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Kinetic analysis of the non-phosphorylated, *in vitro* phosphorylated, and phosphorylation-site-mutant (Asp8) forms of intact recombinant C_4 phospho*enol*pyruvate carboxylase from sorghum

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Abstract: Steady-state kinetic analyses were performed on the non-phosphorylated, *in vitro* phosphorylated and phosphorylation-site mutant (Ser8→Asp) forms of purified recombinant sorghum C_4 phospho*enol*pyruvate (*P*-pyruvate) carboxylase (EC 4.1.1.3 1) containing an intact N-terminus. Significant differences in certain kinetic parameters were observed between these three enzyme forms when activity was assayed at a suboptimal but near-physiological pH (7.3), but not at optimal pH (8.0). Most notably, at pH 7.3 the apparent K_i for the negative allosteric effector L-malate was 0.17 mM, 1.2 mM and 0.45 mM while the apparent K_a for the positive allosteric effector glucose 6-phosphate (Glc6*P*) at 1mM *P*-pyruvate was 1.3 mM, 0.28 mM and 0.45 mM for the dephosphorylated, phosphorylated and mutant forms of the enzyme, respectively. These and related kinetic analyses at pH 7.3 show that phosphorylation of C_4 *P*-pyruvate carboxylase near its N-terminus has a relatively minor effect on *V* and K_m (total *P*-pyruvate) but has a dramatic effect on the extent of activation by Glc6*P*, type of inhibition by L-malate and, most especially, K_a (Glc6*P*) and K_i (L-malate). Thus, regulatory phosphorylation profoundly influences the interactive allosteric properties of this cytosolic C_4-photosynthesis enzyme.

Keywords: C_4 photosynthesis, phospho*enol*pyruvate carboxylase, recombinant enzymes, regulatory protein phosphorylation, sorghum

Abbreviations: CAM, Crassulacean acid metabolism; Glc6*P*, glucose 6-phosphate; *P*-pyruvate or *P*Prv, phospho*enol*pyruvate; *P*-pyruvate carboxylase, phospho*enol*pyruvate carboxylase; PKA, CAMP-dependent protein kinase; S8D and S8C, mutant forms of sorghum *P*-pyruvate carboxylase in which Ser8 was replaced by Asp and Cys, respectively.

Enzymes: NADH-malate dehydrogenase (EC 1.1.1.37); phospho*enol*pyruvate carboxylase (EC 4.1.1.31); cAMP-dependent protein kinase (EC 2.7.1.37); protein-serine/threonine kinase (EC 2.7.1.–); protein phosphatase type 2A (EC 3.1.3.16).

Phospho*enol*pyruvate (*P*-pyruvate) carboxylase is a cytosolic enzyme in bacteria, cyanobacteria, algae and higher plants [1, 2]. During C_4 photosynthesis and Crassulacean acid metabolism (CAM), it plays a cardinal role in the initial fixation of atmospheric CO_2 (as HCO_3^-) into the C_4 dicarboxylic acids L-malate and aspartate [1–3]. In leaf tissue of C_4 and CAM species, *P*-pyruvate carboxylase activity is regulated posttranslationally and interactively by allosteric control by positive [glucose 6-P (Glc6*P*)] and negative (L-malate) effectors [1-3] and a complex regulatory phosphorylation cycle that modulates the enzyme's sensitivity to L-malate [4–6]. *In vitro* and *in vivo* experiments have established the existence of a reversibly light-activated protein-serine/threonine kinase [4–10] and a protein phosphatase type 2A [9] which mediate the interconversion of C_4 *P*-pyruvate carboxylase from a malate 'insensitive' (phosphorylated) light form to a malate 'sensitive' (dephosphorylated) dark form. Recently, site-directed mutagenesis of recombinant C_4 *P*-pyruvate carboxylase from sorghum has demonstrated that the replacement of the phosphorylatable serine (Ser8) by Cys or Asp gives an enzyme which cannot be phosphorylated in *vitro* but is still fully active [11]. This report, in concert with a subsequent study in which the Cys8 mutant was *S*-carboxymethylated by iodoacetic acid [12], demonstrated that phosphorylation mediates its regulatory effect on the malate sensitivity of C_4 *P*-pyruvate carboxylase through the addition of negative charge to the N-terminal domain of the protein.

In addition to this regulatory phosphorylation, plant *P*-pyruvate carboxylase can also be altered by limited proteolysis during purification. It has been reported that maize (C_4 [13]) or *Mesembryunthemum crystallinum* (inducible CAM [141]) *P*-pyruvate carboxylase which had been purified in the absence of the proteinase inhibitor chymostatin had lost a peptide fragment from its N- or C-terminus, in addition to its malate sensitivity and ability to be phosphorylated *in vitro*. Therefore, it has been suggested that the time required for enzyme purification may profoundly influence the regulatory properties of plant *P*-pyruvate carboxylase [4].

Recently, we have observed that a truncated form of maize *P*-pyruvate carboxylase which was missing the first 22 amino acids from its N-terminus, including the target serine at position 15, was more than 10-fold less sensitive to L-malate than the dephosphorylated maize enzyme containing a completely intact N-terminus (unpublished data described in [11, 15]). Interestingly, however, like the Ser8→Asp (S8D) [11] and S-carboxymethylated Ser8→Cys (S8C) [12] mutant *P*-pyruvate carboxylases and the *in vitro* phosphorylated form of the re-

combinant F sorghum Ser8 enzyme [11], no changes in other kinetic parameters were observed at optimal pH [15] (and unpublished data). In addition, there were no differences in the carbon-isotope effects on catalysis by the dephospho, phospho and truncated (Leu23) maize *P*-pyruvate carboxylase forms (O'Leary, M. H., unpublished data). Thus, limited proteolysis of this N-terminal regulatory domain during enzyme preparation may be responsible for much of the observed variation in malate sensitivity (cf. [4, 13, 14]). Indeed, although many detailed kinetic studies have been performed on C_4 and CAM *P*-pyruvate carboxylase (e.g. [16–21]), the documented effect of proteolysis during enzyme preparation [11, 13–15] and the relatively low malate sensitivity of *P*-pyruvate carboxylase often observed in these previous studies [16–19] suggest that the enzyme used in these prior investigations was partially or completely truncated at its N-terminus.

In the present study, we have undertaken the first detailed, steady-state kinetic analysis of non-phosphorylated, *in vitro* phosphorylated, and phosphorylation-site mutant (SSD) recombinant sorghum *P*-pyruvate carboxylase at both near-physiological (7.3) and optimal (8.0) pH in which it has been directly documented that the N-terminus of the purified enzyme is intact. Notably, we have shown that the apparent affinity of *P*-pyruvate carboxylase for both L-malate and its positive allosteric effector, Glc6*P*, changes dramatically and inversely when the enzyme is phosphorylated and subsequently assayed at pH 7.3. In addition, the use of the recombinant sorghum enzyme, instead of dark- leaf (dephospho) *P*-pyruvate carboxylase [22], ensures that we are beginning with the completely non-phosphorylated target enzyme.

MATERIALS AND METHODS

Purification and N-terminal sequencing of recombinant *P*-pyruvate carboxylase. Recombinant wild-type (Ser8) sorghum C_4 *P*-pyruvate carboxylase and the S8D mutant enzyme were purified by FPLC [11] and N-terminally sequenced [11, 12] as previously described.

***In vitro* phosphorylation of recombinant *P*-pyruvate carboxylase.** Purified recombinant wild-type or S8D mutant *P*-pyruvate carboxylase was dialyzed thoroughly against phosphorylation buffer (20 mM Tris/HCl, pH 8.0, 5 mM $MgC1_2$, 1mM di-thiothreitol, 5%, by vol., glycerol) at 4°C. To a 75-µl aliquot of this preparation, containing 200-400 µg purified *P*-pyruvate carboxylase, ATP and/or cAMP-dependent protein kinase (PKA ; Sigma Chemical Co., P-2645) were added, ATP to 1mM and PKA powder to a mass equal to that of *P*-pyruvate carboxylase protein. The reaction was allowed to proceed for up to 75 min at 30°C. Kinetic analysis was immediately performed after the specified time.

Steady-state enzyme kinetics. All 1-ml reactions were initiated with 0.5-1.0 µg *P*-pyruvate carboxylase and initial rates (30–90 s) were determined at 30°C and suboptimal (7.3) or optimal (8.0) pH. *P*-pyruvate carboxylase activity was assayed by coupling to exogenous malate dehydrogenase and following the oxidation of NADH at 340 nm. All reaction mixtures contained 50 mM Hepes/KOH (pH 7.3 or KO), 0.2 mM NADH, 10mM $MgCl_2$, 1mM $NaHCO_3$, 4 U (µmol/min) porcine heart mitochondrial malate dehydrogenase (Sigma Chemical Co., M-2634), and the variable concentrations of Glc6*P* and pH-adjusted *P*-pyruvate and L-malate necessary to perform the kinetic analyses. One unit of *P*-pyruvate carboxylase activity (U) corresponds to the amount of enzyme required to catalyze the oxidation of 1 µmol NADH/min by the coupling enzyme. Apparent K_m and $S_{0.5}$ (total *P*-pyruvate) values were evaluated from Lineweaver-Burk and Hill plots, respectively. Hill coefficients *(h)* were determined from the latter. The concentration range of *P*-pyruvate used to evaluate K_m and $S_{0.5}$ was 0.4–10 mM. Activation constants for Glc6*P* ($A_{0.5}$) at 1mM and 4 mM-*P*-pyruvate were determined using double-reciprocal plots of $1/(V - V_0)$ versus 1/ [Glc6*P*], where v = reaction velocity in the presence of varying concentrations of Glc6*P* and v_0 = reaction velocity in the absence of Glc6*P*. The concentration range of Glc6*P* used to evaluate $A_{0.5}$ was 0.2–4 mM, although activities at concentrations above those giving maximal increase in activation (saturating Glc6*P*) were omitted from the plot. Inhibition constants for L- malate (K_i) were determined from Dixon plots and the pattern of inhibition was evaluated by Lineweaver-Burk analysis. The concentration range of L-malate used to determine K_i was 0.1–1.6 mM at pH 7.3 and 1.5–12.0 mM at pH 8.0, with *P*-pyruvate concentrations varying over 1.5–7.5 mM. $I_{0.5}$ (L-malate) values at pH 7.3 and 2.5 mM *P*-pyruvate were determined from a Job plot [23] using the same concentration range of L-malate as for the K_i determinations. All rates arising from activities in which higher than saturating *P*-pyruvate concentrations were employed were omitted from the K_i and K_m determinations. All kinetic parameters presented are the means of duplicate determinations performed on two separate preparations of each purified enzyme (i.e. n = 4) and are reproducible to within ± 15% SE (or less). All plots were constructed using GraFit 3.0 by R. J. Leatherbarrow (available from Erithacus Software Ltd., Staines, UK).

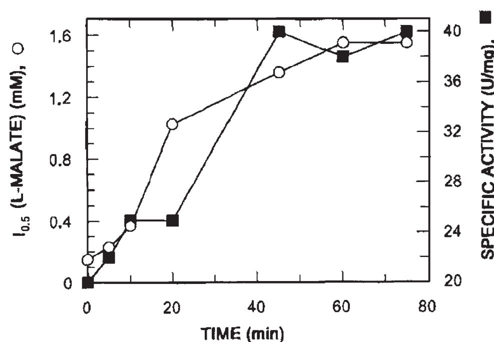

Figure 1. Time course of changes in $I_{0.5}$ (L-malate) and specific activity of recombinant sorghum C_4 *P*-pyruvate carboxylase during *in vitro* phosphorylation by PKA and MgATP. Specific activity (■) was measured at pH 7.3, 2.5 mM *P*-pyruvate; $I_{0.5}$ values (○) were determined at pH 7.3, 2.5 mM *P*-pyruvate and 0–2.0 mM L-malate. Job plots [23] were fitted by linear regression analysis and were reproducible to within ± 10% SE. Specific activity values were reproducible to within ± 5% SE.

RESULTS AND DISCUSSION

The final preparations of recombinant sorghum Ser8 and S8D C_4 *P*-pyruvate carboxylase were assessed to be homogeneous by SDS/PAGE, had final specific activities of approximately 40 U/mg protein (at pH 8.0, 5 mM *P*-pyruvate) and possessed intact N-termini as determined by covalent protein microsequencing [11].

Previous studies have documented that the catalytic subunit of mammalian PKA phosphorylates C_4 *P*-pyruvate carboxylase specifically at the target serine residue near the N-terminus **[24,** 25]. Figure 1 depicts a time course of the ATP- and PKA-dependent changes in $I_{0.5}$ (L-malate) and

Table 1. Steady-state kinetic analysis of purified sorghum recombinant *P*-pyruvate carboxylase enzyme forms at 30°C and pH 7.3 and 8.0. The Ser8 enzyme was phosphorylated *in vitro* by PKA and MgATP for 75 min at 30°C (see Figure 1).The values of $S_{0.5}$ (*P*-pyruvate) and *h* were evaluated using a Hill plot; $I_{0.5}$ (L-malate) was determined at 2.5 mM *P*-pyruvate (*P*Prv), K_a (Glc6*P*) at 1mM and 4 mM *P*-pyruvate. The values for $I_{0.5}$ (L-malate), *h* and K_a (total Glc6*P*) were reproducible to within ±10% SE, $S_{0.5}$ (total *P*-pyruvate), *V* and K_i (L-malate) to within ±5 % SE, and maximal activation to within ±15% SE. K_i (L-malate) values indicate competitive inhibition by L-malate, except for the Ser8-*P* form at pH 7.3 which indicates mixed inhibition.

Assay pH	Enzyme form	$S_{0.5}$ (*P*Prv)	*h*	*V*	K_i (L-Malate)	$I_{0.5}$ (L-Malate)	K_a (Glc6*P*) [activation] at 1 mM *P*Prv	K_a (Glc6*P*) [activation] at 4 mM *P*Prv
		mM		U/mg	mM	mM	mM [fold]	mM [fold]
7.3	Ser8	3.5	1.6	20.0	0.17	0.15	1.30 [19.0]	0.55 [2.3]
	Ser8-*P*	1.4	2.3	40.3	1.2	1.5	0.28 [4.0]	0.06 [1.5]
	Asp8	1.9	1.7	25.2	0.45	0.44	0.45 [4.5]	0.20 [1.8]
8.0	Ser8	1.6	1.1	40.0	4.0	23.5	0.22 [3.5]	0.30 [1.4]
	Ser8-*P*	1.4	1.2	42.0	5.0	26.2	0.22 [2.4]	0.20 [1.2]
	Asp8	1.4	0.9	42.6	4.8	23.0	0.25 [1.2]	0.20 [1.2]

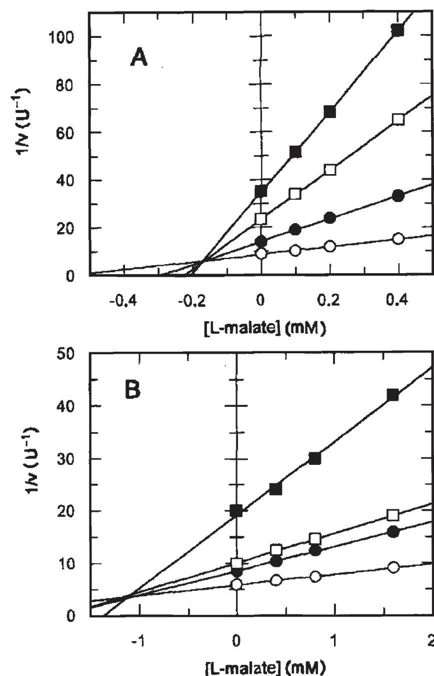

Figure 2. Dixon plot determinations of K_i (L-malate) for dephospho (A) and phospho (B) forms of recombinant sorghum *P*-pyruvate carboxylase at pH 7.3. Kinetic determinations, assays and *in* vitro phosphorylation of *P*-pyruvate carboxylase were as described in the Materials and Methods and Table 1. *P*-pyruvate concentrations (mM) were (A) 7.5 (○),5.0 (●),2.5 (□), 1.5 (■);(B) 5.0 (○),3.5 (●), 2.5 (□),1.5 (■).

specific activity of the recombinant wild-type (Ser8) sorghum *P*-pyruvate carboxylase assayed at pH 7.3 with 2.5 mM total *P*-pyruvate. The $I_{0.5}$ (L-malate) and specific activity of *P*-pyruvate carboxylase increased approximately 10-fold and 2-fold, respectively, in 60 min, after which no further change was observed. When either ATP or PKA was omitted from the *in vitro* phosphorylation mixture no change in $I_{0.5}$ (L-malate) or specific activity was observed (data not shown). Similarly, no change in either parameter occurred when the recombinant S8D phosphorylation-site mutant enzyme was incubated with ATP and/or PKA (data not shown).

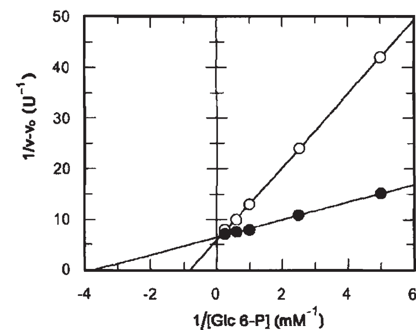

Figure 3. Double-reciprocal plot determination of $A_{0.5}$ (Glc6*P*) at pH 7.3 and 1 mM *P*-pyruvate. Kinetic determinations, assays and *in vitro* phosphorylation of *P*-pyruvate carboxylase were as described in the Materials and Methods and Table 1. Non-phosphorylated (○) and *in vitro* phosphorylated (●) forms of recombinant sorghum *P*-pyruvate carboxylase.

Table 1 summarizes the kinetic analyses of the purified recombinant enzyme forms (Ser8, Ser8-*P*, Asp8) at pH7.3 and pH 8.0, which represent the near-physiological and optimal pH values, respectively, of this cytosolic enzyme. It is readily apparent that when *P*-pyruvate carboxylase is assayed at optimal pH there are no substantial differences in any of the kinetic parameters examined for the three enzyme forms with the exception of the threefold difference in maximal activation by Glc6*P* between dephospho Ser8 and the S8D mutant at 1 mM *P*-pyruvate. In any event, however, the maximal activation for all three enzyme forms at pH 8.0 was 3.5-fold or less. These collective results are consistent with those observed previously at pH 8.0 by researchers in our (Lincoln) laboratory [11, 12].

In contrast, when the enzyme is assayed at a suboptimal but near-physiological pH it is clear that there are substantial differences in certain kinetic parameters (Table 1). Figure 2 depicts the Dixon plots used to determine the K_i (L-malate) values of the dephospho (Figure 2A) and phospho (Figure 2B) forms of recombinant sorghum *P*-pyruvate carboxylase. Phosphorylation of the Ser8 enzyme causes the $I_{0.5}$ (L-malate) to increase from 0.15 mM to 1.5mM at pH 7.3 (Table 1, Figure 1) while the K_i (L-malate) showed a corresponding increase from 0.17 mM to 1.2 mM (Table 1, Figure 2). The S8D mutant *P*-pyruvate carboxylase had intermediate $I_{0.5}$ and K_i values of about 0.45 mM. The $I_{0.5}$ (L -malate) values of the S8D mutant and dephospho forms

of *P*-pyruvate carboxylase at pH 7.3 are consistent with those reported previously [11, 12], although the $I_{0.5}$ value of the present Ser8 enzyme phosphorylated extensively by PKA (rather than maize *P*-pyruvate carboxylase-kinase [11]) is approximately 2.3-times higher. Inhibition of *P*-pyruvate carboxylase by L-malate was competitive in all cases except the phosphorylated form assayed at pH 7.3, which showed mixed inhibition (Table 1).

Figure 3 illustrates the double-reciprocal plot used to determine $A_{0.5}$ (Glc6*P*) for the phospho and dephospho forms of recombinant sorghum *P*-pyruvate carboxylase at pH 7.3. When the enzyme is assayed at 1mM *P*-pyruvate, phosphorylation causes the apparent K_a (Glc6*P*) to decrease from 1.3mM to 0.28 mM (Table 1, Figure 3) and the maximal activation to decrease from 19-fold to 4.0-fold (Table 1). Once again, the S8D mutant enzyme displayed intermediate values when assayed at pH 7.3 and 1mM *P*-pyruvate. At a *P*-pyruvate concentration of 4 mM, which approximates the $S_{0.5}$ and K_m of the Ser8 enzyme form at pH 7.3, substantially lower K_a (Glc6*P*) and maximal activation values were observed for all three enzyme forms (Table 1). We are unaware of other reports that phosphorylation of C_4 *P*-pyruvate carboxylase results in major changes in its interaction with both positive (Glc6*P*) and negative (L-malate) allosteric effectors at near-physiological pH (Table 1, Figures 2 and 3).

Phosphorylation causes a relatively modest increase in the *V* of *P*-pyruvate carboxylase when assayed at pH7.3 (Table 1). The S8D mutant is intermediate between the phosphorylated and non-phosphorylated forms of the enzyme. Phosphorylation also had a relatively minor effect on $S_{0.5}$ (total *P*-pyruvate) at pH 7.3, but not at pH 8.0. As with the other kinetic parameters, the mutant had an $S_{0.5}$ value at pH 7.3 intermediate between the phospho and dephospho forms of the enzyme (Table 1). However, when Lineweaver-Burk plots were used to evaluate K_m (total *P*-pyruvate) no significant effect of phosphorylation or S8D substi-tution was seen at either pH (pH 8.0, all values 1.2–1.3 mM; pH 7.3, all values 3.7–4.3 mM; $n = 6$, SE = ± 5%).

It is evident that phosphorylation of C_4 *P*-pyruvate carboxylase has a dramatic effect on certain of its kinetic properties when the enzyme is assayed at a near-physiological pH. Since *P*-pyruvate carboxylase is regulated allosterically *in vivo* by L-malate and Glc6*P* [1–6], the changes in K_i (L-malate), K_a (Glc6*P*), *V* and possibly $S_{0.5}$ (*P*-pyruvate) should have a tremendous combined effect on *in vivo* *P*-pyruvate carboxylase activity and probably account for the cardinal regulatory role of *P*-pyruvate carboxylase phosphorylation on the rate of leaf photosynthesis by C_4 plants [26]. Interestingly, the S8D mutant when assayed at pH 7.3 always displayed kinetic properties intermediate between the phospho and dephospho forms of *P*-pyruvate carboxylase. This may relate to the fact that phosphorylation introduces a dianionic side chain at position 8, whereas substitution of Asp for Ser8 (or carboxymethylation of the S8C mutant *P*-pyruvate carboxylase by iodoacetic acid [12]) causes the introduction of a monoanionic residue, thus attenuating the influence of negative charge on this N-terminal regulatory domain [11, 12].

In conclusion, we have performed here a detailed steady-state kinetic analysis of C_4 *P*-pyruvate carboxylase at near-physiological pH in which the intactness of the N-terminus has been documented. The use of recombinant sorghum *P*-pyruvate carboxylase has, in addition, enabled us to perform kinetics on the completely non-phosphorylated enzyme since the purified dark-leaf enzyme is not completely dephosphorylated [22]. We have shown that the differences in malate sensitivity between the phospho and dephospho enzyme forms are greater than previously recognized and, more importantly, that regulatory phosphorylation, in addition to desensitizing the enzyme to its negative allosteric effector, also causes a dramatic increase in its apparent affinity for Glc6*P*, its major positive allosteric effector. The changes in the kinetic parameters as a result of phosphorylation all favor increased enzyme activity *in vivo* and should lead to increased flux through the C_4 pathway of photosynthesis.

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