Pyruvate Orthophosphate Dikinase from the Immature Grains of Cereal Grasses

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ABSTRACT

Pyruvate orthophosphate dikinase has been identified in the green grains of eight cereal grasses, most of which are classified as C₄ plants. The wheat (Triticum aestivum L. cv. Lerma Rojo) grain enzyme was further investigated: activity was low in very young grains, increased to a maximum at about 25 days after anthesis, then returned to a low level as the grain matured. It appeared to be located in the aleurone layer. A procedure was developed for obtaining partially purified preparations of pyruvate orthophosphate dikinase from the ears of wheat, oat (Avena sativa L.), barley (Hordeum distichum L.), and rye (Secale cereale L.). These preparations were suitable for measuring activities in both the forward and reverse reaction directions. The affinities of these enzymes for the six substrates (pyruvate, orthophosphate, and ATP in the forward reaction; phosphoenolpyruvate, pyrophosphate, and AMP in the reverse reaction) were determined and found to be similar to the reported affinities of the enzyme from the leaves of the C₄ plant Zea mays. A possible role for pyruvate orthophosphate dikinase in cereal grains is considered briefly.

One of the more interesting enzymological features of the C₄ pathway of photosynthesis is the presence in mesophyll chloroplasts of the enzyme pyruvate Pi dikinase (1, 9, 10) which catalyzes the regeneration of the CO₂ acceptor PEP from pyruvate according to the equation:

\[ \text{Pyruvate} + \text{ATP} + \text{Pi} \rightarrow \text{Mg}^{2+},\text{NH}_4^+ \text{PEP} + \text{AMP} + \text{PPi} \]

For some years it was believed that the enzyme was present only in those higher plants possessing the C₄ pathway (C₄ plants) or CAM (CAM plants) (10, 12, 18), but a preliminary observation by Duffus and Rosie (4) led to the detection of an appreciable level of pyruvate Pi dikinase activity in the immature grains of wheat (15), confirming that at least this C₃ plant has the potential for synthesizing this enzyme.

It seemed worthwhile to extend these investigations in order to confirm whether or not the enzyme exists in similar tissues of other C₃ plants, and also to gain more information on the wheat grain enzyme in order to evaluate its metabolic role.

MATERIALS AND METHODS

Materials. Wheat (Triticum aestivum L. cv. Lerma Rojo) was grown as described previously (15). Green ears of oat (Avena sativa L.), barley (Hordeum distichum L.), rye (Secale cereale L.), and other grains were obtained from the Münster Botanic Gardens or from local fields. Biochemicals and auxiliary enzymes were obtained as stated previously (15). Sephadex was purchased from Pharmacia (Uppsala), and Ca phosphate gel (hydroxyapatite SC) was supplied by Serva (Heidelberg).

Detection of Pyruvate Pi Dikinase Activity in Small Samples of Tissue. Grain or grain part (2 g) was ground with 0.2 g PVP and 2 ml extraction buffer (15) using mortar and pestle, and protein that precipitated between 35% and 50% saturation with ammonium sulfate was obtained as described previously (15). For all experiments other than those in Table I (see legend), this precipitate was dissolved in 1 ml buffer containing 100 mM Tris-HCl (pH 7.0), 10 mM MgSO₄, 10 mM 2-mercaptoethanol, and 1 mM EDTA, dialyzed against 1 L this buffer for 3 h, and then centrifuged at 25,000g for 20 min. The supernatant was assayed in the direction of PEP formation. All operations were at room temperature.

Partial Purification of Pyruvate Pi Dikinase from Cereal Ears. Cereal ears were selected at the stage where grains were near fully grown but still quite green. Ears (600 g) were washed, ground in a manual kitchen mincer (Alexanderwerk No. 8, Remshalden, Germany) with about 700 ml Tris buffer (see above) and 30 g PVP. The homogenate was centrifuged (25,000g, 20 min), and the supernatant was filtered through Miracloth (Calbiochem), then fractionated by addition of solid ammonium sulfate. Protein that precipitated between 40% and 50% saturation was collected by centrifugation (25,000g, 20 min), dissolved in a minimum volume (8 to 15 ml) of Tris buffer, dialyzed for 3 h in 4 L same buffer, then centrifuged at 25,000g for 20 min. Glycerol was added to a final concentration of 10% (v/v). All operations up to this step were at room temperature, but subsequent steps were at 4°C. The preparation was filtered through a column of Sephadex G-200 (5 x 50 cm), using the Tris buffer containing glycerol for equilibration and elution. One peak of enzyme activity was obtained (Fig. 1A); the seven fractions constituting the activity peak were combined and concentrated to approximately 1 ml over a Diaflo XM50 ultrafiltration membrane (Amicon, Lexington, MA), and glycerol was added to 40% (v/v). The final purification step involved chromatography on a column of Ca-phosphate (2.2 x 8 cm) equilibrated with 10 mM K-phosphate (pH 7.0) containing 10 mM MgCl₂, 10 mM 2-mercaptoethanol, 1 mM EDTA, and 10% (v/v) glycerol. The preparation, to which K-phosphate was added to a final concentration of 10 mM, was applied to the column. After washing with 3 bed volumes of the phosphate buffer, enzyme activity was eluted with a linear gradient (0.01–0.50 M) of K-phosphate containing the same additions as noted above. One peak of activity was found (Fig. 1B); this was concentrated as described above, except the concentrated enzyme was then washed over the Diaflo membrane thrice with 50 ml 50 mM Tris-HCl buffer (pH 7.0), containing 10 mM MgCl₂, 10 mM 2-mercaptoethanol, 1 mM EDTA, and 10% glycerol, in order to remove Pi. The
final preparation was free of PEP-carboxylase activity. Providing glycerol was added (40% v/v), it could be stored at -24°C for 1 month with less than 13% loss in activity.

Estimation of Protein. Protein was determined by the biuret procedure as described by Layne (14).

Estimation of Pyruvate Pi Dikinase Activity. Enzyme activity in the direction of PEP formation was measured as described previously (15), except 10 μmol NH₄Cl (20) were included in all reaction mixtures. K⁺, but not Na⁺, could replace this NH₄⁺ requirement. For measurements in the direction of pyruvate formation, reaction mixtures contained (in a final volume of 1 ml) 100 μmol imidazole-HCl buffer (pH 7.0), 50 μmol NH₄Cl, 5 μmol MgCl₂, 1 μmol EDTA, 1 μmol ammonium hexaamidophosphate (to inhibit pyrophosphatase), 5 μmol DTT, 1 μmol PPI, 0.5 μmol AMP, 0.25 μmol NADH, 1 unit lactate dehydrogenase, and 20 μl pyruvate Pi dikinase preparation. After 5 min preincubation at 22°C, reaction was initiated by the addition of 1 μmol PEP. Enzyme activity was calculated from the change in A at 366 nm. The wheat grain enzyme was also assayed by coupling ATP formation to NADP reduction: in these tests reaction mixtures were as above, except NADH and lactate dehydrogenase were replaced by 0.3 μmol NADP and 1 unit each hexokinase and glucose-6-P dehydrogenase, and 5 μmol glucose were included.

RESULTS

Presence of Pyruvate Pi Dikinase Activity in Cereal Grains and Leaves. Besides barley (4) and wheat (15), six other cereal grains were found to contain pyruvate Pi dikinase activity in the developing grains (Table I). Most are C₃ grasses, although activity was also found in the grains of Setaria italica, which is a C₄ grass (16). However, in the developing grains of the C₄ grass Panicum miliaceum (21) and the C₂-C₄ intermediate grass Panicum milioide (3, 18), and in the developing kernels of Zea mays, activity was essentially absent. Similar experiments with developing pea seeds and tomato fruits showed no activity in these tissues.

It is well established that the leaves of C₄ plants possess considerable enzyme activity whereas the leaves of C₃ plants lack activity (10, 11, 15), so it was of interest to look for activity in the leaves of the C₄-C₃ intermediate plant P. milioide using the procedure described in Table I. However, no significant activity could be confirmed. This finding contrasts with that of Rathnam and Chollet (17), but that report does not unequivocally confirm the presence of the enzyme, in that dependence of enzyme activity on Pi and pyruvate was not mentioned. In the present experiments, failure to detect activity was not attributed to enzyme inactivation, since maize-leaf enzyme activity was observed after 1 g maize leaves and 1 g P. milioide leaves were extracted together.

Partial Purification and Kinetic Properties of Grain Pyruvate Pi Dikinase from Wheat, Oat, Barley, and Rye. The preparations of pyruvate Pi dikinase obtained from cereal ears were sufficiently pure and free of interfering enzymes to permit continuous photometric assays in both reaction directions. In previous experiments (15), it was not possible to assay in the direction of pyruvate formation because of high PEP carboxylase activity in those preparations, but in the present experiments PEP carboxylase was eliminated by (a) narrowing the ammonium sulfate fractionation to 40 to 50%, and (b) Ca phosphate gel chromatography: pyruvate Pi dikinase was eluted by 0.05 M phosphate (Fig. 1B), while PEP carboxylase remained on the gel until the phosphate concentration reached 0.2 M (not shown).

The specific activity of pyruvate Pi dikinase in the partially purified wheat preparation was 34 times that in the 40 to 50% ammonium sulfate fraction, and contained 17% of the activity present in that fraction (enzyme activity could not be measured in the crude extract because of excessive blank activities (15)). Enzyme activity in this preparation was shown to be totally dependent on each of the three substrates (pyruvate, ATP, and Pi) in the
direction of PEP synthesis, as reported earlier (15), and also totally
dependent on each of the three substrates (PEP, AMP, and PPI)
in the direction of pyruvate synthesis (Fig. 2). The estimated
apparent $K_m$ values were 24 mM (pyruvate), 24 mM (ATP), 670 mM
(PI), 25 mM (PEP), 10 mM (AMP), and 30 mM (PPI). Almost identical
values for PEP, AMP, and PPI were obtained when the hexoki-
nase-glucose-6-P dehydrogenase assay system was used.

Experiments similar to those in Figure 2 were carried out with
enzyme preparations from oat, barley, and rye, and the respective
$K_m$ values obtained with these three enzymes were similar to those
estimated for the wheat enzyme (above). In addition, the $K_m$
values for these cereal enzymes were close to those reported for
the enzyme from C₄ plant leaves (1, 10, 20) and from the nonpho-
tosynthetic bacteria Propionibacterium shermanii (5) and Aceto-
bacter aceti (19).

### Relationship between Developmental Stage and Pyruvate Pi
Dikinase Activity in Wheat Grains

As a first step toward elucidating the physiological role of pyruvate Pi dikinase in cereal
grains, the activity of the enzyme was followed during wheat grain
development and compared to Chl, protein, and starch contents
(Fig. 3). At early stages (up to 7 days after anthesis) activity was
barely detectable, but thereafter it increased rapidly to a maximum
at about 25 days after anthesis and then declined again to low
levels in the mature grain. The peak activity occurred almost
at 21 days after anthesis and then declined.

Extracts from developing wheat grains were assayed for
activity and the results are given in Table I. The enzyme
activity of the developing wheat grains was higher in the
embryo than in any other tissue at all developmental stages.

### Table I. Pyruvate Pi Dikinase Activity in Various Anatomical Tissues of
Immature Wheat Grains

<table>
<thead>
<tr>
<th>Grain or Tissue</th>
<th>Weight (mg/grain)</th>
<th>Enzyme Activity (nmol/grain·min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>At 21 days after anthesis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Whole grain</td>
<td>53</td>
<td>4.8</td>
</tr>
<tr>
<td>Outer pericarp</td>
<td>4.6</td>
<td>0</td>
</tr>
<tr>
<td>Inner pericarp plus aleurone</td>
<td>11.4</td>
<td>3.5</td>
</tr>
<tr>
<td>Endosperm</td>
<td>35</td>
<td>0.002</td>
</tr>
<tr>
<td>Embryo</td>
<td>1.4</td>
<td>0</td>
</tr>
<tr>
<td>At 26 days after anthesis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Whole grain</td>
<td>65</td>
<td>5.1</td>
</tr>
<tr>
<td>Outer pericarp</td>
<td>5.1</td>
<td>0</td>
</tr>
<tr>
<td>Inner pericarp</td>
<td>5.2</td>
<td>0</td>
</tr>
<tr>
<td>Aleurone plus endosperm</td>
<td>53</td>
<td>4.4</td>
</tr>
<tr>
<td>Embryo</td>
<td>1.8</td>
<td>0</td>
</tr>
</tbody>
</table>

Fig. 2. Substrate saturation curves for wheat grain pyruvate Pi dikinase in both reaction directions. Enzyme was purified and assayed as described under "Materials and Methods," except the concentrations of substrates were varied as shown.

Fig. 3. Pyruvate Pi dikinase activity, and starch, soluble protein and
Chl contents, and fresh weight of wheat grains at various stages of
maturity. Enzyme activity was obtained from small (2 g) samples of grain
and assayed in the direction of PEP formation. Fresh weight is the average
weight of the number of grains in 2 g. Chl (2) in the homogenate, protein
(14) in the supernatant after centrifugation, and starch (6) in 0.2-g samples
of grain were estimated as described in the references. The units shown
are mg/grain for fresh wt, soluble protein, and starch, and µg/grain for
Chl.
to lose moisture (Fig. 3).

**Enzyme Location.** Earlier investigations (4, 15) had suggested that pyruvate Pi dikinase might be in the chloroplast-containing inner pericarp of cereal grains. More detailed studies have now shown that the aleurone layer is the most probable location of the enzyme in grains (Table II). It was not possible to separate cleanly the aleurone layer from developing grains, but it was found that the aleurone layer adhered mostly to the inner pericarp when grains were dissected 21 days after anthesis, but when dissected at 26 days after anthesis it adhered predominantly to endosperm tissue. Enzyme activity was obtained from both aleurone samples, but not from inner pericarp (obtained aleurone-free at 26 days) or from endosperm (obtained aleurone-free at 21 days). No activity was found associated with the outer pericarp or with the embryo (Table II).

**Preliminary Experiments on Cold Inactivation and Light-Mediated Activation.** Light-mediated activation and cold inactivation are characteristic features of pyruvate Pi dikinase from the leaves of C₄ plants (7, 8, 21), but would seem to require some modification for the dikinase located in chloroplast-free cells of a temperate plant. Initial tests with the wheat grain enzyme failed to show any significant change in extractable activity either when the grains were left overnight at -15°C, or when the ears were darkened (by covering with aluminum foil) from anthesis onwards.

**CONCLUDING REMARKS**

The present experiments confirm beyond any reasonable doubt that the developing grains of C₄ cereal grasses contain appreciable levels of pyruvate Pi dikinase, inasmuch as the enzyme activity has now been shown to depend on all substrates in both reaction directions, and the affinity of the enzyme for these substrates is comparable to that of pyruvate Pi dikinase from other established sources.

The metabolic role of the enzyme in the growing grain is not yet known. It may be required to generate PEP which, together with PEP carboxylase, could recapture respiratory CO₂, a good proportion of which is reported to be refixed by the developing grain (13). It now seems even more realistic to consider that this metabolism might be closely associated with amino acid synthesis, since the enzyme is now known to be localized in the aleurone layer. One possibility is that the product of PEP carboxylation (oxaloacetate) is aminated to form aspartate. Alanine, transported into the developing grain (22), could be the source of both the amine group for this aspartate synthesis, and the pyruvate used by pyruvate Pi dikinase. Net synthesis of aspartate from alanine plus CO₂ could therefore be achieved. Current experiments in this laboratory are attempting to evaluate this possibility.

**LITERATURE CITED**

22. _Wallace W, JS Pat_ 1967 Nitrate assimilation in higher plants with special reference to the Cocklebur (Xanthium pensylvanicum Wallr.) Ann Bot 31: 213-228