Plant Pyruvate Dehydrogenase Complex

II. ATP-DEPENDENT INACTIVATION AND PHOSPHORYLATION

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ABSTRACT

ATP inactivated plant pyruvate dehydrogenase complex (PDC) from broccoli (Brassica oleracea) mitochondria. ATP inactivation of the complex was time-dependent and proportional to the ATP concentration. Time-dependent incorporation of $^{32}$P from $[\gamma^{32}$P]ATP into trichloroacetic acid-precipitable protein corresponded to the inactivation of the PDC. It is concluded that plant PDC is phosphorylated and inactivated by a PDC kinase.

The pyruvate dehydrogenase complex (PDC) catalyzes the oxidative decarboxylation of pyruvate by a sequence of reactions. The complex consists of three enzymes: (a) pyruvate dehydrogenase; (b) lipoyl transacetylase; and (c) lipoyl dehydrogenase, acting sequentially in that order. The essentially irreversible nature of the first reaction in the complex and the pivotal position that PDC occupies in metabolism make PDC a logical candidate for regulation.

Reed and colleagues (6, 7) established that mammalian PDC can also be regulated by a phosphorylation-dephosphorylation mechanism. Phosphorylation and the concomitant loss of PDC activity require a Mg-ATP-dependent kinase (5). Dephosphorylation and reactivation of PDC is catalyzed by a Mg$^{2+}$- and Ca$^{2+}$-requiring phosphatase. The phosphorylation-dephosphorylation of PDC has been demonstrated in numerous mammalian tissues using both purified PDC (4, 5) and intact mitochondria (1, 11). This regulatory mechanism has also been demonstrated in Neurospora crassa PDC (14).

Characterization of the plant PDC has been very limited (2, 8–10) and there have been few reports describing the regulation of the plant PDC. There have been no reports to date of inactivation or reactivation of plant PDC by a phosphorylation or dephosphorylation mechanism. We report here our finding of phosphorylation and concomitant inactivation of PDC isolated from the floral bud mitochondria of broccoli.

MATERIALS AND METHODS

Assay for Pyruvate Dehydrogenase Complex. The activity of the PDC was determined by monitoring NADH formation at 340 nm and 27 C with a Gilford model 2000 recording spectrophotometer. The standard assay mixture contained 175 $\mu$mol MOPS-glycylglycine buffer (pH 8.1), 0.10 $\mu$mol thiamine pyrophosphate, 1 $\mu$mol MgCl$_2$, 2.4 $\mu$mol NAD$^+$, 0.12 $\mu$mol lithium CoA, 2.6 $\mu$mol cysteine-HCl, 1 $\mu$mol potassium pyruvate and enzyme complex in a total volume of 1 ml. The pH of the final assay mixture was 7.95. The reaction was initiated by either pyruvate or enzyme. The activity is expressed as $\mu$mol NADH formed/min and is based on the initial rate. A unit of PDC activity is defined as the formation of 1 $\mu$mol NADH/min. Specific activity is expressed as units of activity/mg protein. The NAD$^+$, coenzyme A, and ATP were all purchased from P-L Biochemicals, Milwaukee, Wis., $[\gamma^{32}$P]ATP was purchased from ICN Isotope and Nucl. Trinave, Calif. All other reagents used were of the highest purity commercially available.

Preparation of Mitochondria. Mitochondria were isolated from floral buds of broccoli (Brassica oleracea var. italica) purchased at local markets. The floral buds were shaved from the broccoli heads and crude mitochondria suspensions were prepared by homogenizing floral buds in 2.5 volumes of medium containing 0.5 m sucrose, 0.1 m sodium phosphate buffer (pH 7.8), 0.1% BSA, 5 mm EGTA, and 13.5 mm 2-mercaptoethanol. The brei was filtered through two layers of cheesecloth and two layers of Miracloth (Chicopee Mills, Milltown, N. J.). The filtrate was centrifuged at 400g for 15 min. The resulting supernatant was centrifuged for 30 min at 14,500g to pellet the mitochondria. The mitochondria were gently resuspended in extraction medium, diluted 1:1 with 25 mm K-phosphate buffer (pH 6.8) containing 13.5 mm 2-mercaptoethanol. The mitochondrial suspension was centrifuged as above and the pellet was resuspended in a minimal amount of the diluted extraction medium, but without 2-mercaptoethanol, and stored on ice until used.

Inactivation of PDC. ATP-dependent inactivation of PDC was performed by incubating 0.40 ml mitochondria suspension (8.8 mg protein) in the presence of 1% Triton X-100 at 27 C for 5 min. A 20-$\mu$l aliquot was removed and the PDC activity determined, whereupon 1 $\mu$mol of ATP and 2 $\mu$g oligomycin/mg protein were added to make a total volume of 0.5 ml. At the indicated times, 20-$\mu$l aliquots of the incubation mixture were assayed for PDC activity. The difference between PDC activity at zero time and after ATP addition is a measure of ATP-dependent PDC inactivation or PDC kinase activity.

Phosphorylation of PDC. To demonstrate the phosphorylation of PDC concomitant with the inactivation of PDC, mitochondria were preincubated with 1% Triton X-100 at 27 C for 5 min, and then centrifuged for 15 min at 27,000g. One $\mu$mol of $[\gamma^{32}$P]ATP (28,000 cpm/nmol) was added to the supernatant which contained the PDC activity. At the indicated times, 50-$\mu$l aliquots of the incubation mixture were withdrawn and placed on Whatman 3MM paper (2.5 x 2.5 cm) and dropped into cold 10% trichloroacetic acid for 2 hr. The papers were washed three times with cold 10% trichloroacetic acid, twice with ethanol and ether. The papers were dried and the $^{32}$P incorporated into...
trichloroacetic acid-precipitable protein was measured by scintillation spectrometry.

RESULTS

PDC activity released from Triton X-100-treated mitochondria which were incubated with different concentrations of ATP was determined. Figure 1A shows PDC activity decreased in the presence of ATP as compared to the control without ATP. At 1 mM ATP, there is at least a 40% inhibition in PDC activity after 2 min of incubation. At the lower ATP concentrations (<1 mM), the curves are less convex and the maximum inhibition reached is correspondingly less.

Figure 1B shows the results of incubating partially purified PDC (pellet by centrifuging at 204,000g for 3 hr and redissolving in 0.025 M MOPS buffer [pH 7] with 6 mM dithioerythritol) with various ATP concentrations. The specific activity of the partially purified PDC was 0.26 versus a specific activity of 0.02 for lysed mitochondrial enzyme. Partially purified PDC was sensitive to lower concentrations of ATP than the lysed mitochondrial enzyme. At 200 μM ATP, the partially purified PDC is 40% inactivated at 2 min. ATP concentrations below 50 μM inhibit the isolated PDC only to a very limited degree.

Figure 2 compares PDC activity to 32P incorporation into trichloroacetic acid-precipitable protein. Triton X-100-lysed mitochondria were clearspun at 27,000g for 15 min. The supernatant which contained the PDC activity was incubated in the presence of [γ-32P]ATP as described under "Materials and Methods." As PDC activity decreased, there was a corresponding time-dependent 32P incorporation into trichloroacetic acid-precipitable protein. The control (boiled mitochondria) shows no increase in 32P incorporation with time as the experimental does.

DISCUSSION

In mammalian systems, one of the major control mechanisms involved in regulating the PDC is phosphorylation-dephosphorylation (3, 6, 7). Other mammalian enzymes are also controlled by this mechanism (12). No similar system has previously been demonstrated to be operative in plant tissue for PDC or any other enzyme (2, 8–10, 13). The ATP-requiring, time-dependent inactivation of broccoli PDC as shown in Figure 1, A and B, and the corresponding incorporation of 32P (Fig. 2), demonstrate the presence of a PDC-inactivating system similar to the mammalian PDC kinase. We have not yet been able to reactivate the phosphorylated PDC by increasing the Mg2+ or Ca2+ concentrations as in the mammalian system. However, this may be due to the lability of the phosphatase from our particular tissue. Dialysis does not reactivate the phosphorylated PDC; however, putting the phosphorylation reaction mixture on a Sephadex G-25 column curtails further inactivation.

This report demonstrates the presence of PDC kinase for plant PDC, and suggests that phosphorylation-dephosphorylation of PDC is an essential component in the regulation of dark respiration in plants as it is in mammalian tissues. This report further extends this mechanism of enzyme control into the higher plant kingdom.

LITERATURE CITED

PLANT PYRUVATE DEHYDROGENASE COMPLEX


