Some Properties of Pea Mitochondrial Phospho-Pyruvate Dehydrogenase-Phosphatase

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ABSTRACT

Reactivation of the pea mitochondrial pyruvate dehydrogenase complex was the result of dephosphorylation catalyzed by phospho-pyruvate dehydrogenase-phosphatase, an intrinsic component of the complex. Phosphatase activity was dependent upon divalent metal ions, with Mg$^{2+}$ more effective than Mn$^{2+}$ or Co$^{2+}$. The Michaelis constants for Mg$^{2+}$, Mn$^{2+}$, and Co$^{2+}$ were 3.8, 1.7, and 1.4 millimolar, respectively. Neither the rate nor the extent of activation of the phosphatase by Mg$^{2+}$ or Mn$^{2+}$ was affected by up to 100 units per assay of megamodulin. Calcium ions did not activate pea mitochondrial phospho-pyruvate dehydrogenase-phosphatase, and low concentrations of Ca$^{2+}$ antagonized activation by other divalent cations. Phosphatase activity was inhibited by fluoride and ortho-phosphate but not by molybdate or vanadate. Krebs cycle intermediates, adenylates, polyamines, amino acids, and phosphoamino acids were without effect upon pea mitochondrial phospho-pyruvate dehydrogenase-phosphatase activity in vitro.

Mitochondrial PDC is regulated in part by reversible phosphorylation (4, 19). The PDH component of the complex is subject to multisite phosphorylation on the E$_{1s}$-subunit by an intrinsic kinase, resulting in complete inactivation. Reactivation is the result of dephosphorylation catalyzed by a divalent cation-dependent P-PDH-phosphatase, also an intrinsic component of the complex. We have recently reviewed the regulation of plant mitochondrial PDCs by phosphorylation (11). While it has been shown that P-PDH-phosphatase is a component of plant mitochondrial PDCs (16-18), little detailed information about this activity is available. Herein we characterize the reactivation of pea mitochondrial P-DPC, and present some properties of the phosphatase.

MATERIALS AND METHODS

Unless otherwise indicated, inorganic chemicals were from J. T. Baker. CoA and NAD were from P-L Biochemicals. Other biochemicals and NaMoO$_4$ were from the Sigma Chemical Co. Megamodulin was partially purified from wheat germ or rat brain (8). All other materials were previously described (1, 12).

Pea seedlings were grown, mitochondria isolated, the PDC partially purified, and assayed according to established procedures (1, 12, 16). Incubation of the PDC with ATP resulted in inactivation through phosphorylation catalyzed by the intrinsic PDH-kinase. Typically, incubation with 200 μM MgATP for 5 min at room temperature resulted in complete inactivation. Two different methods were used to measure P-PDH-phosphatase activity. Method one was based upon the divalent-cation dependent reactivation of ATP-inactivated PDC. MgCl$_2$ was added to an aliquot of inactivated PDC giving a final concentration of 10 mM. Following a 40 min incubation at room temperature, PDC activity was assayed as previously described. In method two, [$\gamma$-32P]ATP was used for inactivation of PDC. Inactive, [32P]PDC was then incubated with MgCl$_2$ at 10 mM. At various times aliquots were removed and injected into a microfuge tube containing an equal volume of ice-cold 2% (w/v) TCA. After incubation on ice for 60 min, samples were centrifuged in an Eppendorf microfuge and washed twice by resuspension in ice-cold 10% (w/v) TCA, and repelletized. The washed, TCA-insoluble material was solubilized by heating in 4 M urea, 1% (w/v) SDS, and 2% (v/v) 2-mercaptoethanol. Triplicate aliquots were removed and radioactivity measured by Cerenkov counting using the 32P program of a Beckman LSC-250 spectrometer. Analysis of the TCA-insoluble pea mitochondrial proteins by SDS-PAGE showed that under our assay conditions more than 90% of 32P was incorporated into the E$_{1s}$-subunit of the PDH component of the complex (M, 43,300) (11). Alterations in assay procedures are indicated in the figure legends.

All other methods were previously described (1, 12).

RESULTS

Incubation of pea mitochondrial PDC with MgATP resulted in rapid inactivation. Concomitant with the inactivation was incorporation of 32P from [$\gamma$-32P]ATP into the PDC (Fig. 1). Upon incubation with MgCl$_2$, complex activity was restored and 32P was lost (Fig. 1).

Inactive, phosphorylated PDC was incubated with a variety of cations to test the specificity of the phosphatase (Fig. 2). Monovalent cations, Ca$^{2+}$, and polyvalent cations were unable to activate P-PDH-phosphatase. At lower concentrations, Mn$^{2+}$ and Co$^{2+}$ were as effective as Mg$^{2+}$, but at higher concentrations Mg$^{2+}$ was the best activator of the phosphatase (Fig. 2). Polyamines were unable to activate P-PDH-phosphatase, and had no obvious effect upon the Mg-dependent activation. Initial rate studies of phosphatase activation as a function of divalent cation concentration allowed determination of the Km for Mg$^{2+}$ as 3.8 mM, Km Mn$^{2+}$ 1.7 mM, and Km Co$^{2+}$ 1.4 mM (Fig. 3). Incubation with Mg$^{2+}$ or Mn$^{2+}$ plus 100 units/assay of wheat germ (Fig. 4) or rat brain (data not shown) megamodulin did not affect the rate or extent of reactivation of PDC.

Calcium did not activate P-PDH-phosphatase (Fig. 2), and
antagonized the activation by Mg$^{2+}$ (Fig. 5). A consistently observed stimulation of phosphatase activity by EGTA (Table I) suggests that low levels of Ca$^{2+}$ were present either in the enzyme preparation or as a contaminant in the other reagents. In some instances inclusion of calmodulin resulted in a greater inhibition of Mg$^{2+}$ activation of P-PDC-phosphatase activity than did Ca$^{2+}$ alone, but this potentiation was variable (Table I). Calmodulin plus Ca$^{2+}$ plus EGTA had no effect. Low concentrations of calmodulin antagonists did not effect Ca$^{2+}$ inhibition (Table I), and higher concentrations of the antagonists inhibited PDC activity.

Fluoride and Pi inhibited P-PDH-phosphatase activity in vitro, but molybdate and vanadate, transition-state analogs of Pi, had little effect (Fig. 6). A variety of metabolites thought to be present within the mitochondrial matrix were tested as potential effectors of P-PDH-phosphatase activity, but none had any significant effect (Table II).

**DISCUSSION**

When it was initially demonstrated that plant mitochondrial PDCs could be regulated in part by phosphorylation/inactivation, it was not possible to show dephosphorylation/reactivation (14, 15). While mammalian PDC-kinase is tightly associated with the complex, the P-PDH-phosphatase is only loosely associated, and may be lost during purification (6). Addition of purified mammalian P-PDH-phosphatase to plant P-PDC resulted in reactivation (20). More gentle isolation procedures allowed the demonstration of P-PDH-phosphatase as an intrinsic component of plant mitochondrial PDCs (16-18). Additionally, some properties of the plant phosphatase differ from those of the more thoroughly studied mammalian enzyme, necessitating a thorough characterization.

The divalent cation specificity of pea mitochondrial P-PDH-phosphatase (Fig. 2) was very similar to that of the mammalian enzyme (6). At low cation concentrations Mn and Mg gave similar activation, and Ca did not activate. Cobalt, which appears not to have been tested as an activator of the mammalian phosphatase, was also effective in activating the pea mitochondrial phosphatase (Fig. 2). The $K_m$ Mg$^{2+}$ for the pea phosphatase was 3.8 mM (Fig. 3), in the same range as reported for the bovine heart and kidney enzymes (6).

Recently there has been considerable interest in polyamines as regulatory molecules, which may act in part by modulating the phosphorylation state of certain key enzymes (22). Damuni et al. (3) reported that polyamines stimulated the activity of mammalian P-PDH-phosphatase activity up to 3-fold. This stimula-

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**FIG. 1.** A, Inactivation of pea leaf mitochondrial pyruvate dehydrogenase by phosphorylation, and reactivation by dephosphorylation; B, analysis by immunoprecipitation, SDS-PAGE, and autoradiography of samples taken 10 and 40 min after addition of [γ-32P]ATP.
Ca antagonized the activation of the phosphatase by 10 mM Mg. This was somewhat surprising in that while Ca does not activate the mammalian phosphatase, micromolar concentrations of Ca stimulate both the rate and extent of activation by Mg (5, 21). Ca promotes the binding of P-PDH-phosphatase to the lipoysuccinyltransferase core of mammalian PDC, and activates by lowering the $K_m$ of the phosphatase for P-PDH 20-fold (13). Our results with Ca and pea mitochondrial P-PDH-phosphatase confirm and extend the conclusions of Randall et al. with regard to Ca inhibition. Many of the responses to micromolar concentrations of Ca are mediated by Ca binding proteins such as calmodulin (9). We observed that in 7 of 15 instances, added calmodulin resulted in a greater inhibition than Ca alone. In one instance, added calmodulin seemed to prevent the Ca inhibition and in the other seven instances there was no effect. Calmodulin antagonists such as fluphenazine had little effect upon Ca inhibition of phosphatase activity, but the antagonist studies were complicated by their inhibition of overall PDC activity (J. Miernyk, unpublished data). Our results are similar to early studies with the mammalian phosphorylase kinase (9), where calmodulin turned out to be a regulatory subunit of the complex. Calmodulin could neither be dissociated from phosphorylase kinase by EGTA, nor could the regulatory effects be prevented by calmodulin antagonists. We theorize that only in instances where some calmodulin was lost from the PDC during enzyme isolation was an effect by added calmodulin observed. We have observed by Western blotting, that calmodulin appears to co-sediment with the PDC. Further studies testing the possibility that calmodulin is a regulatory subunit of plant mitochondrial PDCs are in progress.

Fluoride and Pi effectively inhibited P-PDH-phosphatase from bovine heart and kidney (6), and the inhibition of plant P-PDH-phosphatases by 10 mM NaF has been previously reported (16). The concentrations of fluoride and Pi required for 50% inhibition of pea P-PDH-phosphatase activity were approximately 3.5 and 11 mM, respectively (Fig. 6), similar to the responses of the mammalian enzyme (6). Low concentrations of vanadate and molybdate generally inhibit phosphatases (e.g. 2), presumably as transition-state analogs of Pi. Surprisingly, concentrations of vanadate and molybdate up to 20 mM had little effect upon pea mitochondrial P-PDH-phosphatase activity.

A number of metabolites which would be expected to occur in the mitochondrial matrix were tested as potential modulators of P-PDH-phosphatase activity. Krebs cycle intermediates, ADP, AMP, glutamate, alanine, spermine, spermidine, or putrescine had no effect at the indicated concentrations (Table II). In contrast to mammalian PDCs, which are phosphorylated on

![Fig. 3. A double reciprocal plot of divalent cation concentration versus reaction velocity for pea mitochondrial phospho-pyruvate dehydrogenase-phosphatase. $K_m$ values are: Mg (○) 3.8 mM; Mn (▲) 1.7 mM; Co (■) 1.4 mM.](image)

![Fig. 4. The effect of 100 units per assay of wheat germ megamodulin upon Mg or Mn activation of phosphopyruvate dehydrogenase-phosphatase. Original activity was 580 nmol min$^{-1}$ assay$^{-1}$.](image)
serine residues (19), plant PDCs are phosphorylated on threonines. However, neither phosphothreonine, phosphoserine, nor phosphotyrosine had any effect upon phosphatase activity. Of the compounds examined, only PI appeared to be of potential regulatory significance. We have proposed that the overall regulation of PDC activity involves a cascade system (11), one level of which is phosphorylation state. Given the relatively slow rate of reactivation of P-PDC by dephosphorylation and the insensitivity of the phosphatase to mitochondrial metabolites, it seems likely that regulation of PDH-kinase controls phosphorylation state.

Acknowledgments—Dr. W. N. Kuo generously provided the megamodulins used in these experiments, and Squibb Canada supplied the fluphenazine. The technical assistance of B. J. Rapp and N. W. David is gratefully acknowledged.

LITERATURE CITED
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![Fig. 5. Ca inhibition of the Mg-dependent reactivation of inactive, phosphorylated pea mitochondrial PDC. Original activity was 580 nmol min⁻¹ assay⁻¹.](image)

Table 1. Effects of Calcium, Calmodulin, and Related Compounds upon the Activity of Pea Mitochondrial Phospho-Pyruvate Dehydrogenase-phosphatase Activity

<table>
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<th>Compound Tested</th>
<th>Concentration</th>
<th>Percent of Control Activity</th>
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<tr>
<td>CaCl₂</td>
<td>50 μM</td>
<td>60</td>
</tr>
<tr>
<td>CaCl₂ + calmodulin</td>
<td>50 μM + 100 units</td>
<td>20-60</td>
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<tr>
<td>EGTA</td>
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<tr>
<td>EDTA</td>
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</tr>
<tr>
<td>Fluphenazine, (HCl₂)</td>
<td>50 μM</td>
<td>100</td>
</tr>
<tr>
<td>Monofluoromucine, (HCl)</td>
<td>50 μM</td>
<td>100</td>
</tr>
<tr>
<td>CaCl₂ + fluphenazine</td>
<td>50 μM, 50 μM</td>
<td>62</td>
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</tbody>
</table>

![Fig. 6. The effects of fluoride, Pi, ortho-oxonate, and molybdate on pea leaf mitochondrial phospho-pyruvate dehydrogenase-phosphatase activity. Original activity was 354 nmol min⁻¹ assay⁻¹.](image)

Table II. Mitochondrial Metabolites as Potential Effectors of Pea Mitochondrial Phospho-Pyruvate Dehydrogenase-phosphatase Activity

<table>
<thead>
<tr>
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<th>Control Activity</th>
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</thead>
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<td></td>
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<tr>
<td>CoA, acetyl-CoA</td>
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</table>

NADH for fatty acid biosynthesis. Plant Physiol 77: 571-577


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