In Vivo Regulatory Phosphorylation Site in C₄-Leaf Phosphoenol/pyruvate Carboxylase from Maize and Sorghum

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ABSTRACT

Reversible seryl-phosphorylation contributes to the light/dark regulation of C₄-leaf phosphoenolpyruvate carboxylase (PEPC) activity in vivo. The specific regulatory residue that, upon in vitro phosphorylation by a maize-leaf protein-serine kinase(s), leads to an increase in catalytic activity and a decrease in malate-sensitivity of the target enzyme has been recently identified as Ser-15 in ³²P-phosphorylated/activated dark-form maize PEPC (J-A Jiao, R Chollet [1990] Arch Biochem Biophys 283: 300–305). In order to ascertain whether this N-terminal seryl residue is, indeed, the in vivo regulatory phosphorylation site, [³²P]phospho-peptides were isolated and purified from in vivo ³²P-labeled maize and sorghum leaf PEPC and subjected to automated Edman degradation analysis. The results show that purified light-form maize PEPC contains 14-fold more ³²P-radioactivity than the corresponding dark-form enzyme on an equal protein basis and, more notably, only a single N-terminal serine residue (Ser-15 in maize PEPC and its structural homolog, Ser-8, in the sorghum enzyme) was found to be ³²P-phosphorylated in the light or dark. These in vivo observations, combined with the results from our previous in vitro phosphorylation studies (J-A Jiao, R Chollet [1989] Arch Biochem Biophys 269: 526–535; [1990] Arch Biochem Biophys 283: 300–305), demonstrate that an N-terminal seryl residue in C₄ PEPC is, indeed, the regulatory site that undergoes light/dark changes in phosphorylation-status and, thus, plays a major, if not cardinal role in the light-induced changes in catalytic and regulatory properties of this cytoplasmic C₄-photosynthesis enzyme in vivo.

PEPC (EC 4.1.1.31) is the initial carboxylating enzyme in the C₄ pathway of photosynthesis (6). Its catalytic activity, regulatory properties (e.g. sensitivity to feedback inhibition by L-malate and allosteric activation by glucose 6-phosphate) and seryl-phosphorylation status undergo concomitant in vivo changes upon light/dark transitions of the parent leaf tissue in a variety of C₄ plants, including, among others, maize (7, 10, 15, 18), sorghum (5, 21), Porotula oleracea (9), and Setaria verticillata (16). Recent studies with a reconstituted in vitro phosphorylation system, comprised of purified dark-form maize leaf PEPC, a partially purified protein-serine kinase(s) from light-adapted leaves and ATP·Mg, established that the protein kinase-mediated changes in the catalytic activity and malate sensitivity of C₄-PEPC are directly correlated with the concomitant changes in the seryl-phosphorylation status of the target enzyme in vitro (11). The amino acid sequence of the single in vitro regulatory phosphorylation site and its flanking region have been determined as His-His-Ser(P)-Ile-Asp-Ala-Gln-Leu-Arg (12, 19). This N-terminal nonapeptide corresponds exactly to residues 13 to 21 in the deduced primary structure of maize leaf PEPC (8), with Ser-15 being the regulatory phosphorylation site (12, 19). This specific seryl residue is far removed from a species-invariant, active-site lysine residue (Lys-606 in maize PEPC) in the C-terminal region of the ~110-kD subunit polypeptide (14).

In this report, detached maize and sorghum leaves were fed [³²P]orthophosphate, and the ³²P-labeled PEPC was subsequently purified from light- and dark-adapted tissue for isolation and amino acid sequence analysis of the in vivo [³²P]-phosphopeptides. The results indicate that only a single, N-terminal seryl residue is ³²P-labeled in vivo. This specific serine is located at position 15 and 8 in maize and sorghum leaf PEPC, respectively. More importantly, the phosphorylation status of this N-terminal seryl residue undergoes changes in response to light/dark transitions; it is much more phosphorylated in the light than in the dark. These observations, together with previous homologous (11, 12) and heterologous (12, 19) reconstituted in vitro phosphorylation studies, demonstrate that the regulatory phosphorylation of a single, N-terminal seryl residue plays a key, if not cardinal role in the light-induced changes in catalytic activity and malate sensitivity of C₄-leaf PEPC in vivo.

MATERIALS AND METHODS

Materials

Carrier-free [³²P]orthophosphate (pH 2–3 in dilute HCl, 10 mCi/mL) was purchased from Amersham. The sources of all

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³ Abbreviations: PEPC, phosphoenolpyruvate carboxylase; PEP, phosphoenolpyruvate; TPCK, l-tosylamido-2-phenylethyl chloromethyl ketone; IDA, iminodiacetic acid; FPLC, fast-protein liquid chromatography.
other biochemicals and the conditions for growing maize (Zea mays L., cv Golden Cross Bantam) and sorghum (Sorghum vulgare, cv Tamaran) plants were as described (5, 11).

**In Vivo ³²P-Labeling of PEPC**

Two dark-adapted leaves (~0.7 g fresh weight each) were excised from 3-week-old maize plants that had been Pidepleted for 6 d. The detached leaves were fed 390 μCi of carrier-free ³²Pi in 2 mL of tap water in a 10-mL glass vial. The liquid was replaced with tap water as it was depleted by transpiration. Uptake of ³²Pi and labeling of leaf adenylate pools were achieved by illuminating the leaves with a 300-W, low-temperature lamp for 5 h at a light intensity of 600 to 800 μE m⁻² s⁻¹ (400–700 nm). The heat generated by the lamp was dissipated with a hair dryer. After this 5-h labeling period in the light, the leaves were transferred to darkness for 4 h, followed by another 4 h of illumination, and then maintained in the dark overnight. The dark tissue sample was from these leaves and the light sample was taken following a subsequent 90-min period of illumination. A similar labeling protocol was used with detached sorghum leaves.

**Purification of ³²P-Labeled PEPC**

**Maize PEPC**

An individual ³²P-labeled leaf was mixed with 4 g of the corresponding unlabeled leaf tissue and rapidly frozen in liquid nitrogen. The frozen sample was pulverized in a chilled mortar and extracted with 5 volumes of 50 mM Mops-KOH (pH 7.3), 14 mM 2-mercaptoethanol, 5 mM L-malate, 10 mM MgCl₂, 1 mM EDTA, and 2% (w/v) insoluble PVP. The crude homogenate was filtered through two layers of cheesecloth and centrifuged at 35,000g and 4°C for 5 min. The supernatant fluid was fractionated between 8.5 to 15% PEG-8000 as previously described (11). The 8.5 to 15% PEG precipitate was resuspended in 20 mM Tris-HCl (pH 8.0), 5% (v/v) glycerol, 1 mM DTT, 5 mM L-malate, and directly chromatographed twice on a prepacked Mono Q anion-exchange column (0.5 × 5 cm) connected to an automated FPLC system (10, 11).

**Sorghum PEPC**

³²P-Labeled PEPC from 4 to 6 darkened or illuminated sorghum leaves (totaling ~1.4 g fresh weight) was purified by affinity chromatography on an immunoadsorbent column prepared with monoclonal polyclonal antibodies against the sorghum green-leaf PEPC holoenzyme (20). The corresponding unlabeled carrier PEPC was purified as described above for maize PEPC except that the enzyme was extracted by homogenizing chopped leaves in a Waring blender. Purified PEPC was stored in 10 mM Tris-HCl (pH 8.0), 0.5 mM DTT, 5 mM L-malate, 0.1 mM NaCl, and 50% (v/v) glycerol at −20°C.

On a protein basis, the light/dark carboxylase-activity ratio for both purified maize and sorghum PEPC was about 1.6 when assayed (11) in the absence of L-malate at suboptimal levels (7, 9, 10, 16) of pH (7.5) and PEP (2.5 mm) and 5 mM MgCl₂. For maize leaf PEPC, the apparent Ki(malate) values (i.e. the malate concentration required for 50% inhibition of PEPC activity under the above assay conditions) were 0.39 and 0.18 mm for the purified light- and dark-enzymes, respectively. Inhibition of sorghum leaf PEPC by 0.5 mm L-malate was 26 and 72% for the purified light-form and dark-form enzymes, respectively.

**Digestion with Trypsin**

The ³²P-labeled sample of PEPC, containing the corresponding purified carrier enzyme (about 4 mg of total protein), was dialyzed against 0.1 M Tris-HCl (pH 8.0), for 1 h at room temperature. The dialyzed enzyme was then precipitated with 70% saturated ammonium sulfate at 4°C. The protein precipitate, collected by centrifugation at 30,000 g for 10 min, was then dissolved in 3 mL of 0.1 M NH₄HCO₃ (pH unadjusted) and dialyzed against this same solution at room temperature for 3 h. Digestion with TPCK-treated trypsin was performed for 24 h at 37°C (12).

**Purification of ³²P-Labeled Tryptic Phosphopeptides**

**Metal-Ion Affinity Chromatography**

A Fe³⁺-IDA Sepharose 6B column (10-mL bed volume) was prepared and regenerated as previously described (12). The tryptic digest was adjusted to pH 3.0 with 6 N HCl and loaded directly onto the Fe³⁺-IDA Sepharose column that had been equilibrated with 0.1 M acetic acid. The column was then washed, sequentially eluted, and the pH 9.0 eluant rechromatographed as described (12).

**FPLC-Based Reversed-Phase Chromatography**

Peptide fractions containing ³²P-radioactivity from the second Fe³⁺-IDA Sepharose elution-step at pH 9.0 were pooled, brought to 0.1% TFA (pH 3.0) (with 6 N HCl) and chromatographed on a prepacked Pharmacia PepRPC C₁₂/C₈ reversed-phase column (0.5 × 5 cm, 5-μm particle size) connected to an automated FPLC system (12, 14). The column was eluted with a linear gradient of 0 to 60% solvent B (0.1% TFA in CH₃CN) in 30 min at a flow rate of 1 mL/min. Solvent A was 0.1% TFA in water. A₂₁⁴nm peptide-peaks were collected manually, and ³²P-radioactivity was measured by Cerenkov counting.

**Peptide Sequencing**

³²P-labeled phosphopeptides from the reversed-phase chromatography step (see Fig. 2, arrows) were lyophilized, redisolved, and subjected to automated Edman degradation analysis by covalent protein sequencing technology with a MilliGen/Biosearch 6600 ProSequencer System (12, 14).

**Assays**

Protein content was measured according to Bradford (1) using the Bio-Rad dye reagent and crystalline bovine serum albumin as the protein standard. PEPC activity was assayed spectrophotometrically at 340 nm and 30°C by coupling to exogenous NADH-malate dehydrogenase/lactate dehydrogenase as previously described (11).
Denaturing Electrophoresis

SDS-Polyacrylamide vertical slab gels (10% acrylamide) were run as described (4, 17).

RESULTS

In Vivo 32P-Phosphorylation of PEPC

Under the conditions of 32P-labeling of detached maize leaves described in "Materials and Methods," the light/dark changes in catalytic activity and malate-sensitivity of PEPC were similar to those observed previously (10) with attached, Pi-sufficient leaves. Thus, these preliminary experiments indicated that prior Pi depletion of the attached leaves and their subsequent excision had no significant effect on the light-induced regulatory phosphorylation of PEPC in vivo. Following purification, ammonium sulfate precipitation, dialysis and Cerenkov counting of the light and dark enzyme-forms, the incorporation of 32P-radioactivity into maize leaf PEPC was about 14-fold greater in the light than in the dark on a mg-protein basis. This marked light/dark difference is illustrated in Figure 1B where purified light-form maize PEPC shows a much stronger 32P-signal than the corresponding dark-form enzyme. Similar results were obtained with sorghum leaf PEPC phosphorylated in vivo in light and darkness (data not shown), as well as in related 32P-labeling studies with maize (18) and sorghum (5) leaf tissue.

Purification and Sequencing of the In Vivo 32P-Labeled Phosphopeptides

Following exhaustive digestion of 32P-labeled PEPC with TPCK-treated trypsin, the crude digest was chromatographed twice on a Fe3+-IDA Sepharose 6B column for the preparative isolation of phosphopeptides (12 and references therein). Typically, the overall recovery of 32P-radioactivity from this metal-ion affinity chromatography system was in the range of 75 to 85% at the pH 9.0 elution step (data not shown; also see Fig. 1 in ref. 12). The 32P-labeled phosphopeptide fractions that eluted at this step were pooled and further purified by FPLC-based reversed-phase chromatography (Fig. 2). Regardless of the original source of the [32P]phosphopeptide sample (i.e., light versus dark maize and sorghum PEPC), 70 to 80% of the total 32P-radioactivity applied to the reversed-phase column coeluted with an A214 nm peptide peak that had a retention time of about 9.4 min (Fig. 2, arrows; also see Fig. 2 in ref. 12). Based on the absorbance at 214 nm, it is readily apparent that the relative amount of this specific phosphopeptide is much greater in light-form PEPC than in the corresponding dark-form enzyme. Although additional A214 nm peptide peaks were routinely observed with the dark samples (Fig. 2B), none of them contained 32P-radioactivity. Phosphoamino acid analysis (11, 12) of the tryptic [32P]phosphopeptide purified from the in vivo phosphorylated maize light-form enzyme (Fig. 2A, arrow) revealed the exclusive presence of phosphoserine (data not shown [also see refs. 5, 10–12, 18]).

Automated Edman degradation analysis of the purified maize (Fig. 2, arrows) and sorghum [32P]phosphopeptides indicated an identical His-His-Ser(P)-Ile-Asp-Ala-Gln-Leu-Arg amino acid sequence for both the light- and dark-samples (Table I). This in vivo regulatory phosphorylation site is identical to that recently identified from reconstituted in vitro
phosphorylation studies with dark-form maize PEPC and either a maize leaf protein-serine kinase(s) (12) or mammalian protein kinase A (12, 19). This nonapeptide corresponds exactly to residues 13 to 21 and 6 to 14 in the deduced primary structures of maize (8) and sorghum (3) leaf PEPC, respectively (Table I).

**DISCUSSION**

Covalent protein phosphorylation is a major mechanism for posttranslational enzyme modification and plays a ubiquitous role in regulating cellular metabolism in both eukaryotes and prokaryotes. In order to ascertain whether the covalent phosphorylation of a particular target enzyme or protein is of truly physiological significance, the following two general criteria must be met. First, the biological function (e.g. enzyme activity, regulatory properties) of the target protein must be altered by reversible changes in its phosphorylation status in response to external and/or internal stimuli. Second, the relationship between the phosphorylation status and biological function of the target protein must be demonstrated both in vitro and, most especially, in vivo. In the specific case of C₄ leaf PEPC, the results from prior in vivo studies indicated that the degree of seryl-phosphorylation of this cytoplasmic enzyme was greater in the light than in the dark (5, 10, 18). Moreover, this increase in covalent phosphorylation status was correlated with concomitant increases in the target enzyme's activity and, more notably, decreases in its sensitivity to feedback inhibition by L-malate both in vivo (10, 18) and in reconstituted phosphorylation studies in vitro (11, 12, 19). As a result of this latter work, a single, N-terminal serine residue (Ser-15) was pinpointed as the regulatory phosphorylation site in dark-form maize PEPC that was ³²P-phosphorylated/activated in vitro by either a partially purified maize leaf protein-serine kinase (12) or mammalian protein kinase A (12, 19). However, direct experimental evi-

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**Table I. Automated Edman Degradation Analysis of the Tryptic Phosphopeptides Purified by Metal-Ion Affinity and FPLC-Based Reversed-Phase Chromatography from in Vivo Phosphorylated Light (L) and Dark (D) Forms of PEPC**

<table>
<thead>
<tr>
<th>Cycle</th>
<th>Amino Acid</th>
<th>L-Maizeᵇ</th>
<th>D-Maizeᵇ</th>
<th>L-Sorghumᶜ</th>
<th>Position in Deduced Sequence*</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>pmol</td>
<td></td>
<td></td>
<td>Maize Sorghum</td>
</tr>
<tr>
<td>1</td>
<td>His</td>
<td>68</td>
<td>12</td>
<td>9</td>
<td>13</td>
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</tr>
<tr>
<td>3</td>
<td>Serᵈ</td>
<td>___</td>
<td>___</td>
<td>___</td>
<td>15</td>
</tr>
<tr>
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<td>Ile</td>
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<td>13</td>
<td>16</td>
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<tr>
<td>5</td>
<td>Asp</td>
<td>16ᵇ</td>
<td>7ᵇ</td>
<td>7ᵇ</td>
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</tr>
<tr>
<td>6</td>
<td>Ala</td>
<td>89ᵇ</td>
<td>16ᵇ</td>
<td>16ᵇ</td>
<td>18</td>
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<tr>
<td>7</td>
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<td>12ᵇ</td>
<td>16ᵇ</td>
<td>19</td>
</tr>
<tr>
<td>8</td>
<td>Leu</td>
<td>41ᵇ</td>
<td>10ᵇ</td>
<td>13ᵇ</td>
<td>20</td>
</tr>
<tr>
<td>9</td>
<td>Arg</td>
<td>35ᵇ</td>
<td>10ᵇ</td>
<td>9ᵇ</td>
<td>21</td>
</tr>
</tbody>
</table>

* From the deduced amino acid sequences of maize (8) and sorghum (3) leaf PEPC. ᵇ See Figure 2, arrows. ᶜ Quantitative data for the purified phosphopeptide from sorghum dark-form PEPC are not available, but the amino acid sequence of the nonapeptide is identical to that from L-sorghum. ᵈ Note that covalent protein sequencing technology converts phosphoserine residues into the dehydroserine (A₃₁₃nm) and, to a much lesser extent, serine (A₂₆₉nm) adducts (12 and Milligen/Biosearch, personal communication).  * Note that the peptide was covalently attached to the Sequel-n-AA membrane by its free carboxyl groups (12).

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**Figure 3. Proposed molecular mechanism for the light/dark regulation of PEPC activity in vivo in the C₄ mesophyll-cytoplasm by reversible seryl phosphorylation.** The circle and square represent two putative conformational states of the −110-kD PEPC subunit induced by phosphorylation/dephosphorylation of a single, N-terminal serine residue (e.g. Ser-15 or Ser-8 in maize or sorghum PEPC, respectively). PK, PEPC protein-serine kinase that is reversibly light-activated in vivo by some mechanism, indicated by a question mark (?), involving cytoplasmic protein synthesis and degradation as a primary component (4, 15); PP, type 2A protein phosphatase (2). Adapted from Jiao and Cholette (13).
In order to account for the reversible, light-induced increases in phosphorylation-status of Ser-15 and Ser-8 in maize and sorghum leaf PEPC carboxylase, respectively, in vivo regulation of the PEPC protein-serine kinase(s) (11) and/or type 2A protein phosphatase(s) (2) must be inferred. Whereas no information is available concerning the regulatory properties of this okadaic acid-sensitive protein phosphatase (2), we have recently obtained data to indicate that the maize PEPC protein-serine kinase(s) is reversibly light-activated in vivo by some mechanism involving cytoplasmic protein synthesis and degradation as a primary component (4, 15). These recent findings (4, 15) imply that the light signal and ensuing activation/seryl-phosphorylation of the target enzyme in the C₄ mesophyll-cytoplasm involves a cyclic regulatory cascade (Fig. 3).

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LITERATURE CITED