Site-directed mutagenesis of *Flaveria trinervia* phospho*enol*pyruvate carboxylase: Arg^{450} and Arg^{767} are essential for catalytic activity and Lys^{829} affects substrate binding

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Abstract **Phospho*enol*pyruvate carboxylases (PEPC) of all known sequences contain 11 conserved arginine and two lysine residues located in highly conservative regions. Previous chemical modifications show that arginine and lysine residues are essential for catalytic activity. Three conserved residues, Arg^{450}, Arg^{767} and Lys^{829}, in PEPC of *Flaveria trinervia* were converted to glycine. All three mutant PEPC proteins were similarly expressed in *Escherichia coli*. However, mutant Gly^{450} and Gly^{767} PEPCs had no catalytic activity and Gly^{829} PEPC showed increased K_m for PEP and Mg^{2+}. It seems that Arg^{450} and Arg^{767} are essential for PEPC function while Lys^{829} might be associated with PEP and/or Mg^{2+} binding domain.**

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

1. Introduction

Phospho*enol*pyruvate carboxylase (EC 4.1.1.31) is ubiquitous in higher plants, algae and some procaryotes. The native enzyme is a tetramer comprising of four identical subunits each with a molecular weight of about 100 kDa [1]. During catalysis it has an absolute requirement for divalent metal ions, with Mg^{2+} being the most effective physiological cofactor [2]. It has a number of functions in non-photosynthetic tissues or leaves of C_3 plants [3] such as supplying intermediates in the tricarboxylic acid (TCA) cycle [4], providing carbon skeleton for nitrogen fixation in nodules [5,6], and involvement in the regulation of guard cell movement [7,8]. However, the most important function is undoubtably the photosynthetic fixation of CO_2 in C_4 and CAM plants [9,10].

The cDNA and/or gene of PEPC have been isolated from a large number of sources, including monocot C_4, dicot C_4, C_3, CAM and procaryotes. Eighteen nucleotide sequences and their deduced protein sequences are now available [11,12]. All polypeptides contain 11 absolutely conserved arginine residues and two lysine residues. Some of these residues are located in highly conserved regions which may represent functionally important domains of the proteins [11].

Chemical modifications with phenylglyoxal and 2,3-butanedione show that one arginine residue per subunit is essential for catalytic activity [13,14]. Selective modifications of lysine residues by pyridoxal 5′-phosphate [15], eosin isothiocyanate [16], dansyl chloride [17] and fluorescein isothiocyanate [18] suggest that one or two lysine residues per subunit can be modified and that one of these is essential for the activity [15]. The effect of lysine modifications on PEPC activity is both time- and concentration-dependent and that PEP, especially together with Mg^{2+}, prevents the modifications by these reagents suggesting these modifying reagents compete with substrate PEP for the active site or domain. The positions of the amino acid residues involved have not been identified. Modification by pyridoxal 5′-phosphate suggests that Lys^{606} of maize PEPC is involved in PEP binding [19]. However, conversion of this lysine residue (Lys^{600}) in PEPC of *F. trinervia* to threonine increases K_m (HCO_3^-) more significantly than those of PEP and Mg^{2+} indicating that this lysine residue is more likely to be involved with HCO_3^- binding [20].

In the present study, two conserved arginine residues and the other conserved lysine residue are changed to glycine residues by site-directed mutagenesis to determine their roles in catalytic function.

2. Materials and methods

2.1. Materials

All chemicals, bacterial strains, plasmids, DNA restriction enzymes and modifying enzymes, DNA oligonucleotides, site-directed mutagenesis kit, were as described previously [20].

2.2. Site-directed mutagenesis

The protocol was described previously [20]. The 1.3 kb *Sal*I–*Bam*HI fragment of PEPC cDNA of *F. trinervia*, which contains the codons for Arg^{450}, Arg^{767} and Lys^{829}, was subcloned into the multicloning site of pALTER-1 for the production of ssDNA. Note that this fragment was inverted in the vector pALTER-1, the ssDNA produced from the recombinant plasmid was the non-coding strand for the insert region. Oligonucleotides GATTCTTGTCCTATATCAAG, GAATGGCTCCGAGTGATTC and GGGTTACCATTAGCAAAC were used to introduce a single base substitution at the codons for Arg^{450}, Arg^{767} and Lys^{829}, respectively. These mutations convert all three amino acids to glycine.

2.3. PEPC production and activity assay

The wild-type *F. trinervia* PEPC cDNA and the mutant cDNAs were transformed into PEPC-deficient *E. coli* strain PCR1 which was cultured in a minimal medium [21] for PEPC production. For cells carrying mutant cDNAs which encode non-functional PEPC, the minimal medium was supplemented with glutamate (monosodium salt, 1 g/l). The purification of PEPC from transformed PCR1 was described earlier [20]. PEPC activity was determined spectrophotometrically in a coupled system with NADH and malate dehydrogenase at 30°C. The assay buffer consisted of 50 mM HEPES, pH 7.3 or pH 8.0, 10 mM $MgCl_2$, 5 mM $NaHCO_3$, 0.2 mM NADH, 4 U of malate dehydrogenase and the indicated concentrations of PEP. The reaction was initiated by the addition of PEPC, or by the addition of $NaHCO_3$ for studies on HCO_3^- response. One unit of activity corresponds to the oxidation of 1 μmol NADH. Every assay was duplicated and very consistent values were obtained for the two replicates.

To prepare HCO_3^--free buffer, an aliquot (45 ml) of buffer (50 mM HEPES, pH 7.3, 10 mM $MgCl_2$, 4 mM PEP, 0.2 mM NADH; or

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Abbreviations: PEP, phospho*enol*pyruvate; PEPC, PEP carboxylase

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50 mM HEPES, pH 8.0, 10 mM $MgCl_2$, 2 mM PEP, 0.2 mM NADH) was sparged with high purity N_2 in a 50 ml Falcon tube for 16 h at room temperature (4 ml of H_2O was added to compensate for H_2O loss during sparging). The buffer was then sealed in silicon oil and used immediately. An N_2-flushed pippete was used to transfer an aliquot to cuvettes which was then sealed with the silicon oil. The last traces of HCO_3^- in the buffer, PEPC solution and malate dehydrogenase solution (0.6 μl per 1 ml assay volume) was consumed by the addition of PEPC until the absorbance at 340 nm stabilized (which took 2–3 min), then $NaHCO_3$ of required concentration was added last to initiate the reaction.

Enzyme kinetics was calculated by a Varian enzyme kinetic software. Ten percent SDS-PAGE was performed as in [22].

3. Results and discussion

3.1. Site-directed mutagenesis

PEPC sequences from different sources contain a number of absolutely conserved regions and residues [11,12]. Some of these conserved residues or regions are expected to be associated with substrate binding and catalytic function. The conserved positively-charged amino acid residues (11 arginines, 2 histidines and 2 lysines) may be particularly important since both the substrates, PEP and HCO_3^-, are anions at physiological pH. Chemical modification studies show that one arginine and one lysine residues per subunit are essential for catalytic activity [13–16]. Two conserved regions, LDIRQES and LRAIPWiFsWtQtR, have been previously postulated to have functional importance [11]. In this study, the two arginine residues in these two regions, Arg^{450} and Arg^{767}, and a conserved Lys^{829} in *F. trinervia* PEPC (Fig. 1) were converted to glycine. The mutant PEPCs were designated as Gly^{450}, Gly^{767} and Gly^{829} PEPC, respectively.

The cDNA plasmids encoding wild-type or mutant PEPC were transformed into *E. coli* strain PCR1 to produce PEPC proteins. This strain is defective in the *ppc* gene. When grown in a mineral minimal medium containing one sugar (glucose or glycerol) as the sole carbon source, it requires glutamate supplementation in order to grow. Transformation of the cells with cDNA expressing functional PEPC will complement this requirement [21]. Cells which grow in this minimal medium can be regarded as producing active PEPC as represented by cells transformed with wild-type PEPC cDNA.

3.2. Gly^{450} and Gly^{767} mutations

When the cells transformed with mutant plasmids expressing Gly^{450} and Gly^{767} PEPC were cultured in the minimal medium, no growth was observed over a period of more than 24 h. In contrast, the cells containing wild-type PEPC grew well under the same conditions (data not shown). It is possible that no PEPC proteins were expressed from the two mutant plasmids in the host cells or the expressed mutant PEPCs were non-functional.

In order to distinguish between these two possibilities, the minimal medium was supplemented with glutamate to support the growth of cells transformed with the two mutant plasmids. Proteins were partially purified by 5–12% PEG precipitation and fast protein liquid chromatography on a mono-Q column. All three PEPC proteins (wild-type and two mutants) were eluted from the column at 220 mM NaCl. SDS-PAGE showed that both the Gly^{450} and Gly^{767} PEPCs were as efficiently expressed as the wild-type enzyme and their subunits had the same molecular weight (~100 kDa) (data not shown). This evidence strongly suggested that the expressed Gly^{450} and Gly^{767} PEPCs in the cells were inactive. In the in vitro studies, no activity of the partially purified enzymes was detected at pH 7.3 or pH 8.0, at 2.0 or 12 mM PEP even at high protein concentration.

	450	767	829
F. trinervia (C_4)	K L D I R Q E S	L R A I P W I F	E M V F A K G N P
Maize (C_4)	. . . . .	. . . . .	. . . . . D .
Sorghum (C_4)	. . . . .	. . . . .	. . . . . D .
Ice Plant (CAM)	R	. . . . .	. . . . . D .
Tobacco (C_3)	R	. . . . .	. . . . .
Soybean (C_3)	R	. . . . .	. . . . . D .
F. pringlii (C_3)	. . . . .	. . . . .	. . . . . D .
Ice Plant (C_3)	. . . . .	. . . . .	. . . . . D .
E. coli	R I	. . . . .	. . . . . A D L
C. glutamicum	A . . . . . N .	. . . . . V L	A . . M S . A E L
A. nidulans	H	. . . . . V .	. Q T L . . V D L
A. variablis	. . . . .	. . . . . V .	. . T L . . V D M

Fig. 1. Three conserved or conservative regions of all known sequences of PEPC [11,12]. The first two regions contain conserved arginine while the last one contains a conserved lysine. The numbers denote the positions of residues in PEPC of *F. trinervia*.

Arg^{450} and Arg^{767} are thus essential for catalytic activity. In contrast, chemical modification studies indicate that only one arginine residue per subunit is essential for catalytic activity [13,14]. Different results have also been obtained between chemical modification and site-directed mutagenesis in the studies of histidine residues of PEPC. Modification with diethylpyrocarbonate suggested two histidine residues per subunit were essential [23], but site-directed mutagenesis of both conserved histidine residues showed that only one of them was essential [21,24].

The precise roles of Arg^{450} and Arg^{767} are not known at present. However, they may be directly involved in the binding of substrate(s) and/or in catalysis. The observed inactivation of the enzyme upon arginine-glycine conversion could also be due to indirect secondary effects of structural perturbations.

3.3. Gly^{829} mutation

All known PEPC contains only two conserved lysines. Chemical modification studies of lysine residues show that activity was lost upon modification of essential lysine resitues [15,16]. PEP, especially together with Mg^{2+}, blocks the modification and protect the enzyme from inactivation. Calcula-

Table 1
Summary of kinetic parameters of wild-type and mutant PEPCs at pH 7.3 and pH 8.0

	pH 7.3		pH 8.0	
	Wild-type	Gly^{829} PEPC	Wild-type	Gly^{829} PEPC
V_{max} (U mg^{-1} protein min^{-1})	11.36	7.43	11.98	7.40
K_m (PEP) (mM)	0.76	1.67	0.44	1.35
K_m (Mg^{2+}) (mM)	0.95	2.17	0.18	2.22
K_m (HCO_3^-) (mM)	0.072	0.054	0.081	0.070

Assay conditions were 50 mM HEPES, 10 mM $MgCl_2$, 5 mM $NaHCO_3$, 4 mM PEP at pH 7.3 or 2 mM PEP at pH 8.0.

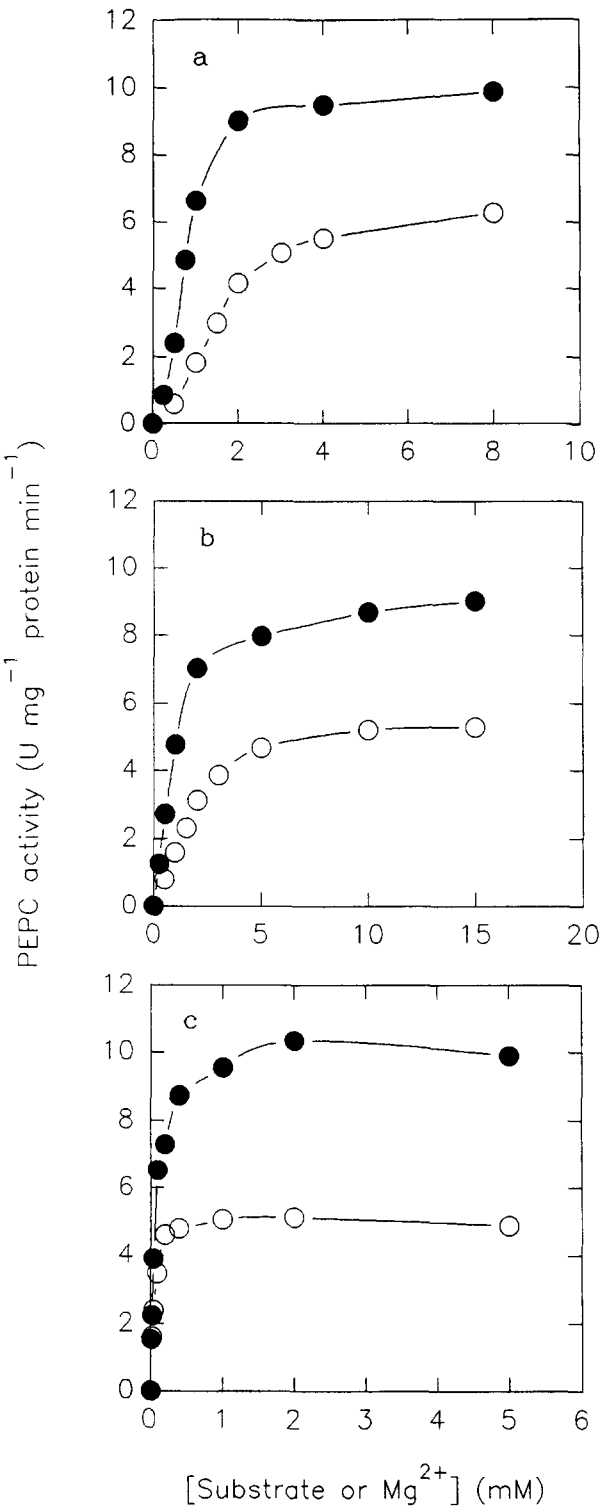

Fig. 2. Kinetic curves of wild-type and mutant Gly^{829} PEPC assayed at pH 7.3. (a) PEP; (b) Mg^{2+}; (c) HCO_3^-; (●) wild-type PEPC; (○) Gly^{829} PEPC.

tions suggest that two lysines per subunit are accessible for chemical modifications and one of these is essential for the catalytic activity [15–17]. We previously reported that one of the conserved lysines, Lys^{600} in *F. trinervia*, is likely to be linked to bicarbonate binding [20], a finding at variance with chemical modification studies [19].

When the second conserved Lys^{829} in *F. trinervia* was changed to glycine. Gly^{829} PEPC complemented the glutamate requirement of PCR1 grown in minimal medium, indicating that active PEPC was produced. However, cells complemented with Gly^{829} PEPC grew significantly slower than those containing wild-type PEPC. The absorbance of an 18 h culture measured at 600 nm was 1.02 and 0.35 for the cells containing wild-type and Gly^{829} PEPC, respectively. After 24 h the absorbance increased to 1.54 and 1.22, respectively. It has been observed that *E. coli* cells containing low PEPC activity take longer to adapt to and grow poorly in minimal medium [20,25]. The decreased cell growth indicated that Gly^{829} PEPC might have lower activity than wild-type enzyme.

Expression and purification of Gly^{829} PEPC was similar to that observed for the wild-type enzyme. However, the kinetic properties of the two proteins were quite different (Fig. 2). At pH 7.3, Gly^{829} PEPC had lower activity and the affinity for PEP and Mg^{2+} decreased by about 1-fold compared to the wild-type but the mutation had little effect on the affinity for HCO_3^-.

The kinetic parameters of the wild-type and Gly^{829} PEPC at pH 7.3 and pH 8.0 are summarised in Table 1. The conversion of Lys^{829} to Gly^{829} affected V_{max} and the affinity for PEP to the same extent at both pH 7.3 and pH 8.0. The K_m for HCO_3^- was not affected at either pH. The mutation had the most significant effect on the K_m (Mg^{2+}) at pH 8.0. This value increased 12-fold at pH 8.0 compared to only 2-fold at pH 7.3. The differential effects on PEP and Mg^{2+} observed here and in other studies [21] suggest that PEP, rather than PEP-Mg^{2+} complex, is the active substrate at least at pH 8–8.5. These results indicated that Lys^{829} may be associated with the domain for PEP and/or Mg^{2+} but it is not obligatory for catalytic activity.

Both conserved lysine residues have been mutated to threonine [20] or glycine (present study) by site-directed mutagenesis. These mutations decrease the affinity for PEP, Mg^{2+} and bicarbonate, but neither is lethal to enzyme function. These results are different from the findings of chemical modifications which show that one lysine residue per subunit is essential for the activity [15]. It is very unlikely that another variant lysine could exert such a vital role in enzyme function. The reason for the discrepancy is not clear. However, it is possible that the loss of activity may by the result of several residues being simultaneously modified by pyridoxal 5′-phosphate [19]. Furthermore, the reagents used to modify lysine residue of PEPC, i.e., pyridoxal 5′-phosphate [15], eosin isothiocyanate [16] and dansyl chloride [17], are large cyclic molecules which could sterically impede the entry of substrate to the active site. In contrast, substitution of lysine by threonine or glycine is non-obstructive and allows the roles of the residues to be assessed directly. The results in this and previous [20] studies indicate that, contrary to previous report [15], neither of the two conserved lysine residues in PEPC is obligatory to the enzyme function.

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