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Regulation of the Phosphorylation of Mitochondrial Pyruvate Dehydrogenase Complex *in Situ*[1]

EFFECTS OF RESPIRATORY SUBSTRATES AND CALCIUM

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ABSTRACT

The activity of the pyruvate dehydrogenase complex (PDC), as controlled by reversible phosphorylation, was studied *in situ* with mitochondria oxidizing dfifferent substrates. PDCs from both plant and animal tissues were inactivated when pyruvate became limiting. The PDC did not inactivate in the presence of saturating levels of pyruvate. Calcium stimulated reactivation of PDC in chicken heart but not pea (*Pisum sativum* L.) leaf mitochondria. With pea leaf mitochondria oxidizing malate, inactivation of PDC was pH dependent corresponding to the production of pyruvate via malic enzyme. When pea leaf mitochondria oxidized succinate or glycine, PDC was inactivated. This inactivation was reversed by the addition of pyruvate. Reactivation by pyruvate was enhanced by the addition of thiamine pyrophosphate, as previously observed with nonrespiring mitochondria. These results indicate a major role for pyruvate in regulating the covalent modification of the PDC.

The mitochondrial pyruvate dehydrogenase complex (PDC[2]) is a large multienzyme complex consisting of three enzymes which coordinate the conversion of pyruvate to acetyl-CoA. It is well established that the activity state of the complex is controlled by two associated regulatory enzymes: PDH kinase and PDH-P phosphatase (16, 17). Phosphorylation results in inactivation and dephosphorylation reactivates PDC. We have recently proposed a model (2, 3) in which the steady state activity of PDC is regulated via the inhibition of inactivation (phosphorylation) by the level of the substrate, pyruvate. Our model predicts that when mitochondria are respiring and producing ATP in the absence of pyruvate, PDC should become inactivated (phosphorylated) and upon addition of pyruvate, PDC should be reactivated (dephosphorylated). We have tested this hypothesis using the substrates pyruvate, malate, succinate and glycine. The results reported here support our model for pyruvate as a primary effector for regulating the covalent modification of PDC. We have also examined the effect of calcium which is a positive effector of PDH-P phosphatase from animal mitochondria. Calcium stimulated animal mitochondrial PDH-P phosphatase as previously established (9). However, calcium had little effect on the reactivation (dephosphorylation) of PDC using pea leaf mitochondria. This is in contrast to a previous report (11) with the partially purified complex where Ca^{2+} inhibited reactivation.

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[2] Abbreviations: PDC, pyruvate dehydrogenase complex; PDH, pyruvate dehydrogenase; TPP, thiamine pyrophosphate; OAA, oxaloacetic acid.

MATERIALS AND METHODS

Materials. Pea (*Pisum sativum* L., cv Little Marvel) seedlings were grown in a growth chamber (10 h photoperiod, 600 μE m^{-2} s^{-1}) at 18°C for 2 weeks before harvesting. Intact pea leaf mitochondria were isolated and purified (devoid of chlorophyll) using two consecutive discontinuous Percoll gradients (7). The pea leaf mitochondrial PDC is *fully active* as isolated. Intact potato (*Solanum tuberosum*) tuber mitochondria were isolated and purified as described (12). Mitochondria were prepared from the hearts of three 2-week-old chickens using a collagenase-facilitated isolation procedure (20).

Assays. Oxygen uptake was measured at 25°C using a Hansatech electrode in a medium consisting of 20 mM Tes-KOH (pH 7.5, unless indicated otherwise), 0.3 M mannitol, 1 mM Na_2EDTA, 3 mM $MgCl_2$, 5 mM potassium phosphate (adjusted to pH 7.5), 0.1% (w/v) defatted BSA, and 10 mM KCl. The reaction mixture contained 0.1 to 0.2 mg/mL of mitochondrial protein in a final volume of 2.5 mL. Cofactor and substrate additions were performed at the times indicated and 0.2 mL aliquots were withdrawn at various times for the immediate assay of PDC activity. PDC was assayed spectrophotometrically (340 nm) at 22°C in 65 mM Tes-NaOH (pH 7.6) with 0.1% (v/v) Triton X-100, 0.5 mM $MgCl_2$, 2 mM β-NAD, 0.2 mM TPP, 0.12 mM LiCoA, 1 mM L-cysteine, and 1 mM Na pyruvate. Assays were initiated with enzyme. Protein was determined by the method of Bradford (1) using BSA as the standard.

The effect of calcium on PDH kinase and PDH-P phosphatase was analyzed *in vitro* by following the inactivation-reactivation cycle of PDC (3). Mitochondria were disrupted by addition of 0.01% (v/v) Triton X-100 in 10 mM Tes-NaOH (pH 7.5), 0.2% (w/v) defatted BSA, 1 mM $MgCl_2$, 0.2 mM Na_2EDTA, and plus/minus 0.5 mM EGTA. Inactivation was initiated by the addition of 0.25 mM ATP.

The formation of pyruvate from malate was determined by quenching 0.4 mL of the oxygen electrode reaction mix with 0.1 mL of 1.6 M $HClO_4$ in a 1.5 mL microfuge tube. The pH was neutralized with 0.2 mL of saturated $KHCO_3$. After centrifugation, the supernatant was assayed spectrophotometrically (340 nm) for pyruvate in the presence of 50 mM Hepes-NaOH (pH 7.2), 1 mM Na_2EDTA, 0.2 mM β-NADH, and 1 unit of lactate dehydrogenase.

Phosphorylation of the PDH 43 kD subunit was analyzed on 8 to 12% gradient SDS-polyacrylamide gels. Autoradiographs were made on Kodak X-Omat AR-2 film with a Cronex Lightning-Plus intensifying screen (DuPont) at −70°C for 72 h.

RESULTS AND DISCUSSION

When intact pea leaf mitochondria are oxidizing pyruvate at a low concentration (0.1 mM) there is an increased level of O_2 consumption upon addition of ADP. This enhanced rate is followed by a decrease to nearly zero which can be reversed only upon addition of more substrate (Fig. 1A and Table I). The same pattern holds true for saturating levels of pyruvate (1 mM) (7) except that there is no increase upon further addition of substrate. This would indicate that with the lower, subsaturating concentration, pyruvate became limiting, but other factor(s) curtailed pyruvate oxidation at saturating pyruvate concentrations. A concentration of 1 mM pyruvate is saturating for both pyruvate-dependent O_2 uptake by intact mitochondria (7) and reactivation of inactivated (phosphorylated) PDC as shown in this report and elsewhere (2). Analysis of the phosphorylation of the 43 kD subunit of PDH (Fig. 1B) indicated that the greatest level of phosphorylation occurred after depletion of pyruvate (sample 2). Subsequent addition of pyruvate resulted in the loss of most of the ^{32}P-label associated with the 43 kD polypeptide (sample 3). When the mitochondria were presented with saturating levels of pyruvate, the 43 kD subunit was only slightly radiolabeled (lane 4, Fig. 1B). The ability of PDC to be partially phosphorylated yet remain active (lane 3, Fig. 1B) could be due to the multiple sites of phosphorylation. For animal PDH, there are three sites with one site controlling 98% of the activity of the enzyme (14). We have determined that plant PDH also contains multiple phosphorylation sites (RJA Budde, DD Randall, in preparation).

When PDC activity and respiration rates (Table I) were compared under the same conditions as Figure 1, PDC was inactivated with 0.1 mM pyruvate (sample 2; 63–90%), but reactivated upon addition of 1 mM pyruvate (sample 3). Reactivation was coordinated with an increased respiration rate (Table I) and partial dephosphorylation of PDC (Fig. 1B, lane 3). When pyruvate was the substrate for pea leaf mitochondria, the activity or activation level of PDC under state 3 conditions (samples 1 and 4) was equivalent to that before addition of ADP. The absolute respiration rate, percentage of inactivation and reactivation of PDC varied from one mitochondrial preparation to another (Table I). However, the trend remained the same for eight different preparations. With 1 mM pyruvate, PDC remained active and only slightly phosphorylated even though respiration almost stops (Fig. 1B and Table I). Thus, pyruvate-dependent respiration at this time point was not inhibited due to inactivation (phosphorylation) of PDC. ADP was not limiting since additional ADP did not alleviate this depressed respiratory rate. *In vitro*, PDC has also been shown to be regulated by product (NADH) and acetyl-CoA) inhibition (10). Pyruvate-dependent respiration was resumed if OAA was added (Table I; experiment 3, sample 6). OAA will scavenge NADH via malate dehydrogenase and serve as an acceptor for acetyl-CoA via citrate synthase thereby relieving the apparent product inhibition of PDC (10).

For both plants and animals, protein phosphorylation is often regulated by calcium (6, 9, 15). For animal PDH, dephosphorylation (PDH-P phosphatase activity) is activated by calcium (9). We isolated chicken heart mitochondria in the presence of EGTA in an attempt to determine how they would compare, plus-and-minus calcium, with the 0.1 *versus* 1 mM pyruvate experiments performed with pea leaf mitochondria. With 0.1 mM pyruvate, PDC began inactivating and would not reactivate with saturating levels of pyruvate unless calcium was added (Fig. 2A). If calcium was added initially (Fig. 2B), the chicken heart mitochondria behaved similarly to the pea mitochondria (Table I). Thus, in contrast to the pea mitochondria, reactivation of PDC in chicken mitochondria was dependent upon calcium. Partial inactivation also occurred with 1 mM pyruvate in the absence of calcium and reactivation was dependent upon the addition of calcium (Fig.

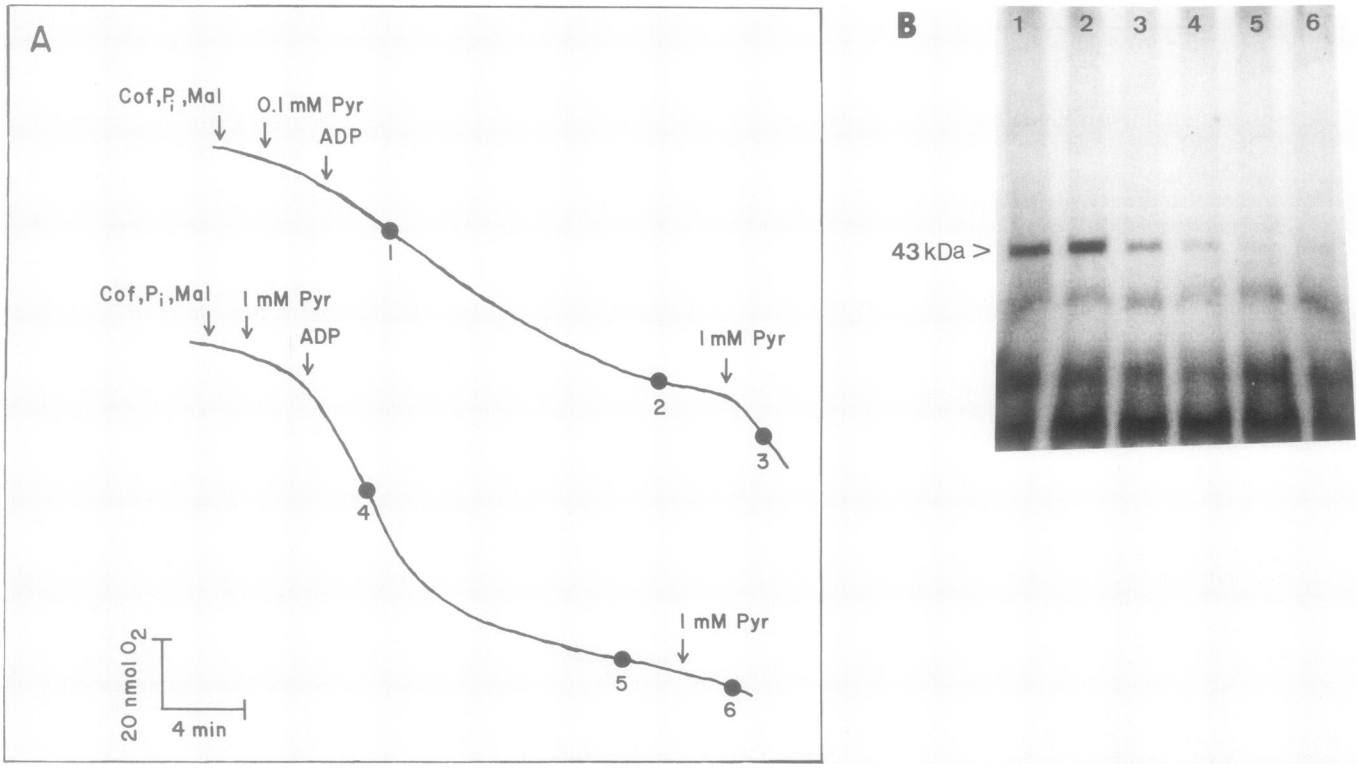

FIG. 1. Effect of pyruvate on the *in situ* activation level and phosphorylation of PDC. Respiring pea leaf mitochondria with 0.1 *versus* 1 mM pyruvate: samples (1–6) were removed at the times indicated (A) for analysis of the phosphorylation (B) of the 43 kD subunit of PDC. Additions: Cof = 1 mM NAD, 0.1 mM CoA and 0.1 mM TPP-cofactors required for pyruvate oxidation, 5 mM ^{32}Pi (1×10^8 dpm/μmol), 0.1 mM 'sparker malate,' 0.2 mM ADP and variable pyruvate as indicated. Initial PDC activity was 0.25 μmol min^{-1} mg mitochondrial protein^{-1}.

Table I. *Effect of Pyruvate Concentration on the Respiration Rate and PDC Activity of Pea Leaf Mitochondria*

Numbers indicate samples taken as illustrated in typical O_2 electrode traces, Figure 1A.

	Sample No.					
	0.1 mM Pyruvate			1 mM Pyruvate		
Mitochondria Preparation	1	2	3	4	5	6
Experiment 1						
nmol O_2/min·mg protein	17[a]	0	19	64	6	8
% Active PDC	100[b]	13	102	100	95	99
Experiment 2						
nmol O_2/min·mg protein	20	4	31	61	5	5
% Active PDC	100[b]	10	109	100	95	99
Experiment 3						
nmol O_2/min·mg protein	36	12	26	47	14	68[c]
% Active PDC	100[b]	37	104	100	100	85

[a] Rates are calculated based on the rate before addition of pyruvate (*i.e.* the background) being zero. [b] With 100% = 0.19 to 0.23 μmol/min·mg protein. [c] Addition of 100 μM OAA instead of additional pyruvate.

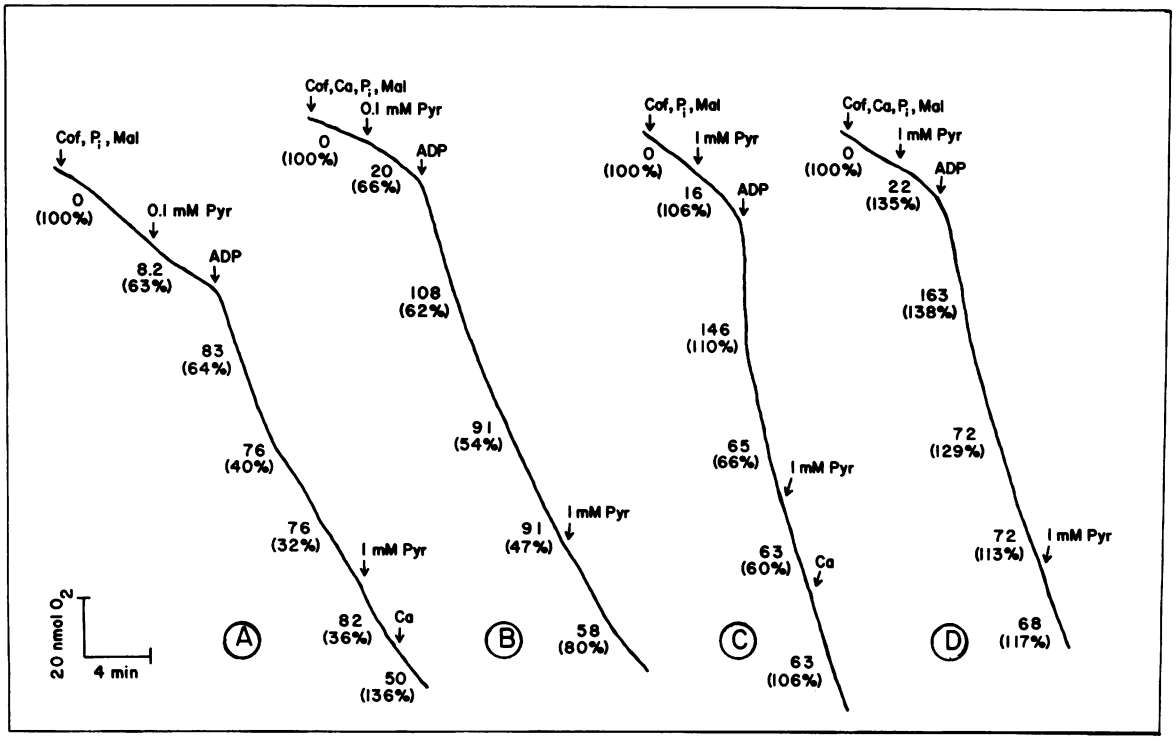

FIG. 2. Effect of calcium and 0.1 (A and B) *versus* 1 mM (C and D) pyruvate on the *in situ* PDC activity from chicken heart mitochondria. Numbers on O_2 electrode traces are nmol O_2 consumed min^{-1} mg mitochondrial $protein^{-1}$. Numbers in parentheses refer to the percent of the initial PDC activity remaining. Initial PDC activity was 0.14 μmol min^{-1} mg mitochondrial $protein^{-1}$. The concentration of calcium was 0.1 mM, all other additions as indicated in Figure 1 legend.

2C). If calcium was added initially with 1 mM pyruvate (Fig. 2D), the chicken heart mitochondria behaved as the pea leaf mitochondria in that PDC remained active at all times. For the chicken mitochondria we observed that inactivation began upon addition of pyruvate (Figs. 2, A and B) in contrast to pea mitochondria (Table I), and endogenous levels of TPP were saturating for pyruvate oxidation (data not shown).

When we isolated pea mitochondria in the presence or absence of 1 mM EGTA we observed no differences from the results shown in Figure 1 and Table I. However, it could be that with intact pea mitochondria the endogenous calcium does not equilibrate with the outer environment containing EGTA. Therefore, we permeabilized the mitochondria by lowering the osmoticum concentration and adding detergent (0.01% Triton X-100) with an additional 0.5 mM EGTA to the preparation purified in the presence of EGTA. The activity of PDC was followed through an inactivation-reactivation cycle (3) initiated with ATP (Fig. 3). Figure 3 shows the typical results where neither inactivation nor reactivation was affected by the addition of EGTA. Thus, pea leaf mitochondria apparently do not require calcium to maintain PDC in an active state or the Ca^{2+} cannot be chelated or removed. This laboratory has previously reported in experiments with partially purified PDC (11) in which calcium inhibited the plant PDH-P phosphatase. The results in this report are consistent with a regulatory role for calcium with animal mitochondria (9) which actively transport calcium (4), while plant mitochondria

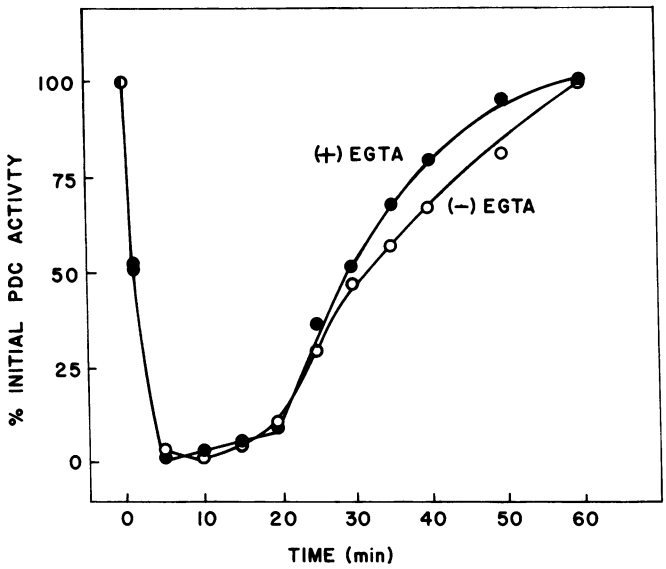

FIG. 3. Effect of EGTA-treatment on the *in vitro* PDH kinase and PDH-P phosphatase activity. Pea leaf mitochondria were isolated plus-or-minus 1 mM EGTA, permeabilized with 0.01% Triton X-100, and the inactivation of PDC (PDH kinase activity) and reactivation of PDC (PDH-P phosphatase activity) was followed by initiating the inactivation-reactivation cycle with 0.25 mM ATP. Aliquots were assayed at the times indicated. Initial PDC activity was 0.22 μmol min^{-1} mg mitochondrial protein^{-1} for both preparations.

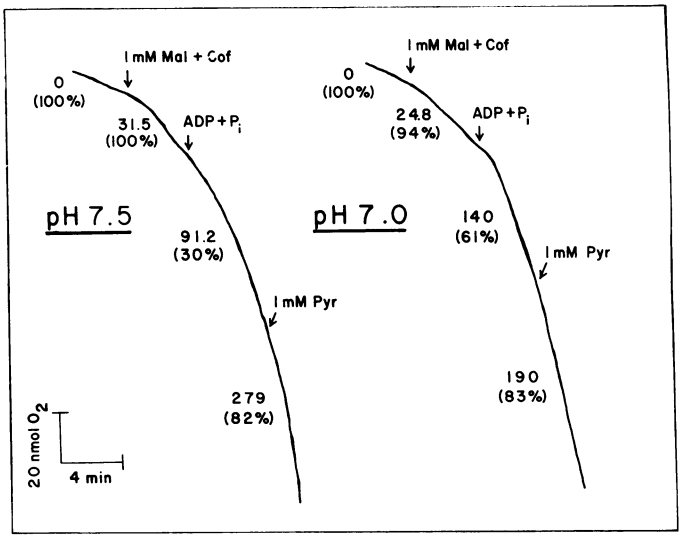

FIG. 4. Effect of pH on the *in situ* inactivation of PDC with pea leaf mitochondria using malate as the respiratory substrate. Numbers on O_2 electrode traces are nmol O_2 min^{-1} mg mitochondrial protein^{-1}. Numbers in parentheses refer to the percent of the initial PDC activity remaining. Initial PDC activity was 0.27 μmol min^{-1} mg mitochondrial protein^{-1}. Additions: 1 mM L-malate, all other concentrations as in Figure 1 legend.

will accumulate calcium only in the presence of nonphysiological levels of calcium (5).

Since pyruvate inhibits the ATP dependent inactivation of PDC we were interested in what effect the oxidation of other substrates would have on the PDC activity. Data obtained using malate as a substrate is of particular interest because there are two means of oxidizing malate, via malate dehydrogenase to OAA or via malic enzyme to pyruvate. Which route predominates is determined by the pH since malic enzyme has a lower pH optimum than malate dehydrogenase (19). Using malate as the substrate, at pH 7.0 and 7.5, PDC inactivation began upon the addition of ADP and reactivation occurred upon addition of pyruvate (Fig. 4). However, inactivation was always less at pH 7.0 in which malic enzyme would be more active thereby producing more pyruvate, which in turn would inhibit PDC inactivation (2, 3, 17). This 0.5 unit drop in pH may also affect cofactor and substrate transport, ATP synthesis, and the ratio of PDH kinase to PDH-P phosphatase activity, all of which would influence the activity of PDC. We measured the amount of pyruvate formed (Table II) and observed a 3.2-fold increase in pyruvate at pH 7.0 *versus* 7.5 and a corresponding 2-fold increase in PDC activity. Addition of TPP resulted in a 22% decrease in pyruvate at both pH values as expected due to pyruvate oxidation being dependent upon the addition of exogenous TPP. Inactivation of PDC was slightly depressed at both pH values when TPP was added. This is consistent with our previous findings (2) in which TPP enhanced the inhibition of inactivation by pyruvate although TPP was not an inhibitor of inactivation under these conditions. Addition of 0.1 mM glutamate (an OAA scavenger) (19) with malate slightly altered (0–10%) the respiration rate and the PDC activity observed (data not shown). This difference is less than that observed from one preparation to another, although all mitochondrial preparations which were coupled showed the same trend towards inactivation (60–90%) upon addition of ADP and reactivation upon addition of pyruvate (80–100% of initial activity). With potato tuber mitochondria oxidizing malate we observed that, upon addition of ADP,

Table II. *Effect of pH on Pyruvate Formation and Inactivation of PDC*

pH	TPP	% of Initial PDC Activity[a]	Pyruvate Formed
			nmol/mg protein
7.5	−	23	269
	+	34	211
7.0	−	47	865
	+	54	673

[a] Percent of initial PDC activity was determined after ADP addition as in Figure 4.

PDC inactivated (greater than 80%) and, upon addition of pyruvate, PDC reactivated (data not shown).

When succinate or glycine were substrates for pea leaf mitochondria the activity of PDC declined following the addition of ADP (Figs. 5 and 6). With succinate alone oxidation quickly declined (Fig. 5A), which was probably due to the inhibition of succinate dehydrogenase by OAA (18). This inhibition or decline in respiration was eliminated by the addition of glutamate (Fig. 5B), an OAA scavenger (8). PDC became inactivated *in situ* when succinate or glycine were substrates for respiration. This inactivation of PDC was partially reversed by addition of pyruvate (25–60% of initial activity) but TPP was required for maximal pyruvate stimulated PDC reactivation (Figs. 5 and 6). We have also observed this phenomena *in vitro* and *in situ* with nonrespiring mitochondria when exogenous ATP was added (2, 3). The rates of glycine oxidation (Fig. 6) were rather low because tetrahydrofolate, a cofactor for glycine decarboxylation was limiting (13). Addition of tetrahydrofolate gave rates which were much too rapid to allow for the assaying of multiple samples during the course of the experiment. The increase in O_2-uptake upon addition of pyruvate in the presence of TPP reflects oxidation of both glycine and pyruvate.

The data reported here and elsewhere (2, 3) demonstrate a major role for pyruvate in controlling the covalent modification of PDC. Although TPP enhances the inhibitory effect of pyruvate on inactivation, we do not foresee a major role for this cofactor in regulating the phosphorylation of PDC because of its very low

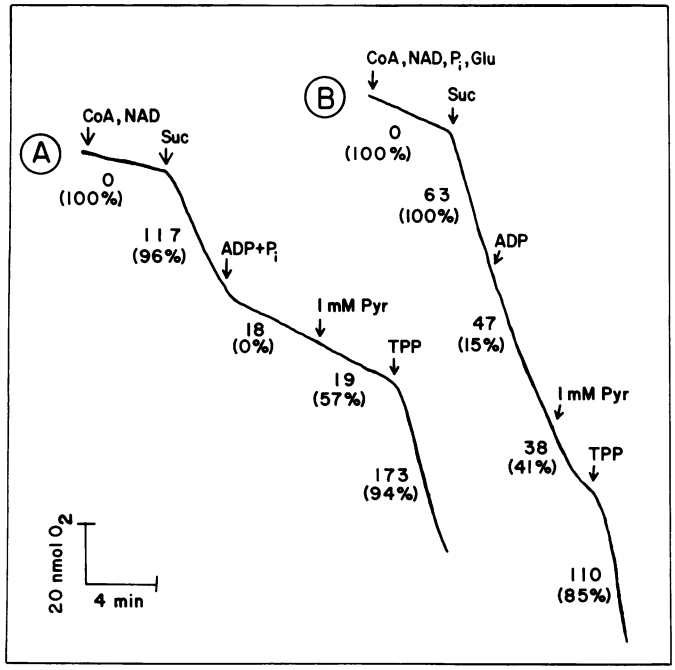

FIG. 5. Effect of succinate oxidation, plus-and-minus glutamate, on the *in situ* inactivation of PDC. Numbers on O_2 electrode traces are nmol O_2 min^{-1} mg mitochondrial $protein^{-1}$. Numbers in parentheses refer to the percent of the initial PDC activity remaining. Initial PDC activity was 0.20 μmol min^{-1} mg mitochondrial $protein^{-1}$. Additions: 1 mM succinate, 0.1 mM L-glutamate, all other additions as indicated in Figure 1 legend.

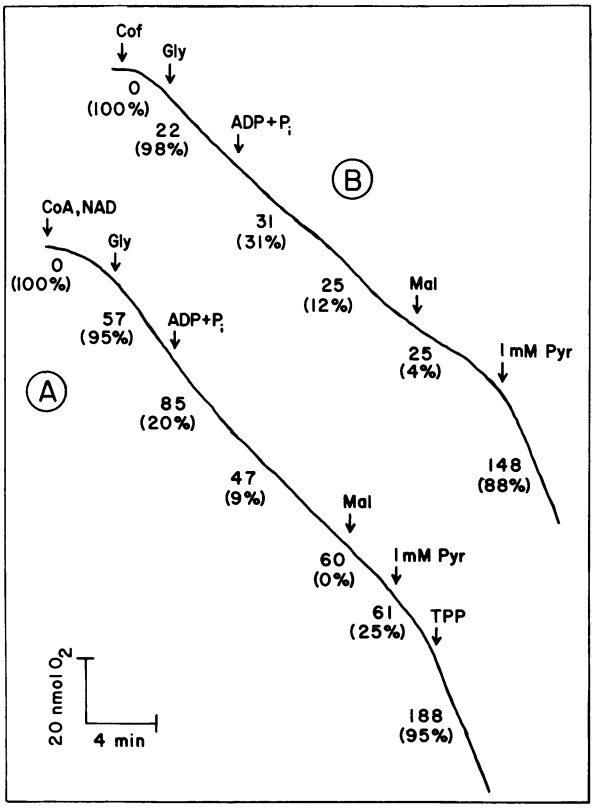

FIG. 6. Effect of glycine oxidation on the *in situ* inactivation of PDC and reactivation by pyruvate plus-and-minus TPP. Numbers on O_2 electrode traces are nmol O_2 min^{-1} mg mitochondrial $protein^{-1}$. Numbers in parentheses refer to the percent of the initial PDC activity remaining. Initial PDC activity was 0.21 μmol min^{-1} mg mitochondrial $protein^{-1}$. Additions: 1 mM glycine, all other additions as indicated in Figure 1 legend.

K_m (80 nM) (10) and that it is not converted into a different form which diffuses away from PDC such as NADH and acetyl-CoA.

CONCLUDING REMARKS

The activity of mitochondrial PDC is under steady state control (3) in which the activity of PDC is dependent upon the ratio of PDH kinase to PDH-P phosphatase activity. We have previously determined that PDH kinase activity *in situ* and *in vitro* is many times greater than PDH-P phosphatase activity (3). Therefore, the steady state favors inactivation of PDC and adjustments of this steady state can be achieved by either inhibiting inactivation and/or stimulating reactivation. Pyruvate in the presence of TPP, as shown here and elsewhere (2, 3) is a very effective inhibitor of inactivation. Previous studies have indicated that pyruvate inhibits inactivation by affecting PDC directly and not by inhibiting PDH kinase (2) or stimulating PDH-P phosphatase (3). Although calcium is an activator of PDH-P phosphatase from animal mitochondria (9) and a potential inhibitor of the plant PDH-P phosphatase (11), EGTA-treatment did not appear to affect the enzyme from pea mitochondria. Pyruvate and malate (which yields pyruvate via the malic enzyme), which are the primary carbon sources for mitochondrial respiration, promote the activation of PDC and hence promote the operation of the citric acid cycle. The question arises as to why does the mitochondria bother with inactivating PDC in the presence of limiting substrate which in itself should limit PDC activity. Perhaps the regulatory phosphorylation is necessary so that under certain conditions pyruvate can be utilized for other reactions/metabolic pathways. Furthermore, mitochondria have an alternate method (product inhibition) for synchronizing PDC activity with the citric acid cycle. Establishing the physiological necessity for the phosphorylation of PDC will require knowledge of the environmental factor(s) which result in phosphorylation.

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