Site-directed mutagenesis of Lys^{600} in phospho*enol*pyruvate carboxylase of *Flaveria trinervia*: its roles in catalytic and regulatory functions

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Abstract **Phospho*enol*pyruvate carboxylases from various organisms contain two conserved lysine residues. In the C_4 dicot *Flaveria trinervia*, one of these residues is Lys^{600}. Converting this Lys^{600} to Arg^{600} or Thr^{600} mainly increased the K_m values and but had minimal effect on the V_{max}. The K_m for PEP, Mg^{2+} increased by up to 3-fold in Arg^{600} and Thr^{600} but the K_m (HCO_3^-) increased 9-fold in Thr^{600}, suggesting that Lys^{600} might be associated with bicarbonate-binding. This lysine was not obligatory for enzyme activity although the wild-type protein showed higher activity at physiological pH and was less inhibited by malate than the two mutants.**

Key words: Phospho*enol*pyruvate carboxylase; Lysine; Substrate-binding site; *Flaveria trinervia*

1. Introduction

Phospho*enol*pyruvate carboxylase (EC 4.1.1.31; PEPC) catalyses the carboxylation of PEP in the presence of bicarbonate and Me^{2+} to form oxaloacetic acid and p_i. This enzyme is widely found in higher plants, algae and prokaryotes. In C_4 plants, it catalyses the initial CO_2 fixation reaction in the mesophyll cells and the fixed CO_2 is translocated in the form of dicarboxylic acid to the bundle sheath cells for decarboxylation with the released CO_2 entering Calvin cycle [1]. This mechanism raises CO_2 concentration in the bundle sheath cells and consequently minimises photorespiration during photosynthesis.

The native enzyme is a tetramer of four identical subunits each with a MW of ~100,000 Da [2]. It is allosteric in nature, and depending on the source of the enzyme, is activated by different chemicals or metabolites. For instance, PEPC from monocot C_4 is strongly activated by glycine and glucose-6-phosphate or triose-phosphate while PEPCs from dicot C_4 plants show little response to glycine [3]. The end product of the carboxylation reaction, i.e. malate or aspartate, generally acts as feedback inhibitors.

The cDNA-coding PEPC has been isolated from a variety of plants and other organisms, including maize, sorghum, *Flaveria trinervia*, *F. pringlii*, tobacco, soybean, *Mesembryanthemum crystallinum* (both CAM and C_3 forms) and prokaryotes, such as *Escherichia coli* [4,5 and references therein]. Alignment of the deduced amino acid sequences of the polypeptides from these organisms reveals some conserved amino acid residues or regions [4,5]. It would be reasonable to expect that the substrate-binding sites are associated with some of these conserved residues and, since the substrates are anions at neutral pH, it is likely that positively charged residues are involved in the binding sites. Chemical modifications indicate that 2 histidine [6], 2 arginine [7] and 4 lysine [8–10] residues per tetramer are essential for the enzyme function. Modifications of these residues, which may form a substrate (PEP) domain [11], result in enzyme inactivation. In vitro mutagenesis has identified two conserved histidine residues as the PEP-binding sites [12,13]. In this study, we have changed Lys^{600} in *F. trinervia*, a site previously thought to be involved in PEP-binding [14], to arginine or threonine by techniques of site-directed mutagenesis, and compared their kinetic and regulatory properties with the wild-type enzyme.

2. Materials and methods

2.1. Materials

PEP (tricyclohexylammonium salt) was supplied by Boehringer. NADH and malate dehydrogenase were from Sigma. All restrictive endonucleases, T_4 DNA ligase, T_4 polynucleotide kinase and the Altered Sites II in vitromutagenesis system were supplied by Promega. RF-purified oligonucleotides were purchased from the Macromolecular Resources (Colorado State University) and were phosphorylated in the laboratory. All other chemicals were AR grade.

2.2. Bacterial strains and plasmids

E. coli PCR1 was defective in the gene for PEPC, *ppc*, and therefore was used for the production of cloned PEPC. Repair minus strain SE1301 and JM109 were used in the first and second round of transformations after mutagenic reaction, respectively. Synthesis and cloning of PEPC of *F. trinervia* were done as described for maize [15]. The entire PEPC cDNA from leaves of the dicot *F. trinervia* was cloned into the open reading frame of plasmid pSI4001 which also carried an ampicillin-resistant gene [16]. Restriction mapping of the cDNA gave an identical map to the published PEPC cDNA sequence of this species [17]. Plasmid pALTER-1 (Promega), which carried a tetracycline-resistant gene and a defective ampicillin-resistant gene, was used for the subcloning and production of single-stranded DNA (ssDNA).

2.3. Site-directed mutagenesis

Mutagenesis was carried out as described by Promega. The 1.3-kb *SalI-Bam*HI fragment of the cDNA, which contained the Lys^{600} codon, was subcloned into the multicloning site of pALTER-1, transformed into JM109 and then infected with R408 helper phage to produce ssDNA for site-directed mutagenesis. The insert was inverted in pALTER-1 so that the ssDNA produced was the non-coding strand for this region. Thus, oligonucleotides CCTGCATCTCTTCCTGAATC and CCTGCATCTGTTCCTGAATC were designed to introduce arginine or threonine mutation at the Lys^{600} site, respectively. One of these mutagenic primers was used, together with a primer which restored the defective ampicillin-resistant gene of pALTER-1, to initiate the synthesis of the complementary strand. The reaction mixture was first transformed into ES1301 from which plasmids were prepared for a second transformation into JM109. The colonies growing on ampicillin (125 μg/ml) plates contained putative mutagenic clones and their plasmid DNA was purified by PEG precipitation [18] and subjected to direct double-stranded DNA-sequencing using fluorescence dye terminator

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Abbreviations: PEP, phospho*enol*pyruvate; PEPC, PEP carboxylase; K_i, the concentration of metabolites inhibiting enzyme activity by 50%.

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method. The efficiency of mutagenesis was over 60%. To confirm that no other nucleotide changes occurred in the *SalI-BamHI* region, this fragment was sequenced by the universal primers T7 and SP6. The mutated fragment was finally cloned back into pSI400l, giving rise to a full-length PEPC cDNA.

2.4. Growth of PCR1 cells

PCR1 cells complemented by wild or mutant PEPC were first grown in LB medium (containing 60 μg/ml ampicillin) overnight at 37°C, 300 rpm. 2 ml of this overnight culture was inoculated to 2 l of minimal medium (containing 60 μg/ml ampicillin and 0.1 mM IPTG). The composition of the minimal medium was the same as in [13] except that glycerol substituted glucose as the carbon source. The cells were cultured at 30°C, 250 rpm for ~22 h, spun down at 4°C and washed once in extraction buffer (100 mM Tris, pH 7.6, 10 mM $MgCl_2$, 5 mM DTT, 1 mM EDTA, 20% glycerol) for partial purification of PEPC.

2.5. Partial purification of PEPC

All steps were carried out on ice or at 4°C. The washed cells were resuspended in the extraction buffer and disrupted by ultrasonication (Branson Sonifier 450). The lysate was fractionated with 5–12% PEG-8000. The pellet was resuspended in buffer A for fast protein liquid chromatography (20 mM Tris, pH 8.0, 10% glycerol, 1 mM DTT, 0.1 mM EDTA) and purified on a Mono-Q column equilibrated with the buffer A [19,20]. The fractions (0.5 ml) containing high PEPC activity were pooled, desalted on a Sephadex-G-25 column and stored in 50% glycerol at −20°C. This purification procedure increased the specific activity ~70-fold.

2.6. Assays of PEPC activity and soluble protein

The activity was determined spectrophotometrically in a coupled reaction with NADH and malate dehydrogenase at 30°C. The rate was calculated from the linear reduction of NADH. Unless stated otherwise, the assay buffer consisted of 50 mM Hepes, pH 7.3, 10 mM $MgCl_2$, 10 mM $NaHCO_3$, 2 mM PEP, 0.2 mM NADH, 4 U of malate dehydrogenase. The reaction was initiated by the addition of PEPC. One unit of activity corresponds to the oxidation of 1 μmol NADH/min. To prepare CO_2-free assay buffer, the buffer was degassed, bubbled with high pure N_2 for 4 h in a 50-ml Falcon tube and sealed in a layer of AR grade silicon oil, and was used immediately. A N_2 flushed pipette was used to transfer the buffer to cuvettes which was sealed immediately with silicon oil. The presence of HCO_3^- in PEPC solution was corrected, assuming that the enzyme contained 1 mM HCO_3^- at pH 8 [3].

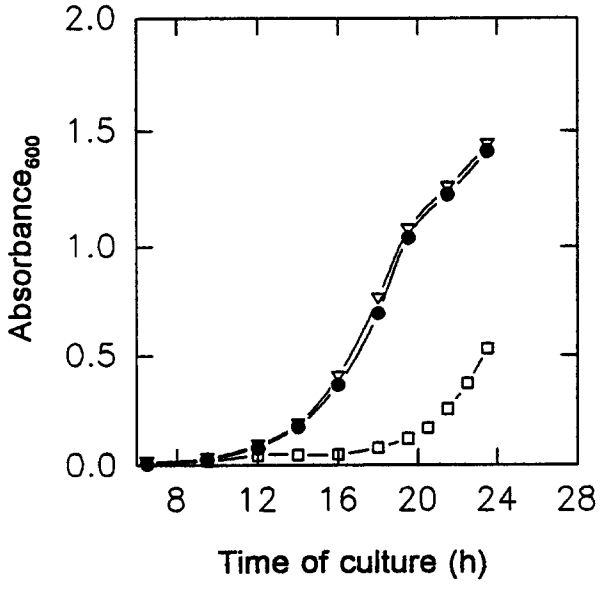

Fig. 1. The growth curves of *E. coli.* PCR1 carrying wild-type or mutant PEPC at 30°C, 250 rpm in 100 ml minimal medium inoculated with 100 μl overnight LB culture. (●), wild-type; (▽), Arg^{600}; (□), Thr^{600}.

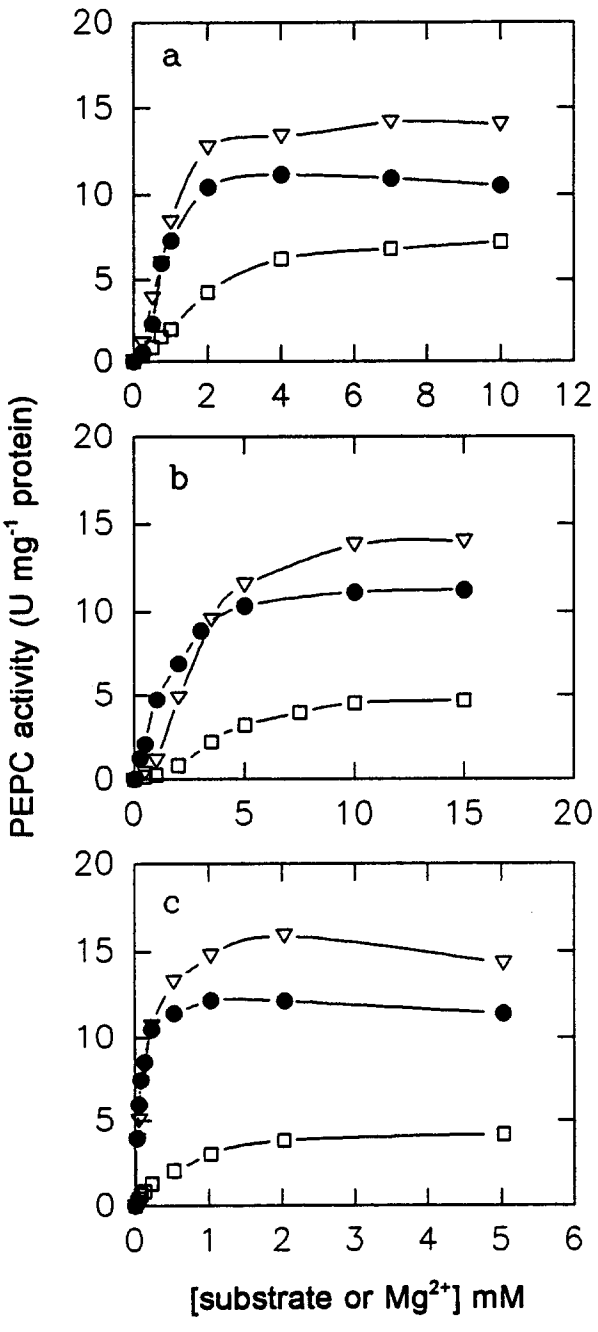

Fig. 2. Kinetic curves of wild-type and mutant PEPCs. (a), PEP; (b), Mg^{2+}; (c), HCO_3^-. (●), wild-type; (▽), Arg^{600}; (□), Thr^{600}.

Soluble protein was determined by the dye-binding method using bovine serum album as the standard [21].

3. Results

3.1. Growth of PCR1 cells transformed with PEPC cDNA

In this study, the conserved Lys^{600} in *F. trinervia* was converted to Arg^{600} or Thr^{600} by site-directed mutagenesis. The substitution of lysine with arginine, due to their similarities in structure and charge status, represented a minor change, while the conversion of lysine to threonine, due to dissimilarities in

Table 1
Summary of the kinetic properties of the wild-type, Arg^{600} and Thr^{600} PEPCs

	Wild-type	Arg^{600}	Thr^{600}
V_{max} (U·mg^{-1} protein)	13.05	17.06	9.44
K_m (PEP) (mM)	0.74	1.08	2.54
K_m (HCO_3^-) (mM)	0.061	0.114	0.602
K_m (Mg^{2+}) (mM)	1.58	3.04	4.41
Hill coefficient (PEP)	2.21	1.80	1.43

structure and charge status, a major change. The plasmid DNA was transformed into an *E. coli* host PCR1 to produce the corresponding PEPC proteins. This host is defective in *ppc* gene, thus requiring glutamate supplementation when grown on a minimal medium containing a sole sugar as the carbon source [22]. Transformation of PCR1 cells with plasmids expressing functional PEPC changes the genotype from glu^- to glu^+. Results showed that both mutant enzymes, like the wild-type, were able to achieve this complementation. However, the cells transformed with Thr^{600} were slow to adapt to the minimal medium. When 100 μl overnight LB culture was inoculated to 100 ml minimal medium (plus 60 μg/ml ampicillin and 0.1 mM IPTG) and grown at 30°C, 250 rpm, the lag phase was 10 h for the wild-type and Arg^{600}, but 16 h for Thr^{600} (Fig. 1). At the exponential phase, however, cells grew at the same rate in all three clones, doubling the A_{600} every 2 h. When 10× more cells were inoculated, the difference in the lag phase disappeared and all three clones grew identically (data not shown). PEPC activity in the crude enzyme extract was 0.182, 0.283 and 0.076 U·mg^{-1} protein in the wild-type, Arg^{600} and Thr^{600}, respectively.

The mutant enzymes were eluted at the same salt concentration (225 mM) as the wild-type enzyme in chromatography on a Mono-Q column, indicating that the overall ionic status of the enzyme had not changed by the mutations (data not shown). Furthermore, when the mutant enzymes were analysed on 10% SDS-PAGE, they moved to the same distance as the wild-type, showing that the subunits were intact as in the wild-type. The gel revealed that the enzyme preparations were ~40% pure (data not shown).

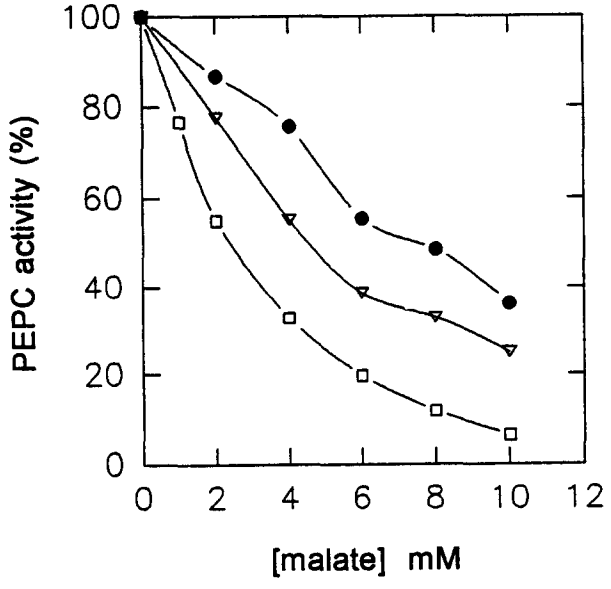

Fig. 3. The inhibition of PEPC activity by malate. The values for 100% activity are the same as in Fig. 2a at 2 mM PEP. (●), wild-type; (▽), Arg^{600}; (□), Thr^{600}.

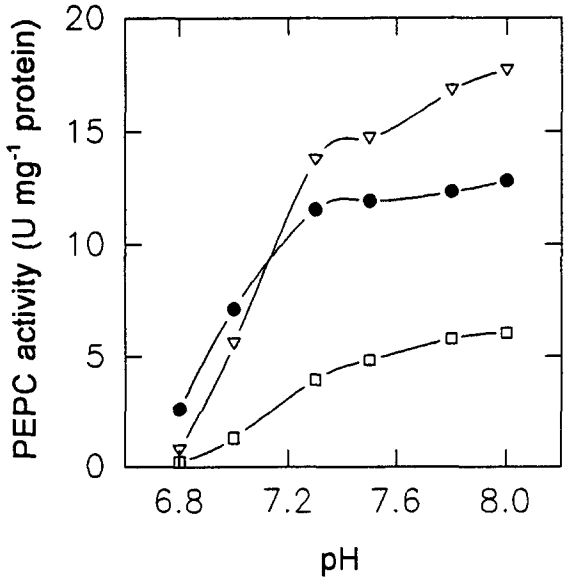

Fig. 4. The pH response curves of wild-type and mutant PEPCs. (●), wild-type; (▽), Arg^{600}; (□), Thr^{600}.

3.2. Kinetic properties of the mutant PEPCs

Fig. 2 shows the response curves of the wild-type, Arg^{600} and Thr^{600} for PEP, Mg^{2+} and HCO_3^- at pH 7.3. The kinetic parameters were summarised in Table 1. At pH 7.3, the K_m(PEP) was 0.74 mM in the wild-type, this value was increased by 46% in Arg^{600} and 2.3-fold in Thr^{600}. At pH 8.0, K_m(PEP) increased from 0.41 mM in the wild-type to 0.57 mM in Arg^{600} and 1.47 mM in Thr^{600} (data not shown), but the magnitude of the difference was similar to that observed at pH 7.3. The $K_m(HCO_3^-)$ value of 0.061 mM determined for the wild-type protein was similar to reported values [3] but considerably smaller than the values of 0.114 and 0.602 mM determined for the Arg^{600} and Thr^{600} enzymes, respectively. The latter value is an order of magnitude greater than that of the wild-type. The $K_m(Mg^{2+})$ in Arg^{600} and in Thr^{600} was 3.04 and 4.41 mM, respectively, compared with a value of 1.58 mM in the wild-type.

3.3. Response of the enzymes to effectors

Dicarboxylic acids, such as malate and citric acid, are strong inhibitors of PEPC from different types of plants [3]. The mutant Arg^{600} and Thr^{600} PEPCs were more sensitive to these inhibitors. The K_i(malate) value decreased from 7.6 mM in the wild-type enzyme to 4.4 mM in Arg^{600} and 2.6 mM in Thr^{600} (Fig. 3). Similar results were obtained with citric acid inhibition (data not shown). Glycine, a strong activator in monocot C_4 PEPC, had no effect while 5 mM glucose-6-phosphate activated all three enzymes by ~10% (data not shown).

3.4. pH profiles of the mutant PEPCs

Activity of PEPC from monocot C_4 and CAM plants increases up to 10-fold when the pH is increased from 7 to 8

[19,23,24]. PEPC from dicot C_4 *F. trinervia* was also responsive to pH changes. The activity was most sensitive to pH variations between 6.8 and 7.3 but was relatively stable between 7.3 and 8.0. The pH profile was altered by the mutation to Arg^{600}. This mutant had lower activity than the wild-type at pH 7 or below, but the activity exceeded that of the wild-type at pH 7.3 or above (Fig. 4).

4. Discussion

Chemical modifications of amino acid residues have shown that some histidine, lysine and arginine residues are essential for the catalytic activity of PEPC [6–10]. Although their precise locations can not be determined by this approach, the conserved residues are likely to be associated with substrate-binding sites and they have served as a guide for site-directed mutagenesis to identify the residues involved. The two conserved histidine residues, corresponding to position 138 and 579 in *E. coli* PEPC, are related to PEP-binding [12,13]. Less is known about the functional roles of the conserved arginine residues, even though 2/11 invariants are located within conserved regions [5].

Lys^{600} in *F. trinervia* is located in a conserved region and found to be invariant in all known eucaryotic and procaryotic PEPCs. Modifications of the enzyme with pyridoxal 5′-phosphate, which specifically reacts with lysine residues, inactivates the maize enzyme, but PEP-Mg^{2+} complex antagonises this inactivation [9]. Further sequencing of the predominant radiolabelled peptide has designated this lysine (Lys^{606} in maize) to be associated with PEP-binding [14]. However, the present study shows that substitution of this lysine by arginine or threonine increased the K_m (HCO_3^-) by up to 9-fold and the K_m for PEP or Mg^{2+} by only 2-fold. These results suggest that this lysine residue is associated with bicarbonate-binding. This is the first time that a residue has been implicated in bicarbonate-binding in PEPC. This conclusion is at variance with the findings based on chemical modification [14]. The exact reason for this discrepancy is not known. But several radiolabelled peptides were produced in the chemical modification study and since only the peptide with dominant radioactivity was rechromatographed and sequenced [14], it is not impossible that the suggested PEP-binding site may represent a misidentification. It is also likely that the bicarbonate-binding site is adjacent to the PEP domain in the polypeptide since the K_m for both substrates are affected by mutation of Lys^{600}.

This study also suggests that Lys^{600} is not obligatory for the catalytic activity of the enzyme. Substitution by arginine or threonine affects the V_{max} only marginally. It is notable that the mutant with arginine substitution has higher activity than the wild-type under optimal conditions. However, compared with Arg^{600}, the wild-type enzyme exhibits greater activity around pH 7 and also has a higher K_i for malate. Apparently, substitution Lys^{600} by Arg^{600} or Thr^{600} reduces the enzyme's capacity under intracellular conditions of around neutral pH [25] and the high concentration of malate in the mesophyll cells of C_4 plants during photosynthesis [26].

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