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## Kinetic analysis of the non-phosphorylated, *in vitro* phosphorylated, and phosphorylation-site-mutant (Asp8) forms of intact recombinant C<sub>4</sub> phosphoenolpyruvate 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<sub>4</sub> phosphoenolpyruvate (*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<sub>i</sub> for the negative allosteric effector L-malate was 0.17 mM, 1.2 mM and 0.45 mM while the apparent K<sub>a</sub> for the positive allosteric effector glucose 6-phosphate (Glc6P) 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<sub>4</sub> *P*-pyruvate carboxylase near its N-terminus has a relatively minor effect on *V* and K<sub>m</sub> (total *P*-pyruvate) but has a dramatic effect on the extent of activation by Glc6P, type of inhibition by L-malate and, most especially, K<sub>a</sub> (Glc6P) and K<sub>i</sub> (L-malate). Thus, regulatory phosphorylation profoundly influences the interactive allosteric properties of this cytosolic C<sub>4</sub>-photosynthesis enzyme.

**Keywords:** C<sub>4</sub> photosynthesis, phosphoenolpyruvate carboxylase, recombinant enzymes, regulatory protein phosphorylation, sorghum

**Abbreviations:** CAM, Crassulacean acid metabolism; Glc6P, glucose 6-phosphate; *P*-pyruvate or PPrv, phosphoenolpyruvate; *P*-pyruvate carboxylase, phosphoenolpyruvate 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); phosphoenolpyruvate 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).

Phosphoenolpyruvate (*P*-pyruvate) carboxylase is a cytosolic enzyme in bacteria, cyanobacteria, algae and higher plants [1, 2]. During C<sub>4</sub> photosynthesis and Crassulacean acid metabolism (CAM), it plays a cardinal role in the initial fixation of atmospheric CO<sub>2</sub> (as HCO<sub>3</sub><sup>-</sup>) into the C<sub>4</sub> dicarboxylic acids L-malate and aspartate [1–3]. In leaf tissue of C<sub>4</sub> and CAM species, *P*-pyruvate carboxylase activity is regulated posttranslationally and interactively by allosteric control by positive [glucose 6-P (Glc6P)] 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<sub>4</sub> *P*-pyruvate carboxylase from a malate 'insensitive' (phosphorylated) light form to a malate 'sensitive' (dephosphorylated) dark form. Recently, site-directed mutagenesis of recombinant C<sub>4</sub> *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 phos-

phorylation mediates its regulatory effect on the malate sensitivity of C<sub>4</sub> *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<sub>4</sub> [13]) or *Mesembryanthemum crystallinum* (inducible CAM [14]) *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<sub>4</sub> 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.

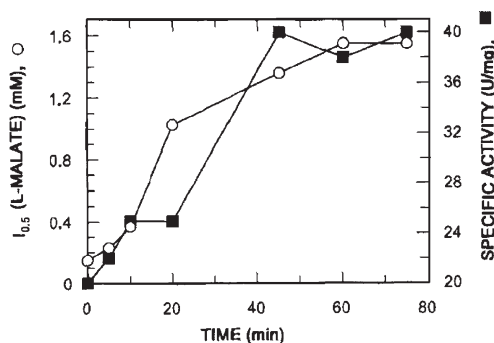
In the present study, we have undertaken the first detailed, steady-state kinetic analysis of non-phosphorylated, *in vitro* phosphorylated, and phosphorylation-site mutant (SSD) recombinant sorghum *P*-pyruvate carboxylase at both near-physiological (7.3) and optimal (8.0) pH in which it has been directly 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, Glc6P, 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<sub>4</sub> *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 MgCl<sub>2</sub>, 1mM di-thiothreitol, 5%, by vol., glycerol) at 4°C. To a 75- $\mu$ l aliquot of this preparation, containing 200–400  $\mu$ g 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  $\mu$ g *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<sub>2</sub>, 1mM NaHCO<sub>3</sub>, 4 U ( $\mu$ mol/min) porcine heart mitochondrial malate dehydrogenase (Sigma Chemical Co., M-2634), and the variable concentrations of Glc6P 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



**Figure 1.** Time course of changes in  $I_{0.5}$  (L-malate) and specific activity of recombinant sorghum C<sub>4</sub> *P*-pyruvate carboxylase during *in vitro* phosphorylation by PKA and MgATP. Specific activity ( $\blacksquare$ ) was measured at pH 7.3, 2.5 mM *P*-pyruvate;  $I_{0.5}$  values ( $\circ$ ) 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  $\pm 10\%$  SE. Specific activity values were reproducible to within  $\pm 5\%$  SE.

oxidation of 1  $\mu$ mol 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 Glc6P ( $A_{0.5}$ ) at 1mM and 4 mM-*P*-pyruvate were determined using double-reciprocal plots of  $1/(V - V_0)$  versus  $1/[Glc6P]$ , where  $v$  = reaction velocity in the presence of varying concentrations of Glc6P and  $v_0$  = reaction velocity in the absence of Glc6P. The concentration range of Glc6P used to evaluate  $A_{0.5}$  was 0.2–4 mM, although activities at concentrations above those giving maximal increase in activation (saturating Glc6P) 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  $\pm 15\%$  SE (or less). All plots were constructed using GraFit 3.0 by R. J. Leatherbarrow (available from Erithacus Software Ltd., Staines, UK).

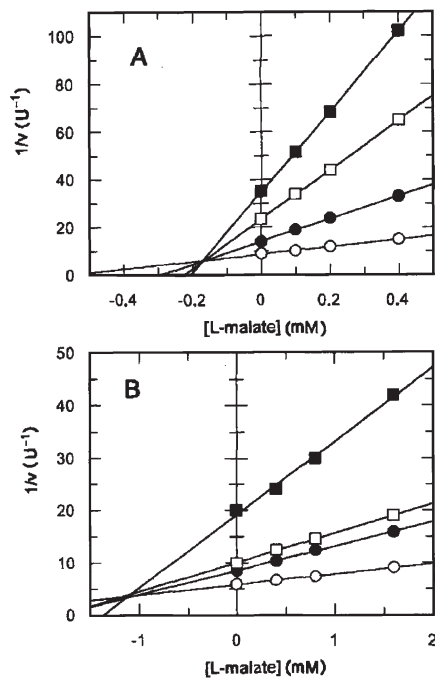
## RESULTS AND DISCUSSION

The final preparations of recombinant sorghum Ser8 and S8D C<sub>4</sub> *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<sub>4</sub> *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

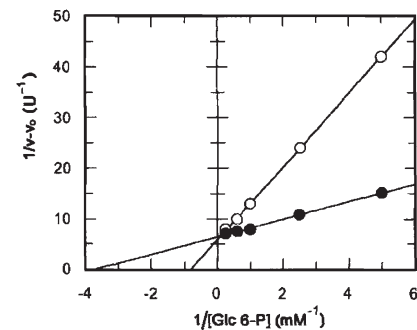
**Table 1. Steady-state kinetic analysis of purified sorghum recombinant *P*-pyruvate carboxylase enzyme forms at 30°C and pH 7.3 and 8.0.** The Ser8 enzyme was phosphorylated *in vitro* by PKA and MgATP for 75 min at 30°C (see Figure 1). The values of  $S_{0.5}$  (*P*-pyruvate) and  $h$  were evaluated using a Hill plot;  $I_{0.5}$  (L-malate) was determined at 2.5 mM *P*-pyruvate (PPrv),  $K_a$  (Glc6P) at 1mM and 4 mM *P*-pyruvate. The values for  $I_{0.5}$  (L-malate),  $h$  and  $K_a$  (total Glc6P) were reproducible to within  $\pm 10\%$  SE,  $S_{0.5}$  (total *P*-pyruvate),  $V$  and  $K_i$  (L-malate) to within  $\pm 5\%$  SE, and maximal activation to within  $\pm 15\%$  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    | $S_{0.5}$ (PPrv) | $h$ | $V$  | $K_i$ | $I_{0.5}$ | $K_a$ (Glc6P) [activation] at |            |
|----------|----------------|------------------|-----|------|-------|-----------|-------------------------------|------------|
|          |                |                  |     |      |       |           | (L-Malate)                    |            |
|          |                | mM               |     | U/mg | mM    |           | mM [fold]                     |            |
| 7.3      | Ser8           | 3.5              | 1.6 | 20.0 | 0.17  | 0.15      | 1.30 [19.0]                   | 0.55 [2.3] |
|          | Ser8- <i>P</i> | 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- <i>P</i> | 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  $A_{0.5}$  (Glc6P) 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 pH 7.3 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 Glc6P 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 1.5 mM 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  $A_{0.5}$  (Glc6P) 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  $K_a$  (Glc6P) 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  $K_a$  (Glc6P) and maximal activation values were observed for all three enzyme forms (Table 1). We are unaware of other reports that phosphorylation of C<sub>4</sub> *P*-pyruvate carboxylase results in major changes in its interaction with both positive (Glc6P) 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 pH 7.3 (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 substitution 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<sub>4</sub> *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 Glc6P [1–6], the changes in  $K_i$  (*L*-malate),  $K_a$  (Glc6P),  $V$  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<sub>4</sub> 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<sub>4</sub> *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 Glc6P, 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<sub>4</sub> pathway of photosynthesis.

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