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Regulation of Steady State Pyruvate Dehydrogenase Complex Activity in Plant Mitochondria[1]

REACTIVATION CONSTRAINTS

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ABSTRACT

The requirements for reactivation (dephosphorylation) of the pea (*Pisum sativum* L.) leaf mitochondrial pyruvate dehydrogenase complex (PDC) were studied in terms of magnesium and ATP effects with intact and permeabilized mitochondria. The requirement for high concentrations of magnesium for reactivation previously reported with partially purified PDC is shown to affect inactivation rather than reactivation. The observed rate of inactivation catalyzed by pyruvate dehydrogenase (PDH) kinase is always greater than the reactivation rate catalyzed by PDH-P phosphatase. Thus, reactivation would only occur if ATP becomes limiting. However, pyruvate which is a potent inhibitor of inactivation in the presence of thiamine pyrophosphate, results in increased PDC activity. Analysis of the dynamics of the phosphorylation-dephosphorylation cycle indicated that the covalent modification was under steady state control. The steady state activity of PDC was increased by addition of pyruvate. PDH kinase activity increased threefold during storage of mitochondria suggesting that there may be an unknown level of regulation exerted on the enzyme complex.

The pyruvate dehydrogenase complex (PDC[2]) is a large multienzyme complex consisting of three enzymes which convert pyruvate to acetyl-CoA. Associated with the mitochondrial complex are two regulatory enzymes (PDH kinase and PDH-P phosphatase) which catalyze the reversible phosphorylation of the E_1-α subunit of PDH (7, 9, 17–20). Phosphorylation results in inactivation and dephosphorylation reactivates PDC (9, 13). Chloroplastic PDC is not regulated by phosphorylation (3, 25).

It has been proposed that the phosphorylation-dephosphorylation of the mammalian PDC is regulated in part by ATP:ADP ratios (21, 23) or adenylate energy charge (9) and pyruvate concentration (7, 9). We have reported that phosphorylation of the plant mitochondrial PDC is unaffected by changes in the ATP:ADP ratio (2). We have proposed a mechanism by which the ATP dependent inactivation can be regulated by pyruvate (2); however pyruvate did not affect reactivation.

Most *in vitro* investigations of PDC regulation have utilized partially purified PDC (13). Those studies would suggest that the complex would always be inactivated (phosphorylated) since the K_m (ATP) for PDH kinase is 2.5 μM (14), and the K_m (Mg^{2+}) for PDH-P phosphatase is 3.8 mM (13). The ATP concentration in plant leaf mitochondria ranges from 0.4 mM in illuminated tissue to 0.2 mM in nonilluminated green leaf tissue (4, 6) and thus, it would seem that the ATP levels would keep the enzyme phosphorylated. Magnesium concentrations of mammalian mitochondria have been reported to be less than 0.5 mM (12) and if the magnesium concentrations of plant mitochondria are similar to those reported for mammalian mitochondria, it would seem that the dephosphorylation would be insufficient to overcome the action of PDH kinase. Therefore, we have undertaken the study of the dephosphorylation (reactivation) of PDC.

In this paper, we have examined the requirement for Mg-dependent reactivation and the effect of pyruvate on the steady state PDC activity using functional, intact mitochondria (*in situ*) or permeabilized mitochondria (*in vitro*). The findings reported here demonstrate that: (a) In contrast to the results with the partially purified complex, high concentrations of magnesium were not required for reactivation; (b) in the absence of pyruvate reactivation will not take place until ATP becomes limiting; (c) the level of PDC activity as modulated through ATP-dependent inactivation, is a steady state system; and (d) pyruvate in the presence of TPP, which inhibits inactivation of PDC directly (2), can control the steady state level of PDC activity.

MATERIALS AND METHODS

Pea (*Pisum sativum* L., cv Little Marvel) seedlings were grown in a growth chamber (10 h photoperiod, 600 μE m^{-2} sec^{-1}) at 18°C for approximately 2 weeks before harvesting. Mitochondria were isolated using rate zonal centrifugation and two consecutive discontinuous Percoll gradients (5). These mitochondrial preparations are *devoid* of Chl and are greater than 97% intact by the Cyt *c* oxidase latency assay (5). Mitochondrial preparations were stored at 4°C in 20 mM Tes-KOH (pH 7.2), 2 mM $MgCl_2$, 1 mM Na_2EDTA, 0.1% defatted BSA, 0.3 M mannitol, and 2 mM DTT. Preparations were used within 48 h unless noted otherwise.

PDC assays were performed spectrophotometrically for 1 min at pH 7.6 and 22°C in 85 mM Tes-NaOH with 0.2% (v/v) Triton X-100, 1 mM $MgCl_2$, 2 mM β-NAD, 0.2 mM TPP, 0.12 mM LiCoA, 1 mM cysteine, and 1 mM Na pyruvate. Assays were initiated with enzyme.

For the inactivation and reactivation of PDC *in situ* the incubation buffer mixture consisted of 10 mM Tes-NaOH (pH 7.5), 0.5 mM $MgCl_2$, 0.2 mM Na_2EDTA, 0.1% (w/v) defatted BSA, 0.3 M mannitol, and 0.5 mM DTT unless indicated otherwise. For the inactivation of PDC activity *in vitro* the mitochondria were permeabilized with an equal volume of 10 mM Tes-NaOH (pH 7.5), 0.2% (w/v) defatted BSA, and 0.01% (v/v)

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[2] Abbreviations: PDC, pyruvate dehydrogenase complex; PDH, pyruvate dehydrogenase; TPP, thiamine pyrophosphate; E_1 and PDH, pyruvate dehydrogenase; PEI, poly(ethyleneimine)-cellulose.

Triton X-100. Control PDC activity levels were determined in the absence of ATP at the beginning and end of each experiment. All experiments were performed in duplicate on at least two different mitochondrial preparations. Inactivation and reactivation rates were measured as the initial rates from slopes of graphs.

The stability of the ^{32}P-labeled ATP in terms of conversion to ADP and ^{32}Pi by ATP-utilizing reactions during the course of the inactivation-reactivation cycle of PDC was analyzed by quenching the reaction in ice cold 0.2 M Na_2EDTA containing 1 M AMP, ADP, and ATP. The percent label remaining in ATP was determined by PEI-TLC analysis (1, 15) using 1 M LiCl. The areas containing ATP, ADP, and AMP plus Pi (Pi migrates with AMP) were visualized with a UV lamp, cut out, placed in a scintillation vial, eluted with 1 mL 0.5 M $MgCl_2$, and counted with scintillation cocktail.

Samples for SDS-PAGE were added to 1 volume of Laemmli (10) sample buffer plus 3 M urea to stop any further reactions. Gradient SDS-PAGE gels were cast with Acrylaide (FMC) as the cross-linker, on Gel Bond (FMC). The Laemmli gel and electrode buffers (10) were utilized. Gels were oven-dried and autoradiographs made on Kodak X-Omat AR film with a Cronex Lightning-Plus intensifying screen (DuPont) at −70°C for 48 h. The relative level of phosphorylation of the 43 kD E_1-α-subunit of PDH was determined with the silver-specific dye: 5-(4-dimethylaminobenzylidene)rhodanine (11).

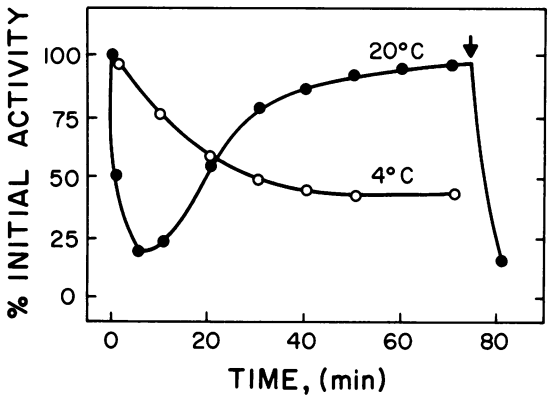

FIG. 1. Time-dependent inactivation and reactivation of pea leaf mitochondrial PDC activity *in situ* as affected by temperature. Intact isolated mitochondria were incubated (pH 7.6) at 4°C (○) or 20°C (●) with 0.5 mM $MgCl_2$ and inactivation initiated (0 min) with 200 μM ATP. At the times indicated aliquots were withdrawn and assayed in duplicate for PDC activity. At 75 min (↓) an additional 200 μM ATP was added to the reactivated (20°C) reaction mixture. Initial PDC activity (100%) was 0.20 μmol min^{-1} mg mitochondrial protein^{-1}.

RESULTS AND DISCUSSION

PDC was inactivated when intact mitochondria are incubated with ATP. The rate of inactivation and the time which the enzyme remains inactive were dependent upon the incubation temperature (Fig. 1). At 4°C the PDC activity slowly declined to a level at which it remained for hours. At room temperature inactivation proceeded more rapidly and to a greater extent, remained at this level for a few minutes, and then reactivation begins. if additional ATP was supplied (Fig. 1, arrow) the enzyme was once again inactivated suggesting that ATP became limiting. The transport of ATP into the mitochondrial matrix is presumably by net import since isolated plant mitochondria are devoid of adenine nucleotides along with many other cofactors (5). The magnesium concentration of the medium was 0.5 mM, but previous work with partially purified PDC had indicated that high levels of magnesium (10 mM) were required for reactivation (13, 18, 19). Thus, the observations illustrated in Figure 1 would suggest that mitochondria (a) may maintain high concentrations of magnesium or alternatively undergo large fluxes in free magnesium concentration to facilitate reactivation; (b) reactivation may be highly temperature sensitive; and/or (c) the reactivation of PDC is dependent upon the ATP concentration. We have examined each of these possibilities.

To discern the effect of magnesium on the inactivation/reactivation of PDC, mitochondria were permeabilized by lowering the osmoticum concentration and adding 0.01% (v/v) Triton X-100 (see "Materials and Methods"). Higher concentrations of detergent (0.1%) had no effect on inactivation (2) but severely inhibited reactivation. When 20 mM $MgCl_2$ was added with ATP to initiate inactivation there was an inhibition of inactivation (Fig. 2A). Reactivation was only slightly stimulated by 20 mM magnesium. When 0.5 mM $MgCl_2$ was added with the ATP a lag of variable time was seen before reactivation occurred (compare Figs. 1–3). Addition of 10 mM NaF (an inhibitor of PDH-P phosphatase) (18) did not alter the observed inhibition of inactivation in the presence of 20 mM $MgCl_2$. Likewise, addition of

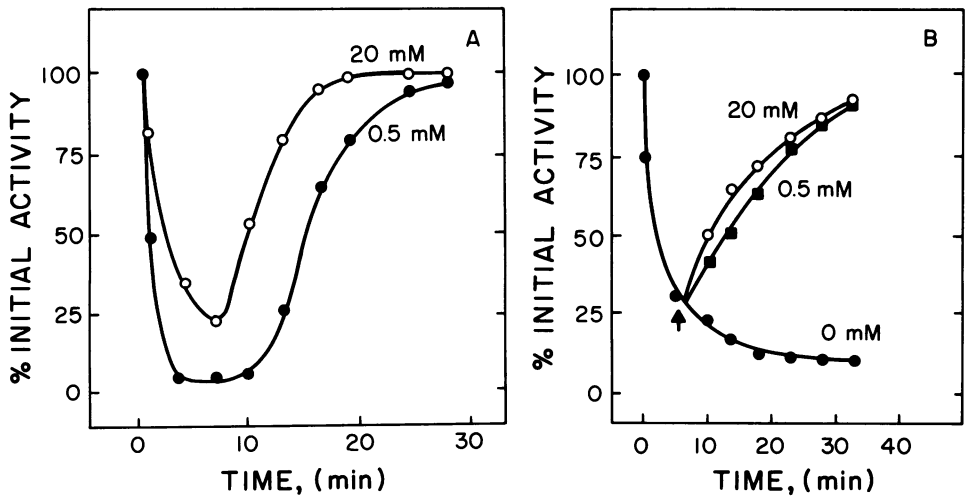

FIG. 2. Effect of magnesium on the inactivation and reactivation of mitochondrial PDC activity *in vitro*. Permeabilized mitochondria were incubated (pH 7.6, 20°C) with 0.5 mM or 20 mM $MgCl_2$ as indicated and inactivation initiated with 200 μM ATP. Aliquots were withdrawn at the times indicated and assayed for PDC activity. A, Different concentrations of magnesium were added initially with the ATP (↓) to discern the effect of magnesium on inactivation and reactivation. Initial PDC activity (100%) was 0.27 μmol min^{-1} mg mitochondrial protein^{-1}. B, PDC was inactivated in the presence of 0.5 mM $MgCl_2$ and at 6 min (↑) the reaction mixture was divided into three parts and the concentration of magnesium changed as indicated (0 mM magnesium provided by the addition of 2 mM Na_2EDTA). Initial PDC activity (100%) was 0.26 μmol min^{-1} mg mitochondrial protein^{-1}.

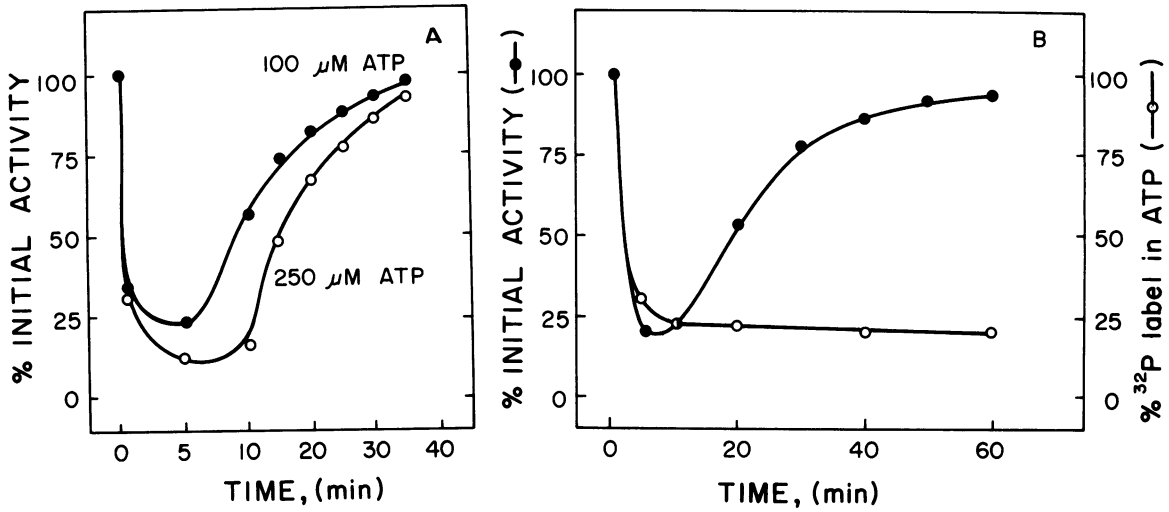

FIG. 3. Effect of ATP concentration on the *in situ* reactivation of mitochondrial PDC activity. Intact mitochondria were incubated (pH 7.6, 20°C) with 0.5 mM $MgCl_2$ and inactivation initiated with ATP. Aliquots were withdrawn and assayed for PDC activity at the times indicated. A, Inactivation and reactivation as affected by 100 μM (●) *versus* 250 μM (○) ATP. Initial PDC activity was 0.28 μmol min^{-1} mg mitochondrial $protein^{-1}$. B, Reactivation as affected by the ATP concentration. Inactivation reactions were initiated with 200 μM [γ-^{32}P]ATP (2×10^7 dpm/μmol). Aliquots were assayed for PDC activity and analyzed for the stability of ATP by PEI-TLC (see "Materials and Methods"). Initial PDC activity was 0.20 μmol min^{-1} mg mitochondrial $protein^{-1}$.

Table I. *Effect of Magnesium Levels on the Inactivation and Reactivation of PDC*

Activity	Expt. No.	Rate[a]		Result
		0.5 mM $MgCl_2$	20 mM $MgCl_2$	
Reactivation	1	0.019	0.022	16% stimulation
	2	0.025	0.027	8% stimulation
Inactivation	1	0.130	0.082	37% inhibition
	2	0.150	0.070	53% inhibition

[a] Units of PDH activated or inactivated per min per mg mitochondrial protein. Initial PDC activity = 0.30 and 0.29 unit per mg mitochondrial protein. (Experiments 1 and 2 are different mitochondrial preparations.) Rates were determined from the initial slopes of graphs as in Figure 2.

glucose and hexokinase (*e.g.*, at 6 min in Fig. 2B) did not effect the rate of reactivation of PDC (data not shown). If the inactivation-reactivation cycle is analyzed first to determine the time at which reactivation begins (*i.e.*, ATP becomes limiting) and the magnesium concentration changed just prior to reactivation, we observed once again a very slight increase in the rate of reactivation and a decrease in the lag with 20 mM $MgCl_2$ (Fig. 2B). When the magnesium was sequestered by addition of 2 mM Na_2EDTA, reactivation did not occur (Fig. 2B). If the initial rates of inactivation and reactivation at 0.5 mM $MgCl_2$ and 20 mM $MgCl_2$ are compared, the effect of 20 mM magnesium was greater on inactivation than reactivation (Table I). Data from several experiments showed that the rates of inactivation of PDC with intact versus permeabilized mitochondria with 0.5 mM $MgCl_2$ were not significantly different (data not shown) suggesting that mitochondria probably do not have high internal levels of magnesium as isolated. This conclusion is compatible with the report that the magnesium concentrations in mammalian mitochondria have been estimated to be less than 0.5 mM (12). The K_a for Mg^{2+} was reported to be 3 mM for PDH-P phosphatase from mammalian mitochondria, and this value was reduced by addition of calcium and treatment with insulin (24). The K_m (Mg^{2+}) for plant PDH-P phosphatase associated with the partially purified PDC has been reported to be 3.8 mM and Mg^{2+} dependent reactivation was inhibited by Ca^{2+} (13). However, using the functional, intact mitochondria or permeabilized mitochondria, reactivation of PDC was not effected by the addition of 100 μM calcium (data not shown).

If the inactivation of PDC was initiated with either 100 or 250 μM ATP there was little effect on the initial rate of inactivation (Fig. 3A). However, with the higher concentration of ATP a longer lag time was observed before reactivation began but there was no effect on the rate or extent of reactivation. Once again, this suggests that reactivation occurs only when ATP becomes limiting. To examine this hypothesis we determined the concentration of ATP throughout the inactivation-reactivation cycle (Fig. 3B). We consistently observed (with permeabilized and intact mitochondria) that reactivation will not begin until at least 75 to 80% of the 200 μM ATP is hydrolyzed. The remaining ATP may be bound to membranes and other proteins of the mitochondria and not available to PDH kinase. The rate of ATP hydrolysis may also explain the variable lag phase before reactivation begins as well as the extent of inactivation. Reactivation was inhibited by oligomycin suggesting that ATP was being utilized by the ATPase in a hydrolytic reaction.

Reversible protein phosphorylation is generally assumed to take place as a steady state system (22). However, the efficiency of a steady state system has been questioned since it results in a futile cycle of ATP hydrolysis. We have examined the possibility that reversible phosphorylation of PDC is under steady state control. The PDC was phosphorylated (inactivated) *in situ* with [γ-^{32}P]ATP. When the enzyme was greater than 90% inactivated, nonlabeled ATP along with an ADP scavenging system and oligomycin to inhibit the ATPase were added (Fig. 4). Without the addition of all of these, PDC would quickly reactivate. PDC was completely inactivated in 10 min and remained so for 60 min at which time it began to slowly reactivate. Aliquots of the reaction were taken and all activities terminated by the addition of the denaturing SDS-PAGE medium and protein phosphorylation was analyzed (Fig. 4 insert).

Comparing the activity of PDC with phosphorylation of the 43 kD E_1-α subunit of PDH indicated that phosphorylation was greatest at maximum inactivation (Fig. 4, 7–10 min). However, although the enzyme remained inactive (as long as ATP was present) the amount of ^{32}P-label on the PDH subunit decreased to virtually background levels. The time frame for ^{32}P-dephos-

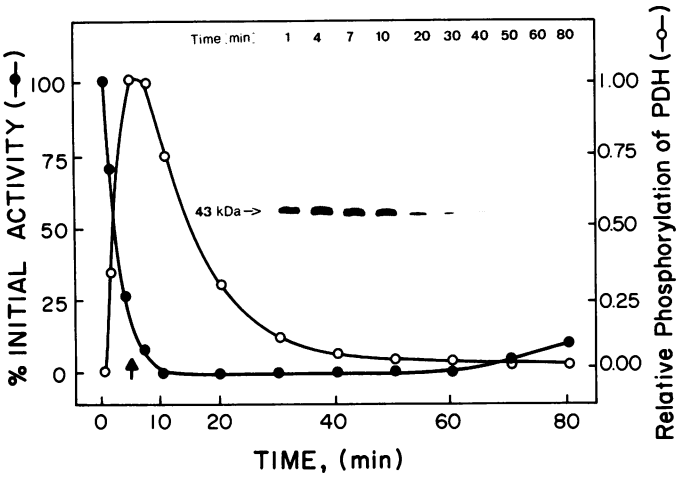

FIG. 4. Steady state analysis of the phosphorylation/dephosphorylation of mitochondrial PDH *in situ*. Intact mitochondria were incubated (25 mM Tes-NaOH; pH 7.5, 20°C) with 0.5 mM $MgCl_2$ and inactivation initiated with 200 μM [γ-^{32}P]ATP (1×10^8 dpm/μmol). At the times indicated aliquots were assayed for PDC activity and quenched for SDS-polyacrylamide gel electrophoresis and analyzed by autoradiography. At seven minutes (↑) 300 μM of nonlabeled ATP, 4 mM phosphocreatine, 6 units of creatine phosphokinase and 5 μg/ml of oligomycin were added to keep PDC inactive. Insert: autoadiograph of the samples assayed for the relative phosphorylation of PDH.

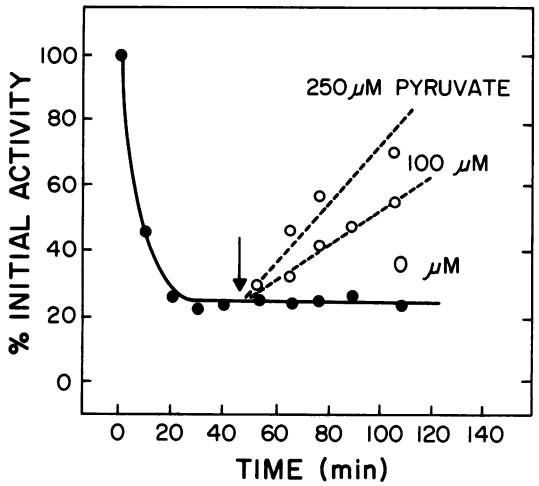

FIG. 5. Effect of pyruvate on the *in situ* PDC steady state activity level. Intact mitochondria were incubated (pH 7.6, 4°C) with 0.5 mM $MgCl_2$, 20 μM TPP and 250 μM ATP. The inactivation reaction was initiated with mitochondria and aliquots withdrawn and assayed for PDC activity at the times indicated. At 42 min (↓) the reaction mixture was divided into three parts and the concentration of pyruvate changed as indicated. Initial PDC activity was 0.25 μmol min^{-1} mg mitochondrial $protein^{-1}$.

phorylation was similar to that observed for reactivation (Figs. 1–3). These observations strongly suggest that PDH kinase and PDH-P phosphatase are both active at the same time. A steady state system such as this implies that when the rate of PDH kinase is greater than PDH-P phosphatase inactivation occurs, when the rates are equal a plateau for inactivation is reached (the lag), and when the rate of PDH kinase is less than PDH-P phosphatase reactivation takes place.

It is unlikely that ATP would become limiting for inactivation *in situ* since the K_m (ATP) is 2.5 μM (14) and ATP levels have been estimated to be between 0.2 and 0.4 mM (4, 6). Our previous work demonstrated that pyruvate in the presence of TPP inhibited inactivation (2). Thus, inhibition of inactivation would be expected to change the relative ratio of kinase: phosphatase activity and allow reactivation. We tested this by inactivating PDC *in situ* (purified, intact, functional mitochondria) at 4°C with ATP followed by the addition of pyruvate (Fig. 5). Reactivation occurred only when pyruvate was present (Fig. 5). Reactivation at 4°C indicates that PDH-P phosphatase is not highly temperature sensitive (Fig. 1), but the oligomycin-sensitive ATPase is probably much slower at 4°C. The effect of pyruvate on the reactivation rate was tested with PDC inactivated at 20°C whereupon PDC reactivates when a limiting concentration of ATP is reached (Figs. 1 and 3). The addition of pyruvate and TPP did not change the rate of reactivation (data not shown), indicating that pyruvate in the presence of TPP exerts its effect on the steady state PDC activity level via inhibition of inactivation and not by stimulation of reactivation.

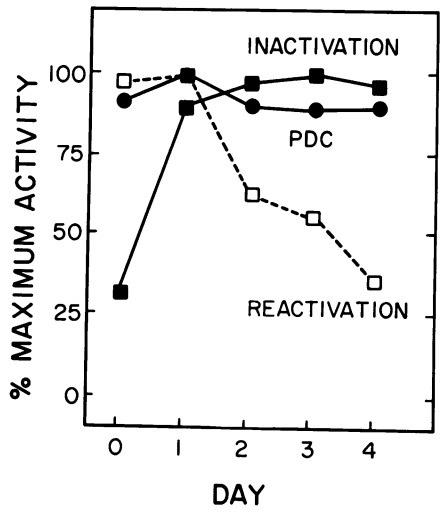

FIG. 6. Relative activities of the pyruvate dehydrogenase complex (●), inactivation rate (■) and reactivation rate (□) as affected by aging of isolated intact pea leaf mitochondria. Activities were measured *in vitro* initially after isolation, and every day thereafter. All activities are initial rates measured from slopes of graphs as in Figure 1 and Table I. Maximum activities (100%) were 0.24 (PDC), 0.17 (inactivation rate), and 0.013 (reactivation rate) units mg mitochondrial $protein^{-1}$.

With a few mitochondrial preparations we have observed that pyruvate was a poor inhibitor of inactivation. These preparations also exhibited very reduced rates of reactivation at 20°C and/or the inability to completely reactivate PDC, yet normal levels of PDC and rates of PDC inactivation were observed. Our model (2) predicts that a deficiency in reactivation would show poor pyruvate inhibition of ATP dependent inactivation, since reactivation or protection from inactivation by pyruvate is maintained by a steady state system with pyruvate acting as a negative effector of PDC inactivation (*i.e.*, PDH kinase) via its interaction with the protein substrate, PDH. In contrast to our studies with plant mitochondria, it has been reported that pyruvate inhibits inactivation of mammalian mitochondrial PDC by directly inhibiting PDH kinase (7).

When isolated mitochondria were stored at 4°C, PDC activity, inactivation, and reactivation responded differently (Fig. 6). Activity of PDC remained fairly constant over the 4 d period, while the rate of reactivation dropped steadily after the first day. In these instances addition of glucose plus hexokinase as an ATP scavenging system did not stimulate reactivation indicating that the loss of ability to reactivate PDC was not an effect of the ATP concentration. Previous work in this laboratory indicated that PDH-P phosphatase was the most labile component of the complex, hence the decline in activity was probably due to its intrinsic lability. More surprising was the threefold stimulation

of the inactivation rate during the first 24 h of storage. Since there was no change in the rate of reactivation between day zero and day one, this increase in the inactivation rate most likely represents an increase in PDH kinase activity. A change in the ratio of kinase to phosphatase activity would potentially alter the steady state level of PDC activity. The basis for activation of the kinase is unknown; however, it has been suggested that PDH kinase may be regulated by thiol-disulfide exchange (16). There are numerous chloroplast enzymes regulated in such a manner (8), but no examples have been established for enzymes in plant mitochondria to our knowledge.

These experiments with intact and permeabilized mitochondria support our previous work in that Mg^{2+} was required for the reactivation (dephosphorylation) of the plant mitochondrial PDC activity. In contrast to our previous findings these results indicate that high concentrations or fluxes of magnesium are not required. In addition, we do not believe that changes in magnesium concentration play a major role in regulating the steady state activity, but pyruvate is apparently a primary component in the regulation of PDC as previously described (2).

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