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Regulation of Pea Mitochondrial Pyruvate Dehydrogenase Complex[1]

Does Photorespiratory Ammonium Influence Mitochondrial Carbon Metabolism?

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ABSTRACT

Inactivation of the pyruvate dehydrogenase complex catalyzed by pyruvate dehydrogenase kinase was studied using intact mitochondria purified from green leaf tissue of pea (*Pisum sativum* L.) and dialyzed mitochondrial extracts. Thiamine pyrophosphate was inhibitory in dialyzed extracts but not in intact mitochondria, except in the presence of high concentrations of Na^+. NH_4^+, at concentrations as low as 20 micromolar, markedly stimulated inactivation in dialyzed extracts. K^+ in the range 1 to 10 millimolar also enhanced inactivation. In contrast, Na^+ was without affect at lower concentrations but was inhibitory at 10 to 100 millimolar levels. The effect of NH_4^+ is discussed in relation to a possible regulatory interaction between photorespiratory NH_4^+ production and the entry of carbon into the tricarboxylic acid cycle by way of the pyruvate dehydrogenase complex.

The mitochondrial pyruvate dehydrogenase complex (PDC[2]) catalyzes a key reaction regulating entry of carbon into the tricarboxylic acid cycle (4) and thereby indirectly affects the rate of dark respiration. Activity of the complex is regulated in part by reversible phosphorylation and in part by product inhibition (4, 12, 17, 19). Phosphorylation, catalyzed by pyruvate dehydrogenase (PDH) kinase, inactivates the complex and dephosphorylation, catalyzed by P-PDH phosphatase, results in reactivation. Both of these regulatory enzymes are associated with the complex.

Plant mitochondrial PDH kinase activity, assayed in intact or detergent-permeabilized mitochondria, purified from green leaf tissue of pea, is inhibited by pyruvate, dichloroacetate and Mg^{2+} but is not affected by ADP, TPP or Ca^{2+} (1–3). In contrast, PDH kinase activity of purified mammalian mitochondrial PDC is inhibited by all of the above effectors (4). In the present study, the work with pea mitochondrial PDC was extended by comparing intact mitochondria with dialyzed mitochondrial extracts. PDH kinase activity is shown to be inhibited by TPP in dialyzed extracts but not in intact mitochondria. Furthermore, K^+ and NH_4^+ are shown to markedly stimulate PDH kinase activity with NH_4^+ being more effective than K^+. The latter result is discussed in relation to a potential role for photorespiratory NH_4^+ in regulating PDC activity and consequently the carbon supply for dark respiration during photosynthesis. This is important because it is not known to what extent dark respiration proceeds simultaneously with photosynthesis, let alone what regulates the balance between these two processes in meeting energy demands (9).

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

MATERIALS AND METHODS

Biochemicals

Biochemicals were purchased from either Sigma (St. Louis, MO) or Pharmacia (Piscataway, NJ). ATP and pyruvate were sodium salts and TPP was the free acid.

Isolation of Intact Mitochondria

Mitochondria, isolated from green leaf tissue of 14 d old pea (*Pisum sativum* L.) cv Alaskan Marvel seedlings, were purified devoid of Chl using two consecutive discontinuous Percoll gradients (7). The buffer in which the purified mitochondria were suspended contained: 0.3 M mannitol, 20 mM Tes-KOH (pH 7.2), 1 mM EDTA, 0.1% (w/v) defatted BSA, 2 mM $MgCl_2$, and 2 mM DTT.

Preparation of Mitochondrial Extracts

Mitochondrial suspensions, which had been stored frozen at −70°C, were thawed and centrifuged at 15,000*g* for 5 min. The supernatant (1–4 ml) was then dialyzed overnight at 4°C against 1 L of 50 mM Tris-HCl (pH 7.2), 0.1% (w/v) BSA, 1 mM $MgCl_2$, and 14 mM 2-mercaptoethanol.

Inactivation of PDC

Inactivation of PDC was initiated by adding ATP to intact mitochondria and mitochondrial extracts at room temperature (*ca.* 25°C). Various effectors, as detailed in figure legends were added as aqueous solutions simultaneously with ATP. Aliquots were removed for PDC assays at the times indicated in the figures.

PDC Assays

PDC activity was assayed by following NADH production at 340 nm using a Gilford-Response spectrophotometer. The assay mixture contained 50 mM Tes (pH 7.5), 2.5% (v/v) Triton X-100 (intact mitochondria only), 3.3 mM $MgCl_2$, 2.5 mM NAD, 0.2 mM TPP, 0.13 mM LiCoA, 2.6 mM cysteine, and 1.5 mM pyruvate.

RESULTS

Inactivation/Reactivation Cycle of PDC in Intact Mitochondria

When ATP was added to intact pea mitochondria maintained at room temperature (*ca.* 25°C) PDC underwent rapid inactivation followed by slower reactivation (Fig. 1). The rate and extent of inactivation, and also the lag-time before reactivation occurred, increased with increasing ATP concentration. These results confirm those of Budde and Randall (1) who showed that maximal inactivation was correlated with maximal phosphorylation of the 43 kD subunit of PDH and that reactivation did not occur until most of the added ATP had disappeared. Since reactivation was blocked by adding oligomycin (an ATPase inhibitor) and an ATP regenerating system, or by incubating the mitochondria at 4°C, it was hypothesized that disappearance of the added ATP was the result of its hydrolysis catalyzed primarily by the mitochondrial F_1-ATPase (1).

Regulation of Inactivation of PDC in Intact Mitochondria

The level of PDC activity is determined by the relative rates of inactivation catalyzed by PDH kinase and reactivation catalyzed by P-PDH phosphatase. Both enzymes are potentially active at the same time and therefore it is necessary to inhibit one of them in order to study effects on the other. Since we were interested in PDH kinase, we used fluoride, a classical phosphatase inhibitor, to inhibit reactivation of PDC (Fig. 2). The fluoride concentration routinely used was 100 mM because lower concentrations were not effective with intact mitochondria (data not shown).

Both pyruvate and TPP markedly inhibited inactivation of PDC when added to intact mitochondria together with ATP and either NaF or KF (Fig. 3). Inhibition by pyruvate confirms the results of a previous investigation of intact and detergent-permeabilized pea mitochondria in which low temperature (4°C) was used to inhibit reactivation (1). However, in that study TPP alone did not inhibit inactivation of PDC but only enhanced the inhibitory effect of pyruvate.

In view of this apparent conflict we attempted to define the conditions which facilitate inhibition by TPP. First of all it should be noted that the ionic strength (due to NaF or KF) was much greater in the present study than in the previous one of Budde and Randall (1). Secondly, the inhibition by either pyruvate or TPP was greater in the presence of the Na^+ salt as compared with the K^+ salt of F^- (Fig. 3).

To examine the relative importance of each of the above factors, we repeated the ATP-dependent inactivation curves either in the absence of high salt or in the presence of Cl^- salts rather than F^- salts of several different monovalent cations. The ATP concentration was increased from 80 μM to 200 μM to make the inactivation rate comparable to that seen in the presence of F^-.

In the absence of high salt, pyruvate still inhibited ATP dependent inactivation of PDC (Fig. 4) whereas TPP did not (Fig. 6A). All three chloride salts tested, stimulated inhibition by pyruvate (Fig. 5). Sodium was the most effective followed by NH_4^+ and then K^+. In contrast, only Na^+ facilitated inhibition by TPP (Fig. 6). Since different monovalent cations gave different effects it was concluded that high ionic strength alone was not the factor facilitating inhibition by TPP.

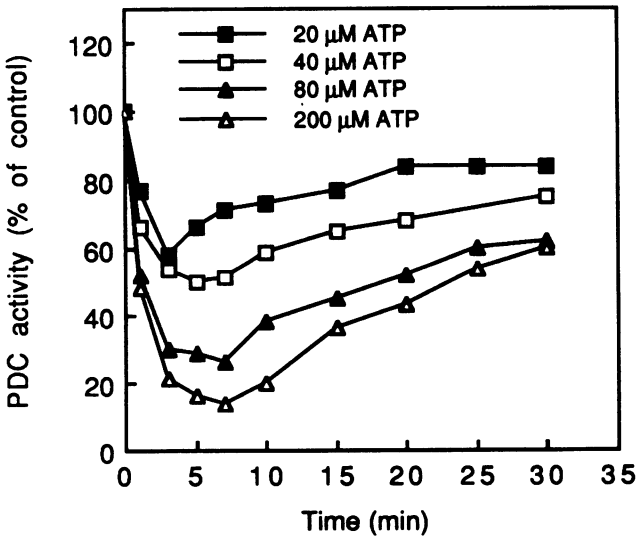

Figure 1. Time course of ATP-dependent inactivation and reactivation of PDC. Intact mitochondria were incubated with the ATP concentrations shown. PDC activity of the minus ATP control varied by less than 10% of the initial activity for the duration of the experiment.

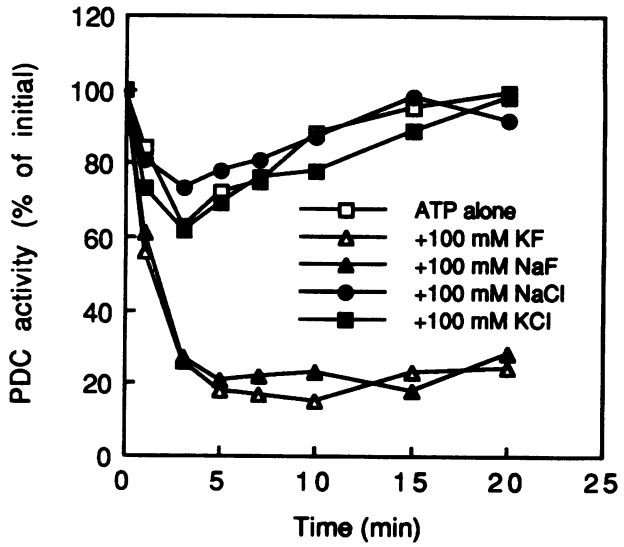

Figure 2. Inhibitory effect of fluoride on reactivation of PDC. Intact mitochondria were incubated with 40 μM ATP plus or minus salts as shown.

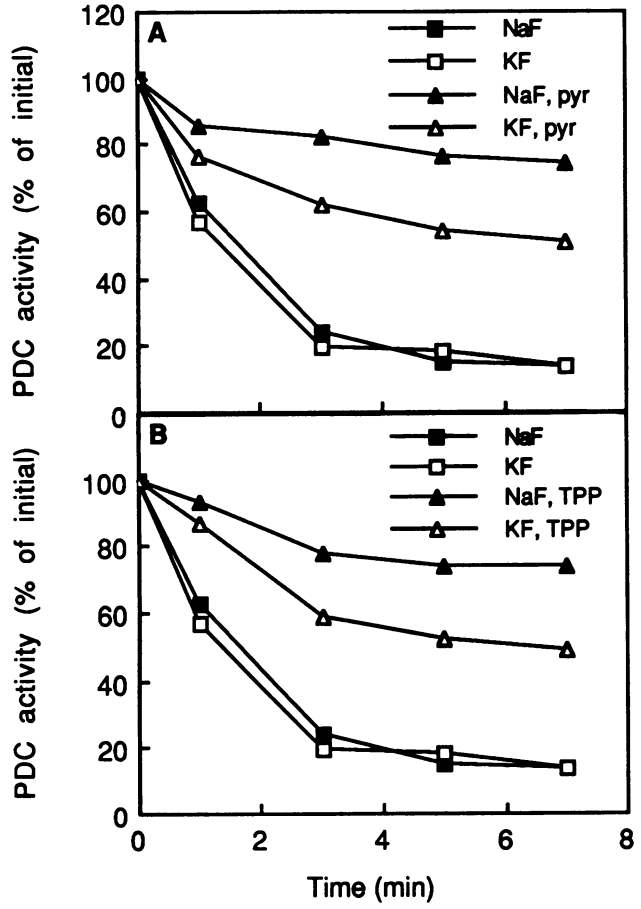

Figure 3. Effects of 1.2 mM pyruvate (A) and 80 μM TPP (B) on ATP-dependent inactivation of PDC in the presence of 100 m*M* fluoride salts. Intact mitochondria were incubated with 80 μM ATP plus effectors as indicated.

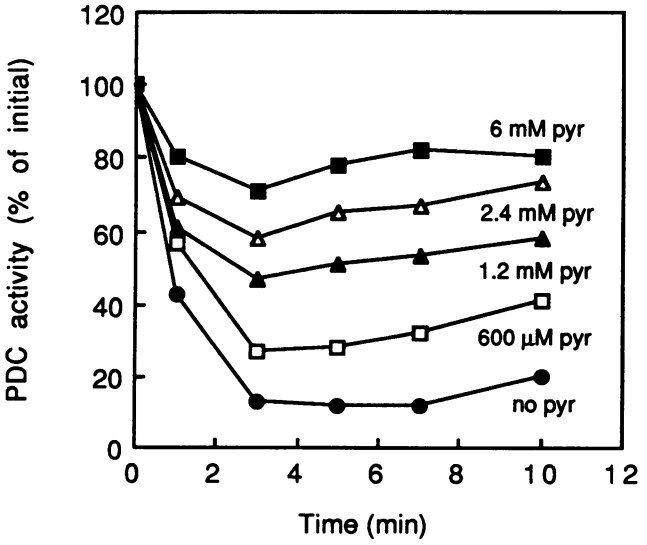

Figure 4. Effect of pyruvate alone on ATP-dependent inactivation of PDC. Intact mitochondria were incubated with 200 μM ATP plus the pyruvate concentrations shown.

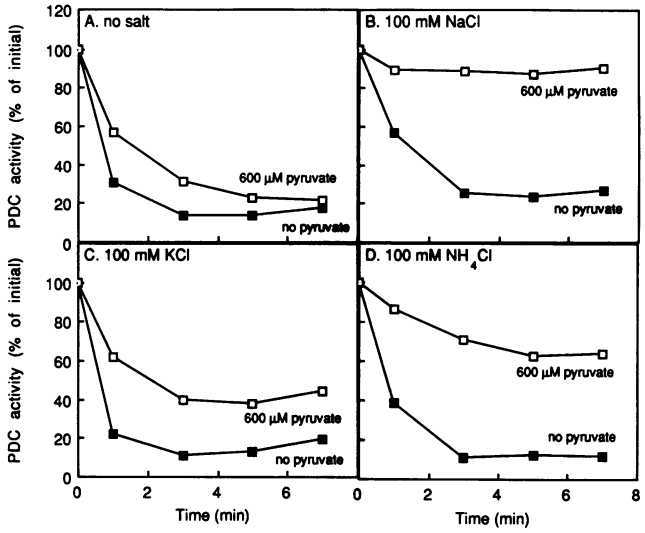

Figure 5. Influence of 100 mM chloride salts on the effect of pyruvate on ATP-dependent inactivation of PDC. Intact mitochondria were incubated with 200 μM ATP plus effectors as shown.

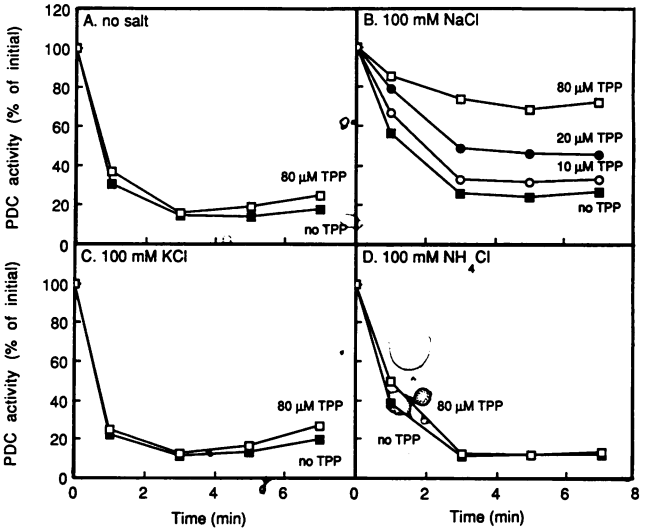

Figure 6. Influence of 100 mM chloride salts on the effect of TPP on ATP-dependent inactivation of PDC. Intact mitochondria were incubated with 200 μM ATP plus effectors as shown.

Regulation of Inactivation of PDC in Dialyzed Mitochondrial Extracts

In order to more thoroughly examine possible monovalent cation effects on the inactivation of PDC, we carried out a series of experiments with dialyzed extracts of freeze-thaw disrupted mitochondria. Intact mitochondria were routinely suspended in a Tes-KOH buffer. In order to remove K^+ from the system, we changed to Tris-HCl for the dialysis buffer. Following thawing and prior to dialysis, broken mitochondrial membranes and any remaining intact mitochondria were removed by centrifugation at 15,000*g* for 5 min. The mitochondrial ATPase, located on the inner membrane, was also most likely removed by this procedure.

Another characteristic of the freeze-thaw mitochondrial

extracts was that they exhibited negligible P-PDH phosphatase activity. This conclusion was based on the observation that addition of glucose plus hexokinase, as an ATP scavenging system, did not result in reactivation of PDC (data not shown). As a control, the ATP scavenging system was added at time zero, simultaneously with the ATP. This resulted in significant inhibition of inactivation. The absence of P-PDH phosphatase activity was fortuitous because it allowed us to study effects on PDH kinase independently of effects of the phosphatase.

The effects of pyruvate and TPP on ATP-dependent inactivation of PDC in dialyzed mitochondrial extracts are shown in Figure 7. Sodium was no longer a requirement for TPP to inhibit inactivation.

Sodium alone, at 10 to 100 mM levels, also inhibited ATP-dependent inactivation of PDC (Fig. 8A). In contrast K^+, at a 10-fold lower concentration, markedly stimulated inactivation (Fig. 8B). Ammonium was also stimulatory but only μM concentrations were required to produce almost the same effect as mM concentrations of K^+ (Fig. 8C). The inhibitory effect of Na^+ could not be explained by stimulation of latent P-PDH phosphatase activity (data not shown).

DISCUSSION

It has been reported (1, 2) that ATP-dependent inactivation of PDC in intact pea leaf mitochondria is inhibited by pyruvate and high concentrations of Mg^{2+} but not by TPP alone or ADP. Here we have extended these observations by comparing intact mitochondria with dialyzed mitochondrial extracts. In dialyzed extracts, ATP-dependent inactivation of PDC catalyzed by PDH kinase was stimulated by NH_4^+ (20–80 μM) and K^+ (0.5–4.0 mM) but inhibited by Na^+ (20–80 mM).

Stimulation of PDH kinase by NH_4^+ suggests the possibility of a regulatory interaction between photorespiratory NH_4^+ production, which occurs in the mitochondria by way of the glycine decarboxylase reaction, and PDC activity. In other

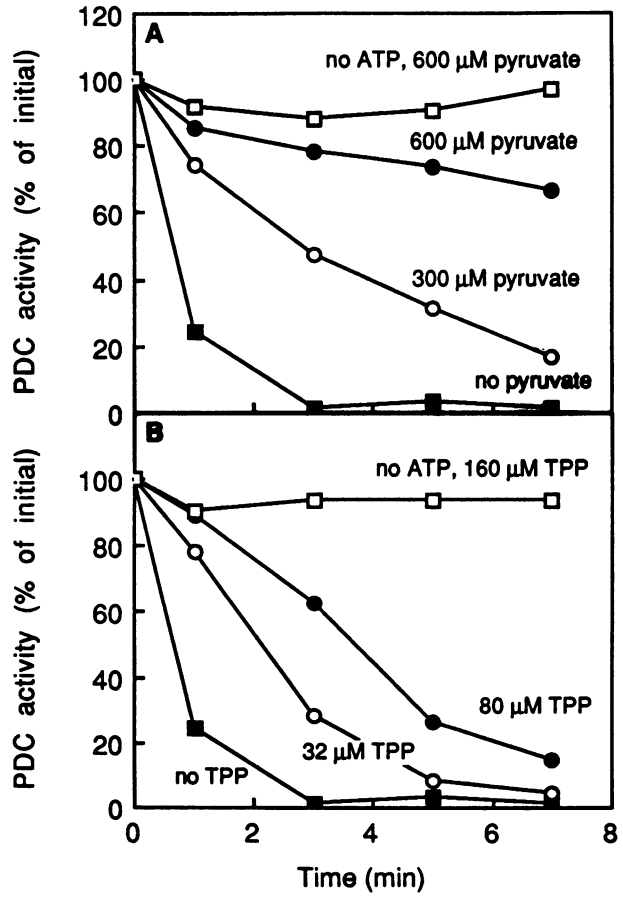

Figure 7. Effects of pyruvate (A) and TPP (B) on ATP-dependent inactivation of PDC in mitochondrial extracts. Extracts were incubated with 200 μM ATP plus effectors as shown.

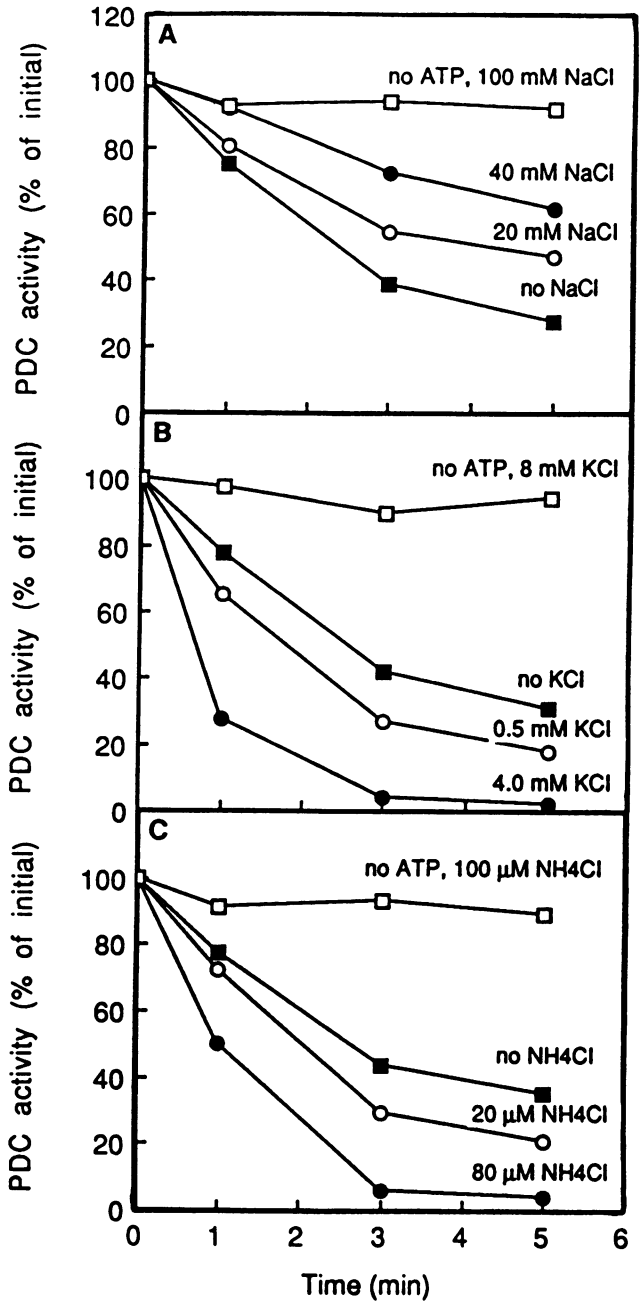

Figure 8. Effects of monovalent cations on ATP-dependent inactivation of PDC in mitochondrial extracts. The ATP concentration was 20 μM.

words, as photorespiration and consequently NH_4^+ production increase in the light, PDH kinase may become more active, making PDC more likely to be inactivated. As a consequence of this, entry of carbon into the tricarboxylic acid cycle by way of PDC will be progressively shut down. This may be at least part of the mechanism determining whether dark respiration occurs simultaneously with photosynthesis, a question which has drawn the attention of plant researchers for some time (9). The NH_4^+ concentration in mitochondria purified from light-grown pea shoots was at least 3.0 mM (25). Therefore, the NH_4^+ concentrations used in the present study are well within physiologically relevant limits.

It has been shown that PDC becomes inactivated in isolated mitochondria oxidizing either glycine or succinate (3). With succinate as the respiratory substrate inactivation of PDC may be attributable to an increase in the mitochondrial ATP level. With glycine, elevated NH_4^+ levels as well as an increase in the level of ATP may be involved in promoting the inactivation of PDC. This effect of NH_4^+ may explain the priority of glycine oxidation over the oxidation of tricarboxylic acid cycle intermediates in isolated mitochondria (5).

Pyruvate kinase isolated from a number of higher plants is activated by NH_4^+ (13, 16, 22). This led Graham (9) to suggest that NH_4^+ produced during photorespiration would diffuse out of the mitochondria, activate pyruvate kinase and thereby stimulate carbon entry into the tricarboxylic acid cycle. Contrary to this, our results and the co-compartmentation of PDC and photorespiratory NH_4^+ production within the mitochondria suggest that tricarboxylic acid cycle activity will initially be inhibited under photorespiratory conditions. Subsequent activation of pyruvate kinase by NH_4^+ could result in an increase in the level of pyruvate and consequent reactivation of PDC (see later).

There are several ways to test the hypothesis that photorespiratory NH_4^+ production promotes inactivation of PDC. First of all, it has been shown that mesophyll mitochondria of C_4 plants lack serine hydroxymethyltransferase and glycine decarboxylase, the enzymes involved in photorespiratory NH_4^+ production (15, 18, 24). Therefore, regulatory phosphorylation of PDC in these mitochondria may not be affected by NH_4^+. Alternatively, PDC may not be inactivated and the tricarboxylic acid cycle may continue to function in the mesophyll cells of C_4 plants. The same may also apply in bundle sheath cells, since although the enzymes catalyzing photorespiratory NH_4^+ production are present in these cells, it is unlikely that appreciable photorespiration occurs because of high CO_2 levels (15, 18, 24).

The NH_4^+ produced by photorespiration is considered to be assimilated through the glutamine synthase/glutamate synthase cycle and GDH is thought not to be involved (11, 14, 21, 23). Therefore, methionine sulfoximine (a glutamine synthase inhibitor) or inhibitors of photorespiration could be used to vary the NH_4^+ levels in mitochondria in leaf discs while simultaneously measuring the *in situ* activity of PDC.

Stimulation of PDH kinase by K^+ may have several explanations. Many enzymes, including kinases, have been shown to be activated by K^+ and to a lesser extent by NH_4^+ (6). Sodium often antagonizes the stimulation by K^+.

Both pyruvate and TPP inhibited PDH kinase in dialyzed mitochondrial extracts. Pyruvate was also inhibitory in intact mitochondria, confirming previous observations (1). In contrast, TPP only inhibited PDH kinase in intact mitochondria when high levels of Na^+ were added. Addition of Na^+ was not necessary in order for TPP to inhibit PDH kinase in dialyzed mitochondrial extracts. The discrepancy between the results obtained with intact mitochondria and dialyzed extracts cannot simply be explained by an inability of mitochondria to take up TPP. Oxygen electrode studies have shown that pyruvate oxidation is almost totally dependent upon the addition of TPP indicating that this cofactor is readily able to cross mitochondrial membranes (3). The most likely explanation lies in the higher level of K^+ in the buffer used to resuspend intact mitochondria, as compared with the dialysis buffer (see "Materials and Methods"). As noted above, K^+ markedly stimulates PDH kinase. Furthermore, K^+ apparently lowers the K_m (ATP) of the kinase (KA Schuller, DD Randall, unpublished data). Consequently, higher concentrations of TPP would be expected to be required to inhibit PDH kinase in the presence of K^+. The observation that high concentrations of Na^+ facilitate inhibition by TPP in intact mitochondria could possibly be explained by externally added Na^+ displacing K^+ inside the mitochondria.

The physiological significance of inhibition by TPP is unclear. This cofactor of PDH is apparently tightly bound to partially purified pea leaf mitochondrial PDC but rapidly dissociates from broccoli bud mitochondrial PDC *in vitro* (12, 20). Purified, intact mitochondria cannot oxidize pyruvate without added TPP (3). Presumably, TPP would have to be dissociated from PDH to be free to regulate PDH kinase.

It has been proposed that pyruvate is the major metabolite regulating the covalent modification of PDC (1). The results of the present study support the importance of pyruvate in this respect. However, it is not immediately obvious as to why pyruvate should regulate covalent modification when it could regulate PDC activity simply through changes in substrate (*i.e.* pyruvate) availability. Perhaps pyruvate is required to maintain PDC in the active form when mitochondrial ATP levels rise. High ATP levels would tend to favour inactivation. There may be conditions, such as a large demand for 2-oxoglutarate for NH_4^+ assimilation, under which tricarboxylic acid cycle flux is required in the presence of high levels of ATP. Rapid subcellular fractionation studies have shown that mitochondrial ATP/ADP ratios are higher under photorespiratory conditions (8). A fuller understanding of this problem awaits information on diurnal fluctuations in mitochondrial pyruvate levels.

In summary, many metabolites affect ATP-dependent inactivation of plant mitochondrial PDC. Pyruvate, TPP and Na^+ are inhibitory while K^+ and NH_4^+ are stimulatory. Other studies have shown that Mg^{2+} is an inhibitor (2). The relative importance of these effectors and the interactions between them are still unclear. Kinetic analyses of PDC activity *in vitro*, coupled with *in situ* data on PDC activity and metabolite levels, should eventually resolve many of the outstanding questions.

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LITERATURE CITED

1. **Budde RJA, Randall DD** (1987) Regulation of pea mitochondrial pyruvate dehydrogenase complex activity: inhibition of ATP-dependent inactivation. Arch Biochem Biophys **258:** 600–606
2. **Budde RJA, Randall DD** (1988) Regulation of steady state pyruvate dehydrogenase complex activity in plant mitochondria: reactivation constraints. Plant Physiol **88:** 1026–1030
3. **Budde RJA, Fang TK, Randall DD** (1988) Regulation of the phosphorylation of mitochondrial pyruvate dehydrogenase complex *in situ*: effects of respiratory substrates and calcium. Plant Physiol **88:** 1031–1036
4. **Denton RM, Randle PJ, Bridges BJ, Cooper RH, Kerbey AL, Pask HT, Severson DL, Stansbie D, Whitehouse S** (1975) Regulation of mammalian pyruvate dehydrogenase. Mol Cell Biochem **9:** 27–53
5. **Dry IB, Day DA, Wiskich JT** (1983) Preferential oxidation of glycine by the respiratory chain of pea leaf mitochondria. FEBS Lett **158:** 154–158
6. **Evans HJ, Sorger GJ** (1966) Role of mineral elements with emphasis on the univalent cations. Annu Rev Plant Physiol **17:** 47–76
7. **Fang TK, David NR, Miernyk JA, Randall DD** (1987) Isolation and purification of functional pea leaf mitochondria free of chlorophyll contamination. Curr Topics Plant Biochem Physiol **6:** 175
8. **Gardeström P, Wigge B** (1988) Influence of photorespiration on ATP/ADP ratios in the chloroplasts, mitochondria, and cytosol, studied by rapid fractionation of barley (*Hordeum vulgare*) protoplasts. Plant Physiol **88:** 69–76
9. **Graham D** (1980) Effects of light on dark respiration. *In* DD Davies, ed, The Biochemistry of Plants, Vol 2. Academic Press, New York, pp 525–579
10. **Hucho F, Randall DD, Roche TE, Burgett MW, Pelley JW, Reed LJ** (1972) α-Keto acid dehydrogenase complexes. XVII. Kinetics and regulatory properties of pyruvate dehydrogenase kinase and pyruvate dehydrogenase phosphatase from bovine kidney and heart. Arch Biochem Biophys **151:** 328–340
11. **Martin F, Winspear MJ, Macfarlane JD, Oaks A** (1983) Effects of methionine sulfoximine on the accumulation of ammonia in C_3 and C_4 leaves. Plant Physiol **71:** 177–181
12. **Miernyk JA, Randall DD** (1987) Some kinetic and regulatory properties of the pea mitochondrial pyruvate dehydrogenase complex. Plant Physiol **83:** 306–310
13. **Miller G, Evans HJ** (1957) The influence of salts on pyruvate kinase from tissues of higher plants. Plant Physiol **32:** 346–354
14. **Morris PF, Layzell DB, Canvin DT** (1988) Ammonia production and assimilation in glutamate synthase mutants of *Arabidopsis thaliana*. Plant Physiol **87:** 148–154
15. **Ohnishi J, Kanai R** (1983) Differentiation of photorespiratory activity between mesophyll and bundle sheath cells in C4 plants. 1. Glycine oxidation by mitochondria. Plant Cell Physiol **24:** 1411–1420
16. **Peterson JB, Evans HJ** (1978) Properties of pyruvate kinase from soybean nodule cytosol. Plant Physiol **61:** 909–914
17. **Randall DD, Williams M, Rapp BJ** (1981) Phosphorylation-dephosphorylation of pyruvate dehydrogenase complex from pea leaf mitochondria. Arch Biochem Biophys **207:** 437–444
18. **Rathnam CKM** (1979) Metabolic regulation of carbon flux during C4 photosynthesis. II. *In situ* evidence for refixation of photorespiratory CO_2 by C4 phosphoenolpyruvate carboxylase. Planta **145:** 13–23
19. **Rubin PM, Randall DD** (1977) Regulation of plant pyruvate dehydrogenase complex by phosphorylation. Plant Physiol **60:** 34–39
20. **Rubin PM, Zahler WL, Randall DD** (1978) Plant pyruvate dehydrogenase complex: Analysis of the kinetic properties and metabolite regulation. Arch Biochem Biophys **188:** 70–77
21. **Somerville CR, Ogren WL** (1980) Inhibition of photosynthesis in *Arabidopsis* mutants lacking leaf glutamate synthase activity. Nature **256:** 257–259
22. **Tomlinson JD, Turner JF** (1973) Pyruvate kinase of higher plants. Biochim Biophys Acta **329:** 128–139
23. **Wallsgrove RM, Keys AJ, Lea PJ, Miflin BJ** (1983) Photosynthesis, photorespiration and nitrogen metabolism. Plant Cell Environ **6:** 301–309
24. **Woo KC, Osmond CB** (1977) Participation of leaf mitochondria in the photorespiratory carbon oxidation cycle: glycine decarboxylase activity in leaf mitochondria from different species and its intra-mitochondrial location. Plant Cell Physiol Spec Issue **18:** 315–332
25. **Yamaya T, Matsumoto H** (1985) Influence of NH_4^+ on the oxygen uptake of mitochondria isolated from corn and pea shoots. Soil Sci Plant Nutr **31:** 513–520