GGC GAG AAG CAC CAC TCC ATC GAC GCG CAG-3'   pTM94
                          N
3'GT GGG CCC GGA CCG CTC TTG GTG GTG AG 5'                      K12N primer
                                  D
                3'CTC TTC GTG GTG CTG TAG CTG CGC 5'            S15D primer
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Fig. 1. Oligonucleotides used as 'mutation primers' for site-directed mutagenesis. They were antisense as to the coding strand. The mutated nucleotides are given in bold-face. The deduced amino acid sequence at the N-terminal domain of maize C_4-form PEPC and nucleotide sequence of the wild-type plasmid is also shown. Ser-15 is the regulatory phosphorylation site of maize C_4-form PEPC.

NADH, 1.5 IU malate dehydrogenase, and the enzyme (0.5–2 μg). The activity was measured spectrophotometrically at 340 nm at 30°C.

2.3. Preparation of maize PEPC-PK and phosphorylation of PEPC

Mature leaves were harvested at 11:00 a.m. from 6 week old maize plants (*Zea mays* L. strain H84) grown in the field. Minced leaves (30 g fresh weight) which were suspended in 5 vol. of an extraction buffer (0.1 M Tris-HCl buffer (pH 8.0), 5% (v/v) glycerol, 14 mM 2-mercaptoethanol, 5 mM benzamidine-HCl, 1 mM PMSF and 5% (w/v) insoluble polyvinylpolypyrrolidone) were disrupted with a Polytron homogenizer (Kinematika). The homogenate was filtered through a layer of nylon mesh (100 μm), and then centrifuged at 6000×*g* for 20 min. The supernatant was fractionated with 35–60% $(NH_4)_2SO_4$. A portion of this sample (about 8 mg protein) was suspended in buffer A (25 mM Tris-HCl (pH 7.4), 0.1 M KCl, 1 mM benzamidine-HCl, 5% (v/v) glycerol and 5 mM DTT), and centrifuged at 100000×*g* for 30 min. The resulting supernatant was subjected to gel-permeation column chromatography using a Superose 12 HR 10/30 column (Pharmacia) preequilibrated with buffer A. Each fraction (0.5 ml) of the eluate was assayed for the PEPC-phosphorylating activity. The most active fraction (containing about 30 kDa proteins) was concentrated by microcon-10 (amicon), and used as the enzyme.

The incorporation of ^{32}P into wild-type and mutant PEPCs was performed in the reaction mixture containing, in a total volume of 15 μl, 20 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 1 mM EGTA, 0.1 mM (1 μCi) [γ-^{32}P]ATP, purified PEPC (2 μg) and the maize leaf PEPC-PK (5 μg protein). After incubation at 30°C for 15 min, the reaction was terminated by the addition of 15 μl of a double-strength sample buffer for SDS-PAGE supplemented with 50 mM EDTA. The sample was subjected to SDS-PAGE [20] and the gel was stained with Coomassie Brilliant Blue and ^{32}P-incorporation into PEPC was detected with a Bio-imaging analyzer BAS-2000 (Fuji) or by autoradiography.

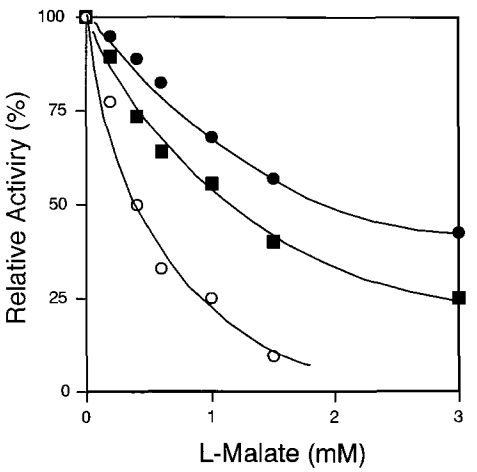

Fig. 2. Effects of increasing concentrations of malate on the activities of WT (open circle), K12N (closed square) and S15D (closed circle) enzymes. Assays were carried out using the standard reaction mixture at pH 7.4 with 1 mM $KHCO_3$ as described in Section 2. The activity of each PEPC in the absence of malate was about 15 μmol/min/mg.

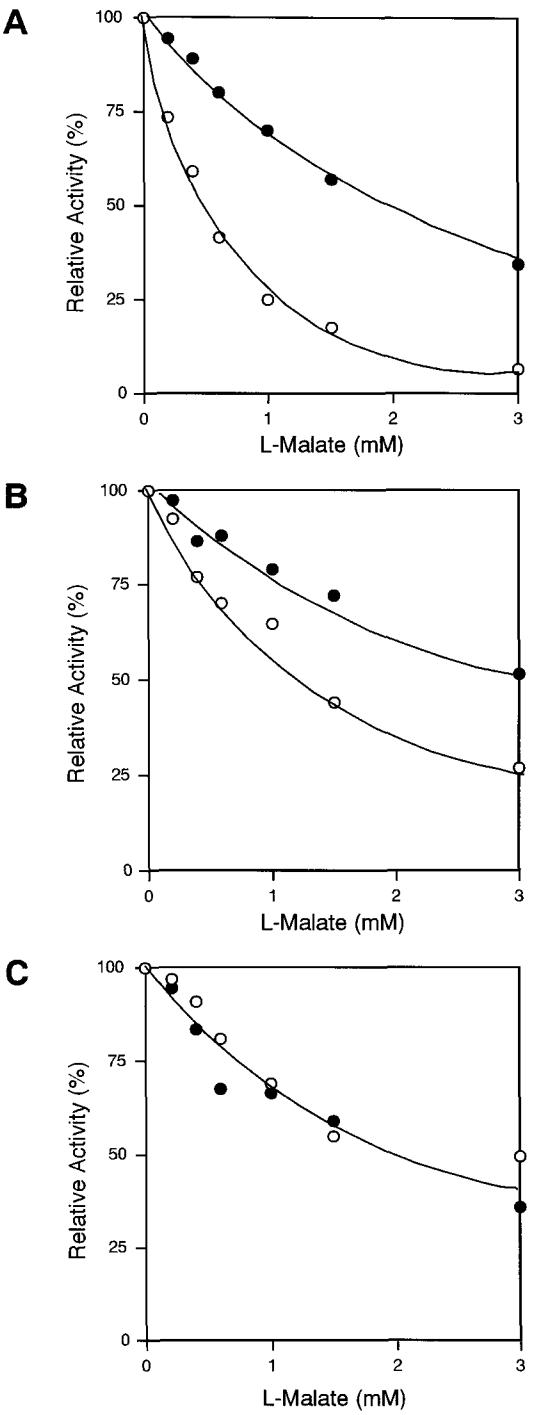

Fig. 3. Effects of phosphorylation by maize PEPC-PK on malate sensitivity of WT (A), K12N (B) and S15D (C) enzymes. Each PEPC was incubated in a reaction mixture with (closed circle) or without (open circle) maize leaf PEPC-PK at 30°C for 3 h. The complete reaction mixture comprised, in 20 μl, 25 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 1 mM DTT, 15% (v/v) ethylene glycol, 0.2 mM ATP, PEPC (20 μg) and partially purified maize leaf PEPC-PK (8 μg protein). After the reaction, aliquots (5 μl) were assayed for the malate sensitivity as described in Section 2. The activities in the absence of malate were 18, 18 and 17 μmol/min/mg for phosphorylated WT, K12N and S15D, respectively.

3. Results and discussion

The specific activity of PEPC in the crude cell extract of the

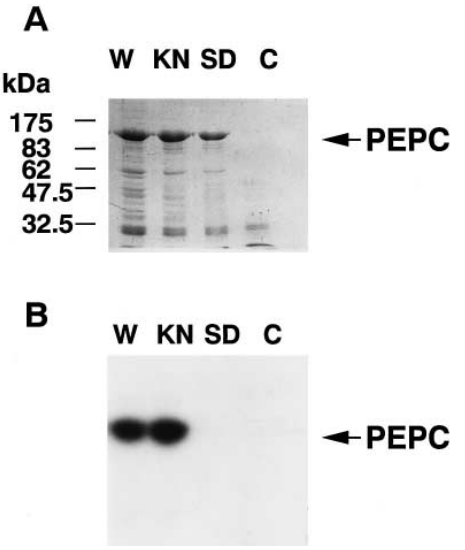

Fig. 4. ^{32}P incorporation into PEPCs by maize leaf PEPC-PK. Each recombinant PEPC was incubated in the reaction mixture containing maize leaf PEPC-PK (5 μg protein) at 30°C for 1 h. The reaction mixture not containing PEPC was also incubated as a control. After the incubation, each reaction mixture was subjected to SDS-PAGE. Lanes loaded with the reaction mixtures containing WT, K12N, S15D and no PEPC are denoted as W, KN, SD and C, respectively. The gel was stained by Coomassie Brilliant Blue (A) and the incorporation of ^{32}P was detected by autoradiography (B).

E. coli transformed with the newly constructed plasmid pTM94 was about 5-fold higher than that of pKM10 [16] (data not shown). The improvement was provided by the use of a high copy number expression vector pTV119N instead of pKK233-2. Recombinant wild-type (WT) PEPC and mutant enzymes, S15D and K12N, were purified and their kinetic properties were investigated. The maximum velocities attainable at infinite concentrations of PEP were almost equal to one another, being about 45 μmol/min/mg at pH 8.0. The half-saturation concentrations of PEP were 1.1, 0.9 and 1.0 mM for WT, S15D and K12N, respectively at pH 7.4. The maximum extents of activation by glucose 6-phosphate, an allosteric activator, were all about 2.5-fold, and half-saturation concentrations were 2.1, 1.3 and 2.1 mM, in the order as above. In contrast, marked variations in the sensitivity to malate, an allosteric inhibitor, were observed as shown in Fig. 2. The concentrations required for 50% inhibition ($I_{0.5}$) were 0.4, 2.0 and 1.2 mM for WT, S15D and K12N, respectively. The data with S15D confirm the previous observation by Wang et al. [6] with S8D of sorghum PEPC. Moreover, the present finding with K12N indicates that the conserved basic residue at −3 position of Ser contributes to the malate-sensitivity as well.

After the three PEPCs were subjected to the phosphorylation reaction with the PEPC-PK which was reported to be solely involved in the regulatory phosphorylation [1], their malate-sensitivity was determined. As shown in Fig. 3, $I_{0.5}$ values for WT and K12N increased to 2.0 and 3.0 mM, respectively, while no change was detected for S15D due to the absence of Ser for the regulatory phosphorylation.

The phosphorylation of PEPCs was also examined by ^{32}P incorporation as shown in Fig. 4. When PEPCs were incubated with the PEPC-PK, strong incorporation was observed with WT but no incorporation with S15D, indicating the specificity of the protein kinase as previously reported by Wang et al. [6] with sorghum PEPC. It is noteworthy that almost the same amount of ^{32}P as WT was incorporated into K12N. This indicates that Lys-12 is not indispensable for the recognition of this protein kinase, though the motif -Lys/Arg-X-X-Ser- is required for many protein kinases [11]. Thus the question arises why the basic residue at −3 position is so strictly conserved among all plant PEPCs. We presume that the residue might be required for the fine adjustment of malate-sensitivity, or for the recognition by another protein kinase which is supplementarily contributed to the regulatory phosphorylation.

It was unequivocally demonstrated that the introduction of a negative charge-shift to the N-terminal domain, which was provided by replacement of Lys-12 by Asn, Ser-15 by Asp, or Ser-15 by phosphorylation, made the enzyme less sensitive to malate. This was further supported by the least malate-sensitivity of K12N phosphorylated at Ser-15. Determination of the molecular mechanism of malate inhibition in detail must await elucidation of the three-dimensional structure of the enzyme.

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