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Phospho*enol*pyruvate carboxylase (PEPC): Mutational analysis of a flexible loop and a putative binding site for an allosteric activator, glucose 6-phosphate (G6P)

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Introduction

Recently the three-dimensional structures of *E. coli* (Ec) PEPC (Kai *et al*. 1999) and *Zea mays* (Zm) C4-form PEPC (Kai *et al*., 2001) have been resolved. We found an untraceable loop structure in both enzymes (^{702}KRRPTGG^{708} in EcPEPC and ^{762}KRRPGGG^{768} in ZmPEPC), bridging above the catalytic site which is located near the C-terminal side of β-barrel (see Fig. 3). Since importance of this loop in catalytic activity in EcPEPC was suggested previously (Kai *et al*., 1999), further mutational analysis of this loop was performed here_with ZmPEPC and EcPEPC. Replacements of P/G and P/T residues in ZmPEPC and EcPEPC, respectively, to R or G residues caused several significant changes in catalytic and regulatory properties.

X-ray analysis of ZmPEPC revealed that an SO_4^{2-} molecule is bound on each enzyme subunit in the crystals formed in the presence of $(NH_4)_2SO_4$ (see Fig. 3). The candidate residues involved in the binding are R183, R184, R231 and R372 (of adjacent subunit). Since SO_4^{2-} molecule is often observed to bind at the site for phosphate group ($-PO_4^{2-}$) of its ligand, we examined whether or not the site is for G6P, an allosteric activator of ZmPEPC. We found that R183 and R184 are essential for G6P activation. Furthermore, we obtained novel kinetic data suggesting the occurrence of two affinity states for PEP and HCO_3^- in wild-type ZmPEPC.

Materials and methods

Preparation of mutant enzymes. Mutant plasmids were constructed by the overlap extension method (Ho *et al*., 1989) by the use of plasmids having maize C4-type PEPC (pTM94, Ueno *et al*., 1997) and having *E. coli* PEPC (pT3, Terada *et al*., 1995) as templates. The mutant enzymes at the SO_4^{2-} binding site are expressed in *E. coli* F15 (a deletion mutant of the *ppc* gene), and crude extracts partially purified by ammonium sulfate fractionation was used as enzymes. Both mutant enzymes of Zm and Ec PEPCs at the flexible loop were expressed by conventional pET system (pET32a) in *E. coli* BL21-Codonplus (DE3)-RIL, and purified by His-bind resin column.

PEPC activity assay. The PEPC activity was assayed by a coupled spectrophotometric method. The standard assay mixture contained, in a total volume of 1.0 ml, 100 mM HEPES-NaOH (pH 7.3), 2 mM PEP, 10 mM $KHCO_3$, 10 mM $MgSO_4$, 0.1 mM NADH, and 1.5 units malate dehydrogenase. When measuring the activity of the bicarbonate-dependent PEP hydrolysis reaction (side-reaction, Fujita, *et al*., 1984), lactate dehydrogenase was used instead of malate dehydrogenase. Measurement of *K*m values for HCO_3^- was carried out essentially according Bauwe (1986) and Dong *et al*. (1997).

Results and discussion

Effects of mutations at flexible loop on kinetic properties. Five mutants of ZmPEPC (P765R/G766R, RR-type; P765R, RG-type; P765G/G766R, GR-type; P765G, GG-type; the insertional mutation of Arg residue between P765 and G766, PRG-type) were constructed. The $A_{0.5}$ values for G6P, were decreased in all mutants. Notably, in the double mutants, RR-type and GR-type, extent of activation by G6P increased. The *K*m values for PEP increased in RR-type and RG-type. The *K*m values for the bicarbonate-dependent PEP hydrolysis activity (side-reaction) increased in RR-type and GR-type. The *K*m values for HCO_3^- increased in PRG-type. In GR-type and GG-type, *V*max decreased.

For EcPEPC, four mutants (P705R/T706R, RR-type; P705R/T706G, RG-type; P705G/T706R, GR-type; P705G/T706G, GG-type) were constructed. The *K*m values for HCO_3^- at pH 8.5 were 0.45, 0.27, 0.49 and 0.23 mM, for the respective mutants in the order described above. Since the value for the wild-type enzyme was 0.42 mM, apparent affinity to HCO_3^- increased significantly in RG-type and GG-type PEPCs.

Although the 4 types of mutant sequences in the loop are identical in both Zm and Ec PEPCs, *K*m values for HCO_3^- were 30- to 40-fold smaller in ZmPEPC than EcPEPC as a whole. This indicates that the loop is not a major determinant site causing this marked difference. However, the mutations in the loop cause significant effects on various enzymatic properties. The effects were not necessarily common to both PEPCs. Simple introduction of a basic residue did not always decrease *K*m value for HCO_3^-.

Table 1. Kinetic and allosteric properties of wild-type and mutant enzymes. *; Vmax=μmol min^{-1} mg $protein^{-1}$, **; The maximal extent of activation by G6P

	PEP		PEP side-reaction		Mg^{2+}		HCO_3^- (pH7.3)	HCO_3^- (pH8.0)	G6P		Malate
	*K*m (mM)	*V*max*	*K*m(mM)	*V*max*	*K*m(mM)	*V*max*	*K*m(mM)	*K*m(mM)	$A_{0.5}$(mM)	G6P Activation(%)**	$_{.5}$ mM)
wild-type(KRRPGGG)	**1.8(0.7)**	23	0.6	2.3	3.1	10.7	0.02	**0.01(0.003)**	2.4	263	1.2
RR-type (KRRRRGG)	**3**	25	**1.1**	1.8	2.9	7.3	0.02	0.03	**1.2**	**400**	1.2
RG-type(KRRRGGG)	**3.1**	15	0.9	2.5	1.9	9.6	0.03	0.01	**1.3**	238	1
GR-type(KRRGRGG)	1.7	**9**	**1.2**	2.7	2.6	4.5	0.02	0.01	**0.8**	**345**	1.1
GG-type(KRRGGGG)	2	**11**	0.8	1.4	3	5.7	0.02	0.01	**1.3**	313	0.8
PRG-type(KRRPRGGG	2.5	25	0.6	2.8	3.9	17.6	**0.07**	**0.07**	**1.7**	270	1

Replacement of Arg residues in putative binding site for G6P. We prepared three mutant enzymes of ZmPEPC whose Arg residues at 183, 184, and 231 were individually replaced to glutamine (R183Q, R184Q and R231Q). The specific activities in the crude extracts of *E. coli* F15 cells expressing wild-type (WT) or each mutant enzyme were 2.7, 0.54, 0.87 and 1.9 μmol min^{-1} mg $protein^{-1}$ for WT, R183Q, R184Q and R231Q, respectively. As shown in Fig. 1a., R183Q and R184Q showed almost complete loss of sensitivity to G6P, while R231Q showed a partial loss of sensitivity.

When the sensitivity to malate, an allosteric inhibitor, was investigated in the presence of 2.5 mM G6P, sigmoidal inhibition curves were obtained for WT and R231Q but hyperbolic inhibition curve for R183Q and R184Q (Fig. 1b.). No significant changes were observed for *K*m values for PEP and Mg^{2+}. Thus simultaneous loss of sensitivity and heterotropic effect strongly suggests the involvement of R183 and R184 in the G6P binding and cooperative conformational change of the enzyme.

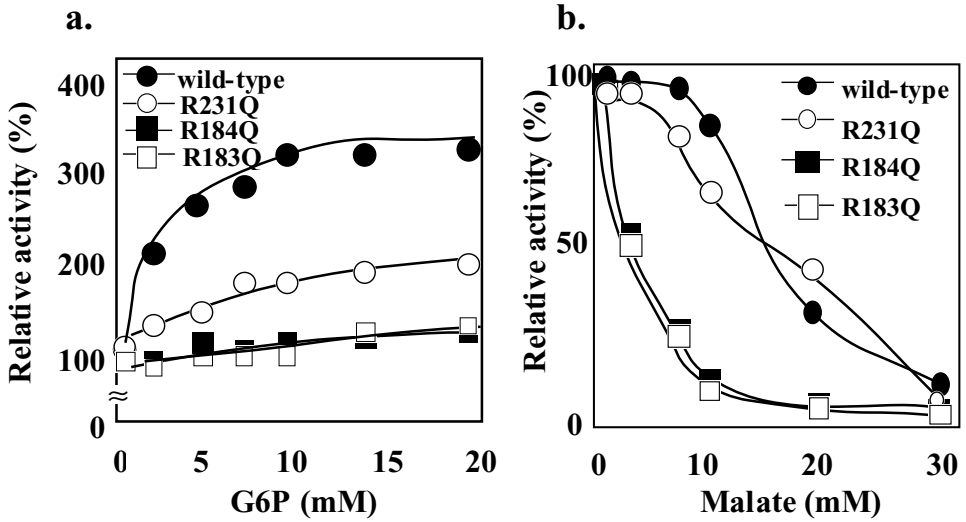

Fig. 1. Effect of mutations in putative G6P binding sites. **a**; the sensitivities to allosteric activater, G6P. **b**; the desensitization to allostericinhibitor, malate induced by G6P.

Occurrence of two affinity states for PEP and HCO_3^-. During the course of these experiments, we obtained the data suggesting the occurrence of two affinity states for PEP (pH7.3) and HCO_3^- (pH.8.0) in wild-type ZmPEPC. Double-reciprocal plot gave two *K*m values for each substrate (Fig. 2). Lower *K*m value was obtained at low concentrations of each substrate, indicating its negative cooperativity. Further studies on this novel phenomenon and its physiological significance seem necessary.

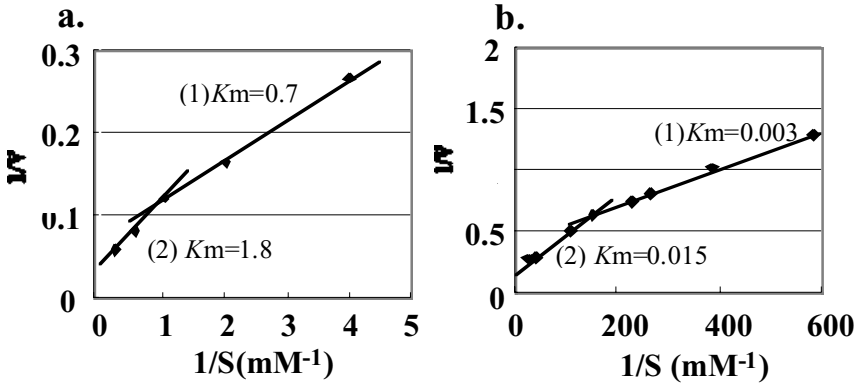

Fig. 2. Occurrence of two affinity states for PEP(a.) and HCO_3^-(b.). a-(1); y=0.0482x+0.073, *K*m=0.7 mM, a-(2); y=0.0793x+0.0439, *K*m=1.8 mM, b-(1); y=0.015x+0.4427, *K*m=0.003 mM, b-(2); y=0.003x+0.1984, *K*m=0.015 mM.

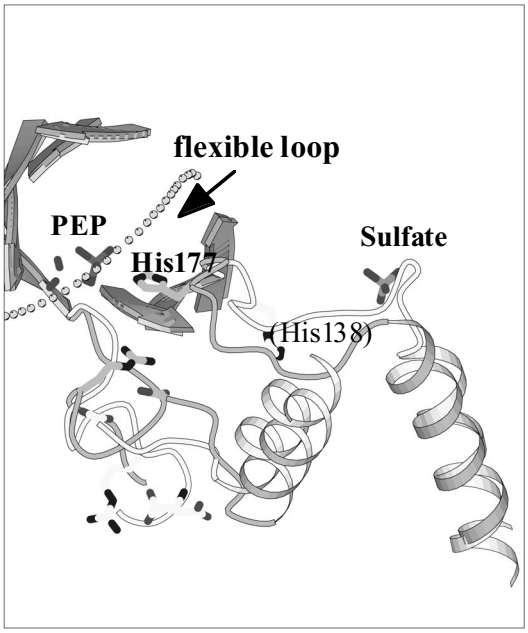

Fig. 3. Comparison of the active-site structure in ZmPEPC (gray) and EcPEPC (white) .

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