# A Ca$^{2+}$-dependent protein kinase phosphorylates phosphoenolpyruvate carboxylase in maize

Noriyuki Ogawa, Satoru Okumura* and Katsura Izui

*Department of Chemistry, Faculty of Science, Kyoto University, Kyoto 606–01, Japan*

In C$_4$ plants the activity of phosphoenolpyruvate carboxylase (PEPC; EC 4.1.1.31) is regulated by phosphorylation/dephosphorylation which is mediated by light/dark signals. The study using protein kinase inhibitors showed that the inhibition pattern of maize PEPC-protein kinase (PEPC-PK) is similar to that of myosin light chain kinase, a Ca$^{2+}$-calmodulin-dependent PK. The kinase activity was also inhibited by EGTA and the inhibition was relieved by Ca$^{2+}$. These results suggest that PEPC-PK is Ca$^{2+}$-dependent in contrast with previous observations by other research groups.

## 1. INTRODUCTION

The regulation of C$_4$ photosynthesis is in part mediated by light/dark regulation of PEPC (phosphoenolpyruvate, EC 4.1.1.31). The enzyme from light-adapted leaves is more active and less sensitive to feedback inhibition by malate than that from dark-adapted leaves. In recent years it has been shown that the PEPC activation/inactivation is mediated by phosphorylation/dephosphorylation of PEPC [1]. Although Jiao and Chollet [2] partially purified PEPC-protein kinase (PK) from maize leaves, which changed some of the enzymatic properties of PEPC, this kinase has not yet been purified completely and its nature is as yet unclear. In particular, it is currently in dispute whether this kinase is Ca$^{2+}$-dependent or not [3–7]. With regard to maize PEPC-PK, Chollet's group reported that it was Ca$^{2+}$-independent [3,4]. In the present study, we examined the effects of kinase inhibitors (K-252a derivatives [10] and W-7 [13]) on PEPC-PK activity, as well as the effect of EGTA. The results strongly suggest that PEPC-PK is a Ca$^{2+}$-dependent kinase.

## 2. MATERIALS AND METHODS

### 2.1. Purification of enzymes

Leaves were harvested from 10-week-old maize plants (*Zea mays* H84) grown in the field. PEPC was purified to homogeneity by a procedure which will be described elsewhere. PEPC-PK was partially purified from maize leaves essentially according to the procedure of Jiao and Chollet [2]. Minor modifications were the inclusion of 0.5 mM phenylmethanesulfonyl fluoride in the extraction buffer to prevent proteolytic modification and the stepwise gradient elution from a Blue–Sepharose CL-6B column. Namely, PEPC-PK was eluted with 0.5 M NaCl after washing with 0.25 M NaCl. The eluate was de-salted by passing through a PD-10 column (Pharmacia) and then concentrated with a Centricon-30 (Amicon). The obtained preparation was stored in small aliquots (30 μl) at −80°C. Protein concentrations were determined by Bradfords' method [11] using bovine serum albumin as a standard.

### 2.2. Phosphorylation assay

The reaction was started by the addition of 100 μM [γ-$^{32}$P]ATP (500 dpm/pmol) to a reaction mixture (40 μl) containing 50 mM Tris-HCl (pH 7.8), 5 mM MgCl$_2$, 10 mM KF, 0.2 mM dithiothreitol, 10 μM leupeptin, 3 μg maize PEPC, 40 μg protein of partially purified PEPC-PK, 1% glycerol and 4% ethylene glycol. After incubation for 60 min at 30°C the reaction was terminated with 40 μl of cold 20% (w/v) trichloroacetic acid containing 100 μM ATP and 5 mM EDTA. The precipitated protein was washed three times with 10% trichloroacetic acid containing 100 μM ATP and 1 mM EDTA, and then rinsed three times with ethanol. Samples were analyzed by SDS-PAGE and autoradiography according to standard protocols. Relative radioactivity of protein bands on the gel were measured by an image analyzer (FUJIX BA-100). K-252a and its three derivatives [10] were generous gifts from Kyowa Medex Co. W-7 was obtained from Biomol. Res. Labs. Inc.

## 3. RESULTS

Maize PEPC was efficiently phosphorylated when incubated with a PK from the same origin and [γ-$^{32}$P]ATP. The site of phosphorylation was shown to

**A**

**B**

**1 2 3 4 5 6**      **1 2 3 4 5 6**

PEPC ▶

Fig. 1. Inhibition of PEPC-PK by K-252a derivatives. SDS-PAGE analysis of phosphorylation assays described in section 2 with (lanes 1–6, respectively) no inhibitor, 2.5% (v/v) DMSO (solvent of K-252a derivatives), 10 $\mu$M K-252a, 10 $\mu$M KT5720, 10 $\mu$M KT5823, 10 $\mu$M KT5926. (A) Coomassie blue staining. (B) Autoradiograph.

be the same (Ser[15]) as the site phosphorylated by a mammalian cyclic AMP-dependent PK (A-kinase) [12] as judged from the behaviors of phosphorylated peptide on HPLC and TLC (data not shown). Furthermore, the conversion of PEPC to a more activated state was also brought about by the phosphorylation. To characterize PEPC-PK, the effect of a series of kinase inhibitors on PEPC-PK was investigated. K-252a produced by *Norcardiopsis* sp. is known to potently inhibit various types of PK's in competition with ATP [8,9]. The three derivatives from K-252a, namely KT5720, KT5823 and KT5926, selectively inhibit A-kinase, cGMP-dependent protein kinase and myosin light chain kinase (MLCK), respectively. K-252a potently inhibits these three types of kinase and phospholipid-sensitive $Ca^{2+}$-dependent protein kinases with almost equal $K_i$ values. Figs. 1 and 2 show that K-252a and KT5926 strongly inhibited PEPC-PK activity in a concentration-dependent manner, and the concentration required for 50% inhibition ($IC_{50}$) was 5.0 and 2.5 $\mu$M, respectively. KT5720 and

KT5823 did not cause significant inhibition. The inhibition pattern was similar to that of MLCK, which belongs to the $Ca^{2+}$-calmodulin-dependent PK family. Furthermore, we tested the effect of a calmodulin antagonist, W-7, which also inhibits PK's [13]. W-7 inhibited PEPC-PK (Fig. 3B) with an $IC_{50}$ value of 125 $\mu$M. This value is comparable to that reported for the inhibition of soybean $Ca^{2+}$-dependent PK [14].

To test whether or not PEPC-PK activity is dependent on $Ca^{2+}$ the effect of EGTA was investigated (Fig. 3A). EGTA at a concentration of 10 $\mu$M strongly inhibited the PEPC-PK activity (residual activity was 14% of controls). Addition of both 20 $\mu$M $CaCl_2$ and 10 $\mu$M EGTA abolished the EGTA effect and kept PK activity as high as controls. The $I_{50}$ of EGTA was ca. 2 $\mu$M (data not shown). These results provide direct evidence for the $Ca^{2+}$ dependence of PEPC-PK.

## 4. DISCUSSION

Echevarria et al. [5] reported that there is a $Ca^{2+}$-calmodulin-dependent PEPC-PK in sorghum leaf crude extract. On the other hand, PEPC-PK preparations partially purified through Blue-dextran agarose either from maize or sorghum were reported by serveral groups to be active even under $Ca^{2+}$-depleted conditions [3,4,6,7]. Although we tried to find out the cause of the discrepancy between these results and ours by the use of the same reaction conditions as described in [4] and by changing various reaction conditions including reaction time, glycerol concentration (0–20%), pH (6.0–8.0) and omission of KF, leupeptin or ethylene glycol, the inhibition of PEPC-PK by EGTA was consistently observed in our experiments. Another possibility to be examined is whether partial proteolysis of PEPC-PK, which might have occurred during preparation, caused desensitization to $Ca^{2+}$ as has been observed for smooth muscle MLCK [15]. Our positive results suggest that
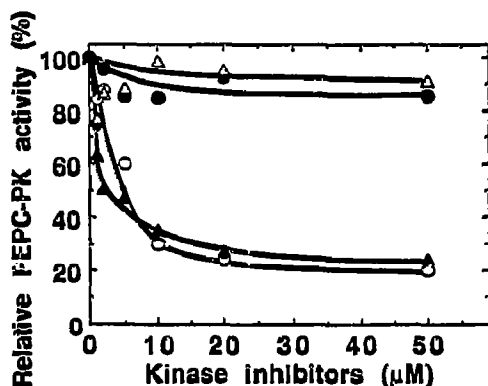
Fig. 2. Effect of increasing concentrations of inhibitors on PEPC-PK activity. Assay conditions were as described in section 2 except for the addition of inhibitors. The reaction mixture contained K-252a (○), KT5720 (●), KT5823 (△) or KT5926 (▲).
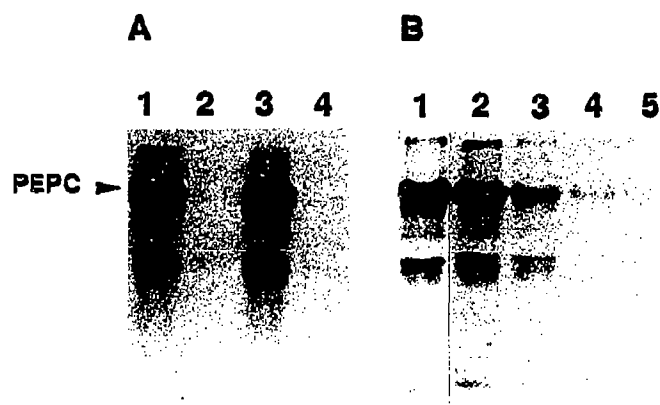
[Graph: Relative PEPC-PK activity (%) vs Kinase inhibitors ($\mu$M), y-axis 0–100, x-axis 0–50]

**A**　　　　**B**



Fig. 3. Effect of $Ca^{2+}$ and W-7 on PEPC-PK activity. Autoradiograph of phosphorylation assays described in section 2 with (A; lanes 1-4, respectively) no addition, 10 $\mu$M EGTA, 10 $\mu$M EGTA + 20 $\mu$M $CaCl_2$, 10 $\mu$M EGTA + 20 $\mu$M $MgCl_2$, and (B; lanes 1-5, respectively) no addition, 10 $\mu$M, 100 $\mu$M, 500 $\mu$M and 1 mM of W-7.

$Ca^{2+}$ is still a candidate for the mediator of the light/dark signal for PEPC-PK.

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