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Kinetic analysis of the non-phosphorylated, in vitro phosphorylated, and phosphorylation-site-mutant (Asp8) forms of intact recombinant C₄ phosphoenolpyruvate carboxylase from sorghum

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Abstract: Steady-state analyses were performed on the non-phosphorylated, in vitro phosphorylated and phosphorylation-site mutant (Ser8→Asp) forms of purified recombinant sorghum C₄ phosphoenolpyruvate (P-pyruvate) carboxylase (EC 4.1.1.31) 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 Kᵢ for the negative allosteric effector l-malate was 0.17 mM, 1.2 mM and 0.45 mM while the apparent Kᵢ 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 C₄ P-pyruvate carboxylase near its N-terminus has a relatively minor effect on V and Kᵢ (total P-pyruvate) but has a dramatic effect on the extent of activation by Glc6P, type of inhibition by l-malate and, most especially, Kᵢ (Glc6P) and Kᵢ (l-malate). Thus, regulatory phosphorylation profoundly influences the interactive allosteric properties of this cytosolic C₄-photosynthesis enzyme.

Keywords: C₄ photosynthesis, phosphoenolpyruvate carboxylase, recombinant enzymes, regulatory protein phosphorylation, sorghum

Abbreviations: CAM, Crassulacean acid metabolism; Glc6P, glucose 6-phosphate; P-pyruvate or PPv, 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 C₄ photosynthesis and Crassulacean acid metabolism (CAM), it plays a cardinal role in the initial fixation of atmospheric CO₂ (as HCO₃⁻) into the C₄ dicarboxylic acids l-malate and aspartate [1–3]. In leaf tissue of C₄ 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 C₄ P-pyruvate carboxylase from a malate ‘insensitive’ (phosphorylated) light form to a malate ‘sensitive’ (dephosphorylated) dark form. Recently, site-directed mutagenesis of recombinant C₄ 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 C₄ 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 (C₄ [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-
corresponds to the amount of enzyme required to catalyze the mitochondrial malate dehydrogenase (Sigma Chemical Co., 10 mM MgCl₂). The reaction mixture contained 50 mM Hepes/KOH (pH 7.3), 0.2 mM NADH, and ATP within ± 5% SE. Specific activity values were reproducible to within ± 10% SE. Specific activity values were reproducible to within ± 5% SE.

Figure 1. Time course of changes in I₅₀ (L-malate) and specific activity of recombinant sorghum C₄ P-pyruvate carboxylase during in vitro phosphorylation by PKA and MgATP. Specific activity (■) was measured at pH 7.3, 2.5 mM P-pyruvate; I₅₀ 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.

oxidation of 1 μmol NADH/min by the coupling enzyme. Apparent Kₘ and S₀.₅ (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 Kₘ and S₀.₅ was 0.4–10 mM. Activation constants for Glc6P (A₀.₅) at 1 mM and 4 mM P-pyruvate were determined using double-reciprocal plots of 1/(V – Vₒ) versus 1/ [Glc6P], where v = reaction velocity in the presence of varying concentrations of Glc6P and Vₒ = reaction velocity in the absence of Glc6P. The concentration range of Glc6P used to evaluate A₀.₅ 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 (Kᵢ) 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 Kᵢ 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. I₅₀ (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 Kᵢ determinations. All rates arising from activities in which higher than saturating P-pyruvate concentrations were employed were omitted from the Kᵢ and Kₘ 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).

RESULTS AND DISCUSSION

The final preparations of recombinant sorghum Ser8 and SBD C₄ 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 C₄ 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 I₅₀ (L-malate) and

MATERIALS AND METHODS

Purification and N-terminal sequencing of recombinant P-pyruvate carboxylase. Recombinant wild-type (Ser8) sorghum C₄ P-pyruvate carboxylase and the SBD 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 SBD mutant P-pyruvate carboxylase was dialyzed thoroughly against 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) sorghum enzyme, instead of dark-leaf (dephospho) sorghum pyruvate carboxylase and the S8D mutant pyruvate carboxylase were determined from a Job plot [23] using the same concentration range of L-malate as for the Kᵢ determinations. All rates arising from activities in which higher than saturating P-pyruvate concentrations were employed were omitted from the Kᵢ and Kₘ 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).
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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).

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), \( K_i \) (Glc6P) at 1 mM and 4 mM P-pyruvate. The values for \( I_{0.5} \) (l-malate), \( h \) and \( K_i \) (total Glc6P) 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.

<table>
<thead>
<tr>
<th>Assay pH</th>
<th>Enzyme form</th>
<th>( S_{0.5} ) (PPrv)</th>
<th>( h )</th>
<th>( V )</th>
<th>( K_i )</th>
<th>( I_{0.5} )</th>
<th>( K_i ) (Glc6P) [activation at 1 mM PPrv]</th>
<th>( K_i ) (Glc6P) [activation at 4 mM PPrv]</th>
<th>( K_i ) (Glc6P) [activation at 1 mM PPrv]</th>
<th>( K_i ) (Glc6P) [activation at 4 mM PPrv]</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.3</td>
<td>Ser8</td>
<td>3.5</td>
<td>1.6</td>
<td>20.0</td>
<td>0.17</td>
<td>0.15</td>
<td>1.30 [19.0]</td>
<td>0.55 [2.3]</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ser8-P</td>
<td>1.4</td>
<td>2.3</td>
<td>40.3</td>
<td>1.2</td>
<td>1.5</td>
<td>0.28 [4.0]</td>
<td>0.06 [1.5]</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Asp8</td>
<td>1.9</td>
<td>1.7</td>
<td>25.2</td>
<td>0.45</td>
<td>0.44</td>
<td>0.45 [4.5]</td>
<td>0.20 [1.8]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8.0</td>
<td>Ser8</td>
<td>1.6</td>
<td>1.1</td>
<td>40.0</td>
<td>4.0</td>
<td>23.5</td>
<td>0.22 [3.5]</td>
<td>0.30 [1.4]</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>Ser8-P</td>
<td>1.4</td>
<td>1.2</td>
<td>42.0</td>
<td>5.0</td>
<td>26.2</td>
<td>0.22 [2.4]</td>
<td>0.20 [1.2]</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Asp8</td>
<td>1.4</td>
<td>0.9</td>
<td>42.6</td>
<td>4.8</td>
<td>23.0</td>
<td>0.25 [1.2]</td>
<td>0.20 [1.2]</td>
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</tr>
</tbody>
</table>

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 (●), 2.5 (+), 2.5 (+). 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 \( I_{\text{app}} \) 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 \( K_{\text{m}} \) (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 \( K_i \) (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 SSD 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 \( K_{\text{m}} \) of the Ser8 enzyme form at pH 7.3, substantially lower \( K_i \) (Glc6P) and maximal activation values were observed for all three enzyme forms (Table 1). We are unaware of other reports that phosphorylation of C4 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 pH7.3 (Table 1). The SSD 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 pH7.3, 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 phos- pho and dephospho forms of the enzyme (Table 1). However, when Lineweaver-Burk plots were used to evaluate \( K_{\text{m}} \) (total P-pyruvate) no significant effect of phosphorylation or SSD 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; \( n = 6, \text{SE} = \pm 5\% \)).

It is evident that phosphorylation of C4 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), \( K_i \) (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 C4 plants [26]. Interestingly, the SSD 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 diatomic side chain at position 8, whereas substitution of Asp for Ser8 (or carboxymethylation of the SCC 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 C4 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 C4 pathway of photosynthesis.

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