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Maize leaf phosphoenolpyruvate carboxylase: phosphorylation of Ser^{15} with a mammalian cyclic AMP-dependent protein kinase diminishes sensitivity to inhibition by malate

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The so-called light-activation of phosphoenolpyruvate carboxylase (PEPC) (EC 4.1.1.31) involved in C_4 photosynthesis is known to be mediated by phosphorylation. A cyclic AMP-dependent protein kinase from bovine heart was found to be able to phosphorylate PEPC. The phosphorylation was accompanied by the changes in kinetic properties, which were very similar to the reported light activation. The phosphorylated amino acid residue was identified as Ser and the position of this Ser on the primary structure [(1988) FEBS Lett. 229, 107–110] was determined to be Ser^{15}.

Phosphoenolpyruvate carboxylase; C_4 photosynthesis; Phosphorylation; Cyclic AMP-dependent protein kinase; Malate sensitivity; (Maize)

1. INTRODUCTION

PEPC (EC 4.1.1.31) catalyses the key step in the fixation of atmospheric CO_2 into malate in the mesophyll cells of plants which carry out C_4 photosynthesis. For C_4 plants such as maize and sorghum, this enzyme is in a more active form in extracts from illuminated leaves than from leaves in darkness, as indicated by changes in specific activity under suboptimal assay conditions or sensitivity to feedback inhibition by malate [1]. Recently, it was shown that the conversion of maize PEPC between light and dark forms are mediated by phosphorylation and dephosphorylation of PEPC at its seryl residue [2,3]. In vitro phosphorylation of PEPC by an endogenous protein kinase and accompanying changes in the kinetic properties were also reported [4]. Although the complete primary structure of maize PEPC involved in the C_4 photosynthesis was deduced from the nucleotide sequence of the cDNA by us [5,6], the site of the seryl residue which undergoes phosphorylation has not yet been elucidated. In this study, we found that the maize PEPC was a good substrate for mammalian A-kinase in vitro and that the phosphorylation caused the changes in kinetic properties, which were very similar to the phosphorylation observed in vivo [3]. Furthermore, the phosphorylated amino acid residue was characterized and the sequence of the ^{32}P-labeled phosphopeptide derived from PEPC was determined.

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Abbreviations: A-kinase, cyclic AMP-dependent protein kinase; API, *Achromobacter* protease I; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; HPLC, high performance liquid chromatography; Mes, 2-(*N*-morpholino)ethanesulfonic acid; PEPC, phosphoenolpyruvate carboxylase; PTH, phenylthiohydantoin; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis

2. MATERIALS AND METHODS

2.1. *Purification and assay of enzymes*

Leaves were harvested from 10-week-old maize plants (*Zea mays* H84) grown in the field. PEPC was purified to a specific activity of 20–27 U/mg protein by a procedure which will be described elsewhere. Enzyme activity was determined spectrophotometrically at 30°C. The standard assay mixture contained 0.1 M Tris-HCl (pH 8.0), 10 mM $MgSO_4$, 10 mM $KHCO_3$, 0.1 mM NADH, 1.5 IU malate dehydrogenase, 2 mM PEP, and 20% ethyleneglycol in a total volume of 1 ml. One unit of PEPC activity corresponds to the oxidation of 1 μmol of NADH/min by the coupling enzyme. Protein concentration was measured by the method of [7] using bovine serum albumin as a standard.

The catalytic subunit of cAMP-dependent protein kinase (A-kinase) was purified to homogeneity from bovine heart by the procedure of Sugden et al. [8] with some modifications. The specific activity of the enzyme, assayed with histone IIA (Sigma) as a substrate [8], was 3.1–3.7 U/mg.

2.2. *Preparation of phosphorylated PEPC*

3 mg (30 nmol subunit) of PEPC at a concentration of 0.5 mg/ml were incubated at 30°C for 1 h with the catalytic subunit of A-kinase (5.6×10^{-2} U/ml) in a reaction mixture containing 12 mM Mes-NaOH (pH 6.0), 5 mM magnesium acetate, 0.2 mM [γ-^{32}P]ATP (62.9 cpm/pmol). The reaction was terminated with an equal volume of 20% (w/v) cold trichloroacetic acid. The precipitated protein was washed twice with 10% trichloroacetic acid and twice with acetone, and then lyophilized.

2.3. *Phosphoamino acid analysis*

A portion of the ^{32}P-labeled PEPC preparation obtained in section

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2.2. was subjected to SDS-PAGE and the distribution of the radioactivity was detected by autoradiography. A block of the gel containing PEPC was excised, lyophilized, and treated directly with trypsin in 50 mM NH_4HCO_3 for 12 h at 30°C. The supernatant was lyophilized and acid-hydrolyzed in 6 N HCl at 110°C for 2 h. The hydrolysate was analyzed on a cellulose thin-layer plate (Merck 5716) by electrophoresis (pH 1.9) followed by ascending chromatography (1-butanol/pyridine/glacial acetic acid/H_2O, 75:50:15:60 (v/v)) in the second dimension as described [9]. The standard phosphoamino acids were detected by staining with ninhydrin.

2.4. *Proteolytic digestion of ^{32}P-labeled PEPC and purification of the liberated phosphopeptide*

Phosphorylated PEPC was dissolved in 4 M urea and 50 mM Tris-HCl (pH 8.0) and digested with *Achromobacter* protease I (API) (Wako Pure Chem. Industries, Ltd.) for 15 h at 30°C. The molar ratio of enzyme to substrate was 1:30. After digestion, the solution was directly subjected to an HPLC on a Waters μ-Bondapak C_{18} column. Buffer A was 0.1% trifluoroacetic acid, and buffer B was acetonitrile containing 0.07% trifluoroacetic acid. The gradient used was 0–10 min, 0% B (i.e. 100% A); 10–60 min, 0–50% B; 60–70 min, 50–80% B; 70–80 min, 80% B. The flow rate was 1 ml/min and effluent was monitored at 215 nm. Each fraction (1 ml) was counted for the radioactivity and the pooled ^{32}P-containing fractions were dried, redissolved in 0.1 ml of 50 mM NH_4HCO_3 and 10 mM $CaCl_2$, and further digested with 1.6 μg of diphenyl carbamyl chloride treated trypsin (Sigma) for 9 h at 30°C. The reaction was stopped with 10% trifluoroacetic acid and subjected to HPLC as described above.

2.5. *Amino acid sequence analysis*

Automatic Edman analyses were performed with a gas-phase sequencer (Model 477A, Applied Biosystems). Phenylthiohydantoin (PTH) derivatives were identified by an on-line analyzer (Model 120A, Applied Biosystems).

3. RESULTS

3.1. *Phosphorylation by a mammalian protein kinase*

After the incubation of maize PEPC with A-kinase, the reaction mixture was subjected to SDS-PAGE followed by autoradiography. As shown in fig.1a, PEPC was found to be heavily labeled with ^{32}P. The other faint bands could be due to the phosphorylation of proteins contaminated in the PEPC preparation. Quantitative measurement of radioactivity distribution with an image scanner (Radioanalytic Imaging System, AMBIS System) showed that about 94% of the total radioactivity was attributable to the band of PEPC. No protein kinase activity was detected in the PEPC preparation used in this study. Two-dimensional separation of ^{32}P-labeled amino acid which was liberated from the phosphorylated PEPC demonstrated that the phosphorylation occurred exclusively on the seryl residue (fig.1b).

When the time course of phosphorylation was followed with the reaction mixture containing 5 μM PEPC, 0.2 μM A-kinase and 0.2 mM [γ-^{32}P]ATP at 30°C, the reaction proceeded linearly with time up to 20 min and thereafter the rate gradually decreased to level off after 60 min. The amount of phosphate incorporated into PEPC reached 0.5 mol/mol subunit (data not shown).

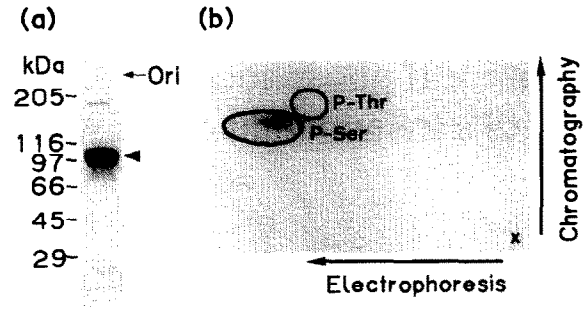

Fig.1. Phosphorylation of PEPC with A-kinase (a) and two-dimensional separation of phosphorylated amino acids from ^{32}P-labeled PEPC (b). (a) Autoradiogram of phosphorylated proteins separated by SDS-PAGE. The arrowhead indicates the position of PEPC as revealed by protein staining. (b) Autoradiogram of phosphorylated amino acids. Circled areas represent the location of the authentic phosphoamino acids as detected by ninhydrin. x, origin.

3.2. *Effect of phosphorylation on the kinetic properties*

The PEPC phosphorylated by A-kinase was compared with the nontreated PEPC for the catalytic and regulatory properties. As shown in fig.2a, the half-saturation concentration of PEP ($S_{0.5}$) was decreased from 2.0 mM to 1.2 mM by the phosphorylation, but the maximal velocity (V_{max}) and Hill coefficient ($n = 2.0$) were not affected significantly. The results indicate that under suboptimal assay conditions in which pH was 7.0 and the concentration of PEP was in the range of 1–3 mM, a slight activation could be seen by phosphorylation, in accordance with the previous observations in vivo [2,3]. Fig.2b shows the saturation curves of malate, a feedback inhibitor. Upon phosphorylation by A-kinase, the shape of the curve changed from hyperbolic ($n = 1.4$) to sigmoidal ($n = 2.7$) and the concentration required for 50% inhibition ($I_{0.5}$) increased from 0.9 mM to 3.0 mM. The sensitivities to glucose 6-phosphate and glycine, allosteric activators,

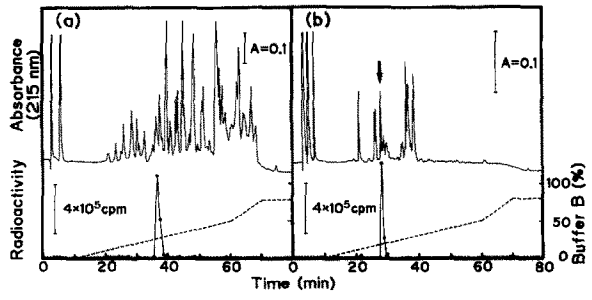

Fig.2. Effect of phosphorylation of PEPC on the substrate dependency (a) and the sensitivity to malate (b). PEPC (0.5 mg/ml) was incubated in a reaction mixture containing 12 mM Mes-NaOH (pH 6.0), 0.2 mM ATP and 5 mM magnesium acetate with (●) or without (○) 5.6×10^{-2} U/ml of A-kinase for 1 h at 30°C. Samples were assayed for PEPC activity with an assay mixture containing 0.1 M Hepes-KOH (pH 7.0), 10 mM $MgSO_4$, 10 mM $KHCO_3$, 0.1 mM NADH, 1.5 IU malate dehydrogenase, and indicated concentrations of PEP (a) or of malate in the presence of 2 mM PEP (b).

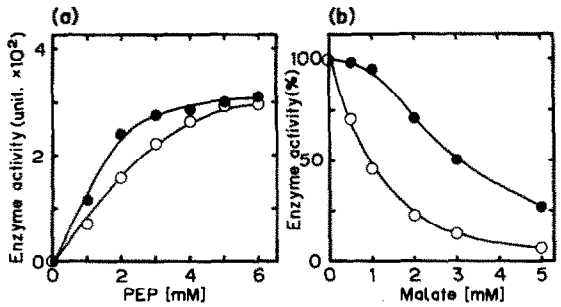

Fig.3. Reverse-phase HPLC of phosphopeptide fragments. PEPC phosphorylated by A-kinase was digested with API and the resulting digest was subjected to HPLC (a). The radioactive fractions from (a) were combined and further digested with trypsin and then the digest was separated by HPLC (b). The UV-peak coincident with the radioactivity peak is indicated by an arrow.

were also investigated. But the shapes of the saturation curves were not changed significantly by the phosphorylation (data not shown). These results indicate that the changes in the kinetic properties caused by mammalian A-kinase were quite similar to those caused by endogenous protein kinase of maize [4].

3.3. *Identification of the phosphorylation site*

To isolate phosphopeptide, labeled PEPC was first digested with a lysine-specific protease (API) and the resulting peptides were separated by HPLC as shown in fig.3a. A single radioactive peak was detected at a retention time of 37 min and the peak fractions were collected for further digestion with a lysine/arginine-specific protease (trypsin). When the digest was separated by HPLC, the major radioactive peak appeared at a retention time of 28 min, coinciding with a peptide peak monitored by absorbance at 215 nm (fig.3b). This fraction was subjected to sequence analysis. From the data shown in table 1, the sequence of the peptide was determined as HHSIDAQLR. The occurrence of phosphorylated seryl residue in the peptide was indicated by the appearance of dithiothreitol adduct of PTH-serine and the absence of PTH-serine [10]. The amino acid composition of the peptide was confirmed by the method of [11]. The sequence of this peptide exactly coincided with the sequence from the 13th to 21st amino acid residue on the primary structure of PEPC [6]. Thus it can be concluded that the serine residue phosphorylated by A-kinase is Ser^{15}.

Table 1

Sequence analysis of phosphopeptide

Cycle	Residue	pmol
1	His	53
2	His	77
3	(P-Ser)	–
4	Ile	173
5	Asp	77
6	Ala	150
7	Gln	100
8	Leu	155
9	Arg	57

Data were obtained from a gas-phase sequencer (ABI 477A); 500 pmol of peptide were applied to the glass fiber filter. P-Ser refers to position of seryl-phosphate residue as identified by presence of the PTH-serine-dithiothreitol adduct as the major detected species

4. DISCUSSION

It was clearly established that the changes in kinetic properties of PEPC were caused by phosphorylation of Ser^{15} located near the N-terminal end. It seems highly possible that the same seryl residue is involved in the phosphorylation in vivo. The maximal extent of phosphorylation by A-kinase was about 0.5 mol/mol subunit of PEPC as measured by ^{32}P incorporation. According to [3], PEPC is phosphorylated to extents of 0.6 and 0.4 mol/mol subunit under light and dark conditions, respectively, and hence PEPC used in our experiment was supposed to have already been phosphorylated to a considerable extent in vivo. Therefore, the observed limitation of ^{32}P incorporation could be accountable if assumed that Ser^{15} is involved in the phosphorylation in vivo and only a half of the total Ser^{15} is left available for further phosphorylation in vitro. The amino acid sequence around the phosphorylated seryl residue (*), EKHHS*IDAQLR, was quite different from the so-called consensus sequences for the phosphorylation by A-kinase [12], which contain arginyl residue at the 2nd or 3rd position upstream of the seryl residue. It seems of interest to test whether the synthetic peptide containing this sequence can be also phosphorylated by A-kinase or not. Two other mammalian protein kinases, Ca^{2+}/phospholipid-dependent protein kinase and Ca^{2+}/calmodulin-dependent protein kinase, which have different substrate specificities from A-kinase, were found less effective for the phosphorylation of PEPC.

PEPC is known also as a key enzyme in the Crassulacean acid metabolism (CAM), and is reported to be converted to more active form at night through phosphorylation [13,14]. Inspection of the primary sequence of PEPC from *Mesembryanthemum crystallinum*, a kind of CAM plant [15] revealed that, although the sequence of phosphopeptide found in the maize PEPC is not conserved, an analogous sequence DRLTSIDAQ LR is located near the N-terminal end. It may be of interest to test whether this seryl residue is involved in the phosphorylation or not.

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