Biochimica et Biophysica Acta, 485 (1977) 336—349

BBA 68283

PLANT PYRUVATE DEHYDROGENASE COMPLEX PURIFICATION, CHARACTERIZATION AND REGULATION BY METABOLITES AND PHOSPHORYLATION

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(Received April 26th, 1977)

Summary

The pyruvate dehydrogenase complex was purified from mitochondria of cauliflower, *Brassica oleracea* var. botrytis floral buds to a specific activity of 5.4 μmol of NADH/min per mg of protein. The pyruvate dehydrogenase complex required CoASH, NAD^+, thiamine pyrophosphate and Mg^{2+} for the oxidative decarboxylation of pyruvate. The kinetic analysis of the complex gave a series of parallel lines for all substrates. Product interaction patterns showed that NADH is competitive with NAD^+; acetyl-CoA is competitive with CoASH; and NADH and acetyl-CoA uncompetitive with pyruvate. These kinetic patterns suggest a multisite ping-pong mechanism as described by Cleland ((1973) J. Biol. Chem. 248, 8353). The noncompetitive inhibition of NADH versus CoASH, and acetyl-CoASH versus NAD^+ are not predicted by this mechanism.

Regulation of the complex was more sensitive to the NADH/NAD^+ ratio than acetyl-CoA/CoASH ratio. Hydroxypyruvate and glyoxylate inhibited the complex noncompetitively versus pyruvate. The pyruvate dehydrogenase complex was inactivated and phosphorylated by ATP. The ATP dependent inactivation is believed to be enzyme catalyzed by a pyruvate dehydrogenase complex kinase. However, no evidence was found for a plant pyruvate dehydrogenase complex phosphatase. The results suggest that the cauliflower pyruvate dehydrogenase complex is regulated by a phosphorylation-dephosphorylation mechanism.

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Abbreviations and Symbols: EGTA: ethylene-glycol 2-bis(β-aminoethylether)-*N*,*N*′-tetraacetic acid; MOPS: Morpholinopropane sulfonic acid.

Introduction

Pyruvate undergoes oxidative decarboxylation by the pyruvate dehydrogenase multienzyme complex. This complex is composed of at least 3 different enzymes: pyruvate dehydrogenase (EC 1.2.4.2), dihydrolipoyl transacetylase (EC 2.3.1.12) and dihydrolipoyl dehydrogenase (EC 1.6.4.3). They catalyze the overall reaction:

$$CH_3COCOOH + CoASH + NAD^+ \rightarrow CH_3CO{-}CoA + NADH + H^+ + CO_2. \quad (1)$$

This enzyme complex has been purified from several mammalian [1—4] and microbial [5—8] sources. These multienzyme complexes range in molecular weight from 1.1 million to several million and are localized in the mitochondria of non-plant eucaryotes. The biochemical properties of the pyruvate dehydrogenase complexes have been reviewed by Reed et al. [9] and Denton et al. [10].

The multiple metabolic fates of pyruvate and the irreversibility of the first enzymatic reaction of the complex make it necessary that the pyruvate dehydrogenase complex activity be regulated. Several regulatory mechanisms have been found which involve metabolite or product inhibition [9—15]. Reed et al. [9,16] have also established a specific, enzyme catalyzed, phosphorylation mechanism for regulating mammalian pyruvate dehydrogenase complex. Others have since demonstrated this mechanism of pyruvate dehydrogenase complex control in other tissues [10]. The phosphorylation catalyzed by a specific $Mg \cdot ATP^{2-}$-dependent kinase inactivates the mammalian complex, while dephosphorylation catalyzed by the specific Mg^{2+}-dependent phosphatase reactivates the mammalian complex [17].

Reports of studies on pyruvate dehydrogenase complex from higher plants are limited. The biochemical properties and regulation of this enzyme have been assumed to be like the mammalian and microbial enzyme [18]. Extracts of pea epicotyl mitochondria have yielded a pyruvate oxidase that was inhibited by glyoxylate [19]. Crompton and Laties [20] reported that pyruvate dehydrogenase complex from crude extracts of potato tuber mitochondria was inhibited by NADH, acetyl-CoA, and glyoxylate. Pyruvate dehydrogenase complex in proplastids from castor bean endosperm is the only reported non-mitochondrial location of pyruvate dehydrogenase complex in eucaryotes [21] which we have also observed (unpublished observation). We recently isolated the enzyme complex from broccoli floral buds [22] and have made a preliminary report on the occurrence of the phosphorylation-inactivation mechanism with this enzyme [23].

Variations in the biochemical and regulatory properties of non-plant pyruvate dehydrogenase complex and the limited information concerning this very important enzyme in plant tissue indicate that a detailed investigation is essential to understanding its role and regulatory functions in plant tissues. The role of pyruvate dehydrogenase complex in regulating the interaction between respiration, carbohydrate metabolism, and the unique metabolic events in plants must be established. This report describes the isolation, purification and the biochemical and regulatory properties of the pyruvate dehydrogenase complex from cauliflower, *Brassica oleracea* var. botrytis, mitochondria.

Methods

Materials

NAD^+ NADH, CoASH, acetyl-CoA and ATP were purchased from P-L Biochemicals. Potassium pyruvate (Type III), thiamine pyrophosphate, glyoxylate, hydroxypyruvate, glycine, serine, 3-phosphoglycerate and phosphoenolpyruvate, fraction V and crystalline bovine serum albumin, dithiothreitol, 2-mercaptoethanol and EGTA were purchased from Sigma. Polyethylene glycol 6000 was obtained from J.T. Baker. [γ^{23}P]ATP was obtained from ICN. All other chemicals were of the highest purity commerically available.

Assay for pyruvate dehydrogenase complex

The initial rate of the overall PDC reaction 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 μmol of MOPS/glycylglycine (pH 8.1), 0.21 μmol thiamine pyrophosphate, 1 μmol $MgCl_2$, 2.4 μmol NAD^+, 0.12 μmol lithium CoASH, 2.6 μmol cysteine · HCl, 1 μmol potassium pyruvate, and enzyme complex in a total volume of 1 ml. The final pH of the assay mixture was 7.9. The reaction was initiated with enzyme unless otherwise indicated. A unit of enzyme activity was defined as one μmol of NADH formed per min and was based on the initial rate. Specific activity was defined as units per mg protein. Protein concentrations were determined by the method of Lowry et al. [24] using crystalline bovine serum albumin as the standard.

Isolation of mitochondria

Mitochondria were isolated from cauliflower floral buds by a modification of a procedure of Jung and Hansen [25]. Cauliflower was obtained from local markets and the outer 2—4 mm of the floral buds removed with a razor blade and kept at 4°C until processed. Mitochondria were isolated from 600 g of floral buds by homogenizing 100 g batches in 200 ml of extraction medium (0.4 M sucrose, 50 mM potassium phosphate, pH 7.7 and 5 mM EGTA) for 30 s with a Polytron (speed 7). The pooled brei was filtered through 4 layers cheesecloth and 2 layers Miracloth (Chicopee Mills), and centrifuged at 400 × *g* for 10 min. The supernatant was centrifuged 30 min at 14 500 × *g* and the pellet suspended in 250 ml of extraction medium containing 20 mM 2-mercaptoethanol. This suspension was then centrifuged at 14 500 × *g* for 20 min, and the resulting pellet suspended in 20 mM potassium phosphate, pH 7, containing 20 mM 2-mercaptoethanol. This mitochondrial suspension was centrifuged at 14 500 × *g* for 20 min and the pellet suspended in a minimal amount of 20 mM potassium phosphate pH 7. Mitochondria to be used for inactivation and phosphorylation were shell-frozen in a solid CO_2/isopropanol bath. Dithiothreitol (8 mg/10 ml) was added to mitochondria used for pyruvate dehydrogenase complex isolation prior to shell freezing. The frozen mitochondria were stored at −20°C. Intact mitochondria were isolated by the same extraction filtration and centrifugation procedure. The last mitochondrial pellet was resuspended in minimal amount of 20 mM potassium phosphate, pH 7, and stored at 4°C.

Processing of kinetic data

Kinetic data were plotted as reciprocal velocities versus reciprocal substrate

concentration to determine linearity. When linear, the data were fitted to Eqn. 2, by the least squares method as described by Cleland [26]. The combined data for each experiment were then fitted to the appropriate rate equation. Initial velocity data were fitted to Eqn. 3 for a ping-ping mechanism, and inhibition data were fitted to Eqns. 4, 5 and 6 for linear competitive, linear uncompetitive and linear noncompetitive respectively; the pattern reported is the one which gave the best fit. The lines in each figure are calculated from the computer fit to Eqn. 2.

$$v = \frac{VA}{K + A} \tag{2}$$

$$v = \frac{VAB}{K_b A + K_a B + AB} \tag{3}$$

$$v = \frac{VA}{K(1 + I/K_{is}) + A} \tag{4}$$

$$v = \frac{VA}{K + A(1 + I/K_{ii})} \tag{5}$$

$$v = \frac{VA}{K(1 + I/K_{is}) + A(1 + I/I_{ii})} \tag{6}$$

Inactivation of pyruvate dehydrogenase complex

ATP-dependent inactivation of the complex was performed by incubating 0.5-ml aliquots of enzyme at 25°C with 0.5—1 μmol of ATP. Aliquots of this reaction mixture were removed just prior to ATP addition (zero time) and at various time intervals after ATP addition and assayed for pyruvate dehydrogenase complex activity. Controls were treated identically except they lacked ATP. When intact mitochondria were used as the enzyme source, the mitochondria were incubated at 25°C with 1% Triton X-100 for 5 min and the pyruvate dehydrogenase complex activity determined prior to addition of ATP. The differences in activity between zero time and times after ATP addition is a measure of ATP dependent pyruvate dehydrogenase complex inactivation or pyruvate dehydrogenase complex 'kinase' activity.

Phosphorylation of pyruvate dehydrogenase complex

Incorporation of ^{32}P from [$\gamma^{32}P$]ATP was used to show phosphorylation of the pyruvate dehydrogenase complex. The conditions for inactivation were the same as described above except that the [$\gamma^{32}P$]ATP (specific activity 28 000 cpm/nmol) was present. Aliquots (50 μl) of the incubation mixture were placed on Whatman 3 MM paper (2.5 × 2.5 cm) and dropped into cold 10% (w/v) trichloroacetic acid for 2 h. The papers were washed 3 times with cold trichloroacetic acid, twice with ethanol, twice with diethylether and then dried. The ^{32}P incorporated into trichloroacetic acid precipitable protein was measured by a liquid scintillation spectrometer. Parallel measurements to determine pyruvate dehydrogenase complex activity were performed and control incubations using boiled enzyme were carried out to correct for non-specific ^{32}P incorporation.

Results

Pyruvate dehydrogenase complex purification

The complex was extracted and purified by the procedure summarized in Table I.

Step I. The shell-frozen mitochondrial suspension was thawed and diluted with 2.5 vols. of 25 mM potassium phosphate, pH 6.5, containing 3.2 mM dithiothreitol. The suspension was stirred at 4°C for 15 min and centrifuged at 30 000 × *g* for 15 min. Most of the enzyme was recovered in the supernatant which was designated the mitochondrial extract.

Step II. The mitochondrial extract was centrifuged at 204 000 × *g* for 3 h to pellet all of the pyruvate dehydrogenase complex activity (no measureable pyruvate dehydrogenase complex activity was found in the supernatant). The amber colored pellet was gently suspended in a small volume of 25 mM MOPS, pH 7.0, containing 3.2 mM dithiothreitol, and centrifuged at 27 000 × *g* for 20 min to remove any insoluble material. At this point recovery of pyruvate dehydrogenase complex from the mitochondrial extract was 50—80%.

Step III. The enzyme solution from Step II was warmed to 20°C and 10 μmol of $MgCl_2$ added per ml. The solution was centrifuged at 27 000 × *g* for 15 min to remove the precipitate that formed. The supernatant was fractionated by dropwise addition of 1/10 vol. of 50% (w/v) solution of polyethylene glycol 6000. The solution was equilibrated for 15 min prior to centrifuging at 27 000 × *g* for 20 min. The pellet, which contained the pyruvate dehydrogenase complex activity, was suspended in a minimal volume of 25 mM MOPS, pH 7 containing 3.2 mM dithiothreitol, and centrifuged as above to remove the insoluble material. The pyruvate dehydrogenase complex remained in the supernatant and was designated the polyethylene glycol fraction.

Step IV. The polyethylene glycol fraction was further purified by layering 1-ml aliquots on 37-ml linear gradients of 10—40% (v/v) glycerol in 25 mM MOPS, pH 7, 3.2 mM dithiothreitol, 0.1 mM thiamine pyrophosphate, and 1 mM NAD^+. The gradients were centrifuged in an SW-27 rotor at 20 000 rev./min for 9 h in a Beckman Model L2-65B ultracentrifuge. The gradients were fractionated into 1-ml fractions and enzyme activity and protein levels deter-

TABLE I

SUMMARY OF PURIFICATION OF PYRUVATE DEHYDROGENASE COMPLEX FROM CAULIFLOWER FLORAL BUDS

Fraction	Volume (ml)	Activity (units *)	Protein (mg)	Specific activity ($\frac{\mu mol\ NADH\ formed}{min \cdot mg\ protein}$)	Recovery (%)
Freeze-thawed mitochondria (crude)	34	55	760	0.07	100
I. Mitochondrial extract	104	45	291	0.15	82
II. Ultracentrifugation	9	33	55	0.60	60
III. PEG ** fractionation	3	20.3	6.4	3.2	37
IV. Glycerol gradient	1.2	2.3	1.5	5.4	14

* μmol NADH formed per min per ml enzyme.

** PEG: polyethyleneglycol.

mined. (Fig. 1) The peak fractions were pooled, as indicated, and precipitated by addition of 1/10 vol. of polyethyleneglycol (50% w/v) in the presence of 10 mM $MgCl_2$. The precipitated complex was collected by centrifuging at 27 000 $\times g$ for 15 min and the pellet dissolved in a minimal volume of 25 mM MOPS, pH 7.0, containing 3.2 mM dithiothreitol, and 0.1 mM thiamine pyrophosphate. All further experiments reported utilized pyruvate dehydrogenase complex purified through the glycerol gradient.

Substrate and cofactor requirements

The substrate specificity and cofactors required by the purified complex are shown in Table II. The complex is reasonably specific for pyruvate. Oxidation of 2-oxobutyrate was at 1/6 the rate of that of pyruvate and no activity was detected with 2-oxoglutarate. The complex required Mg^{2+} as a divalent cation. The NAD^+, CoASH and thiamine pyrophosphate were absolute requirements for pyruvate dehydrogenase complex activity.

The effect of pH

Pyruvate dehydrogenase complex activity was optimal between pH 7 and 8 as shown in Fig. 2. The pH optimum was quite broad in MOPS/glycylglycine buffer while narrower and more alkaline with MOPS or HEPES buffers alone. The points shown in Fig. 2 are the pH values of the reaction mixture after assay. The pH of the reaction mixture decreased 0.2 pH unit from the buffer.

Kinetic analysis

Initial velocity patterns were obtained by varying the concentrations of one

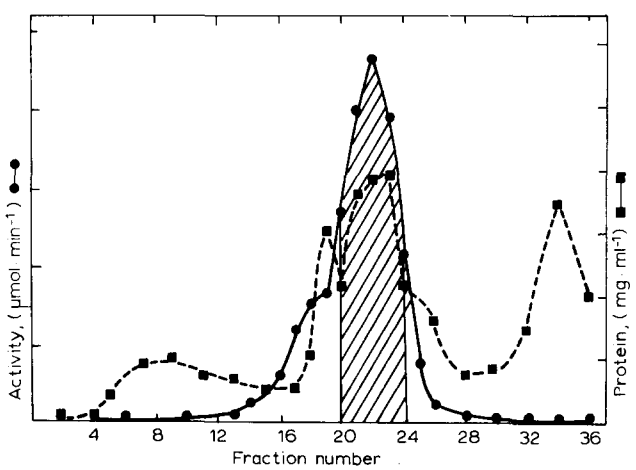

Fig. 1. Glycerol gradient. The polyethylene glycol-fractionated enzyme was layered on a 10—40% (v/v) glycerol gradient, centrifuged for 9 h at 20 000 rev./min in Beckman SW27 Rotor. The gradient was fractionated from the bottom in 1-ml fractions. Pooled fractions were precipitated with 10% polyethylene glycol for Step IV of Table I as indicated by shaded area. Protein (■) and pyruvate dehydrogenase complex activity (●).

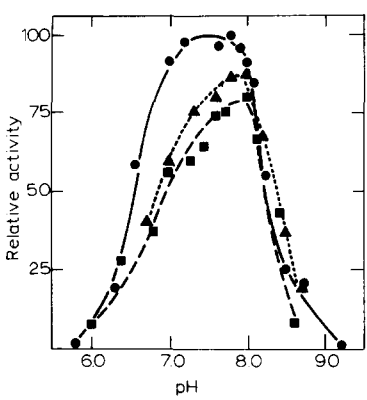

Fig. 2. The pH activity curve of the pyruvate dehydrogenase complex. The pyruvate dehydrogenase complex activity was determined in enzyme initiated reactions using the standard pyruvate dehydrogenase complex assay conditions with either 175 μmol MOPS (▲); 175 μmol HEPES (■), or 90 μmol MOPS plus 90 μmol glycylglycine (●). The pH values indicated were measured after the reaction had been monitored.

TABLE II

SUBSTRATE AND COFACTOR REQUIREMENTS

Pyruvate dehydrogenase complex from polyethyleneglycol fractionation step was passed through a G-25 Sephadex column equilibrated with 50 mM MOPS, pH 7.0, 3.2 mM dithiothreitol, and 5 mM EDTA. The complete reaction mixture contained 174 μmol of MOPS/glycylglycine, pH 8.0, 0.21 μmol of thiamine pyrophosphate, 1 μmol of $MgCl_2$, 2.4 μmol of NAD^+, 0.12 μmol of CoA, 2.6 μmol cystein · HCl, 20 μg pyruvate dehydrogenase complex and 1.0 μmol of pyruvate in a total volume of 1 ml. Reactions were initiated with pyruvate after 1 min incubation of enzyme in assay mixture. The final EDTA concentration in the reaction mixture was 50 μM.

Assay mixture	Relative activity
Complete	100
— Pyruvate	0
+ 2-oxogluturate, 2.0 mM	0
+ 2-oxobutyrate, 2.0 mM	18
— NAD^+	0
+ 2.5 mM $NADP^+$	0
— CoASH	0
— Thiamine pyrophosphate	0
— $MgCl_2$	0
+ 1.0 mM $MgCl_2$	100
+ 0.5 mM $MgCl_2$	100
+ 0.1 mM $MgCl_2$	80
+ 0.05 mM $MgCl_2$	0
+ 1.0 mM $MnCl_2$	4
+ 1.0 mM $CaCl_2$	0
+ 0.5 mM $CaCl_2$	0
+ 0.1 mM $CaCl_2$	0

of the substrates, pyruvate, CoASH or NAD^+, at fixed concentrations of a second substrate. The third substrate was present at a constant concentration close to the saturation level. Fig. 3A, B, C shows the resulting 3 families of parallel lines. The apparent Michaelis constants obtained by fitting the data to Eqn. 3 were estimated to be 207, 125 and 7 μM for pyruvate, NAD^+ and CoASH respectively.

Regulation

Product inhibition analysis. Analysis of the interactions between various substrate-product combinations were also performed and the data processed as described in Methods. NADH (apparent K_i = 34 μM) and acetyl-CoA (apparent K_i = 13 μM) exhibit competitive inhibition patterns versus NAD^+ and CoASH respectively as shown in Fig. 4A and B. The data best fit Eqn. 4 for linear competitive inhibition. The double reciprocal plots of enzyme activity, as a function of pyruvate concentration in the presence of NADH or acetyl-CoA showed uncompetitive inhibition (Fig. 5A and B) and the data best fit Eqn. 5. However, the data from NADH versus CoASH best fit Eqn. 6 for noncompetitive inhibition (Fig. 6A). Similarly, acetyl-CoA exhibited a non-competitive inhibition pattern with NAD^+ (Fig. 6B).

Metabolite effectors. Several other metabolites that could potentially regulate or effect the pyruvate dehydrogenase complex activity were examined by either preincubation with the enzyme or under normal assay conditions where the reaction was initiated with enzyme. Hydroxypyruvate and glyoxylate,

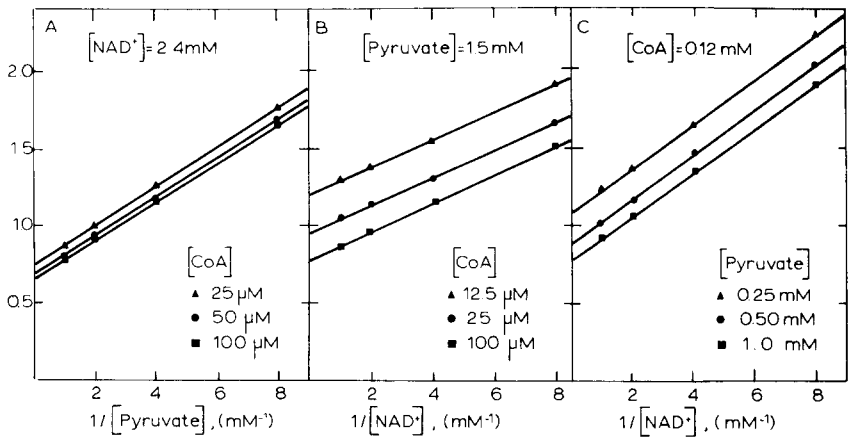

Fig. 3. Initial velocity patterns of the pyruvate dehydrogenase complex from cauliflower floral buds. The specific activity of the pyruvate dehydrogenase complex was 5.4. A. Double reciprocal plot of [CoASH] versus varying concentrations of pyruvate. The reaction was initiated by 25 μg of pyruvate dehydrogenase complex. The NAD^+ concentration in the assay was 2.4 μM with (■) 100 μM CoASH; (●) 50 μM CoASH; (▲) 25 μM CoASH. B. Double reciprocal plot of CoASH versus varying concentrations of NAD^+. The assay was performed as described above except 12.5 μg of pyruvate dehydrogenase complex was used to initiate the reaction. The pyruvate concentration was constant at 1.5 mM with (■) 100 μM CoASH; (●) 25 μM CoASH; (▲) 12.5 μM CoASH. C. Double reciprocal plot of pyruvate versus varying concentrations of NAD^+. The assays were performed as described above with 12.5 μg of pyruvate dehydrogenase complex. CoASH concentration was constant at 0.12 mM with (■) 1 mM pyruvate (●) 0.5 mM pyruvate; (▲) 0.25 mM pyruvate.

which are both substrate analogs as well as common plant metabolites, inhibited the complex non-competitively against pyruvate (Fig. 7A and B). These data were processed as described in Methods, and were found to fit best the equation for linear non-competitive inhibition.

Phosphorylation. The purified complex could be inactivated by ATP. Fig. 8 shows the time dependence of this process, and the incorporation of ^{32}P from

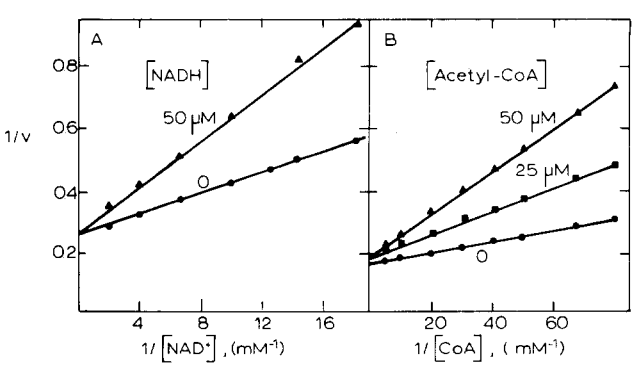

Fig. 4. Product inhibition of the cauliflower pyruvate dehydrogenase complex. A. NADH vs. NAD. The assay was initiated by 15 μg enzyme (specific activity of 3) with 0.12 mM CoASH, 1.5 mM pyruvate present in the standard pyruvate dehydrogenase complex assay. The NADH concentrations were 0, (●); and 50 μM (▲). The lines drawn are based on the computer generated slope and intercept from Eqn. 4. B. Acetyl-CoA vs. CoASH. The assay was initiated with 10 μg pyruvate dehydrogenase complex, (specific activity of 3) in the presence of 1.5 mM pyruvate and 2.4 mM NAD^+ in the standard assay. The acetyl-CoA was present at zero acetyl-CoA (●); 25 μM acetyl-CoA (■); 50 μM acetyl-CoA (▲).

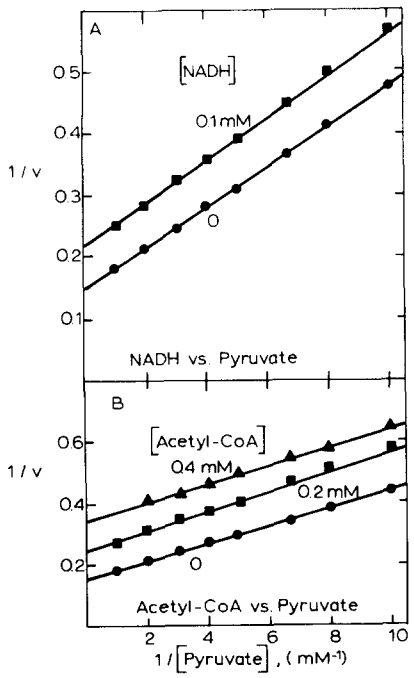

Fig. 5. Substrate-product interaction studies. A. Double reciprocal plots of NADH vs. pyruvate. The reactions were initiated by addition of 10 μg of pyruvate dehydrogenase complex (specific activity = 3). The standard pyruvate dehydrogenase complex assay was used with 2.4 mM NAD^+ and 0.12 mM CoASH in the presence of no NADH (●) or 0.1 mM NADH (■). B. Double reciprocal plot of acetyl-CoA vs. pyruvate. The reactions were initiated with 10 μg pyruvate dehydrogenase complex (specific activity = 3) with 2.4 mM NAD^+ and 0.12 mM CoASH in the presence of no acetyl-CoA (●), 0.2 mM acetyl-CoA (■), or 0.4 mM acetyl-CoA (▲).

[γ^{32}P]ATP into trichloroacetic acid-precipitable protein. The inactivation was not reversed by extended dialysis, passage through G-25 Sephadex, addition of 10—20 mM $MgCl_2$, 10—100 μM $CaCl_2$ or potato acid phosphatase [27].

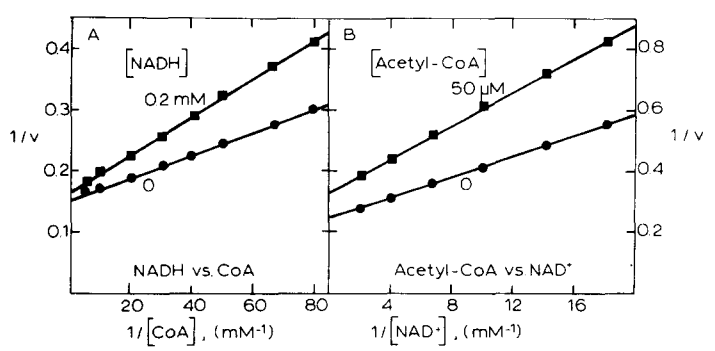

Fig. 6. Substrate-product interaction studies: A. Double reciprocal plot NADH vs. CoASH. The reaction was initiated by addition of 10 μg enzyme using the standard pyruvate dehydrogenase complex assay with 1.5 mM pyruvate and 2.4 mM NAD^+ with either no NADH (●) or 0.2 mM NADH (■). B. Double reciprocal plot of acetyl-CoA vs. NAD^+. The reaction was initiated by addition of 10 μg pyruvate dehydrogenase complex (specific activity = 3). The standard pyruvate dehydrogenase complex assay was used with 1.5 mM pyruvate and 0.12 mM CoASH in the presence of no acetyl-CoA (●) or 50 μM acetyl-CoA (■).

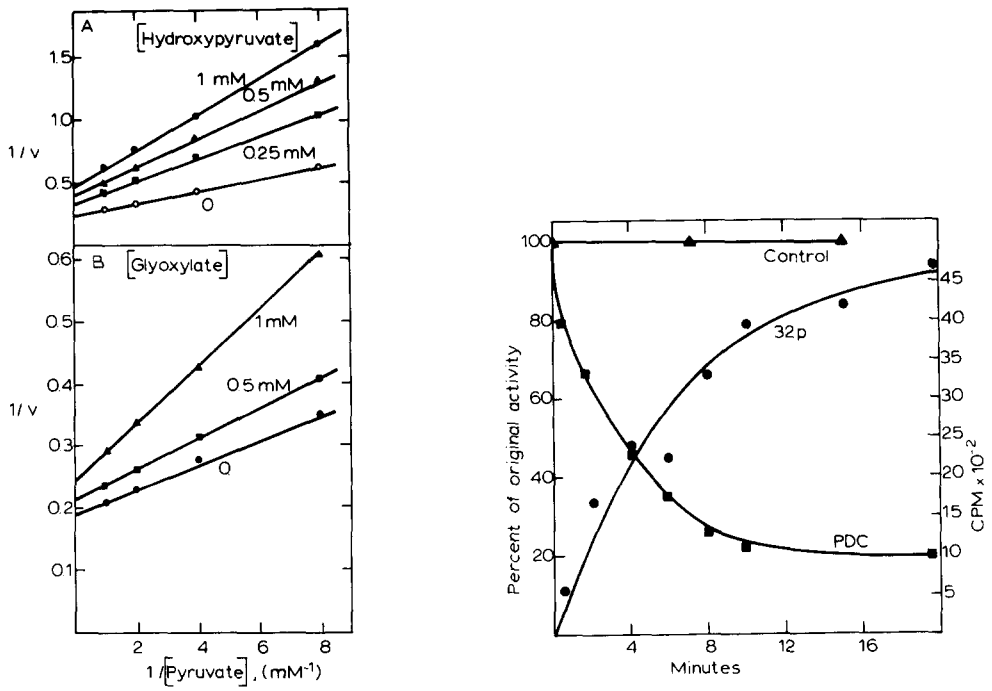

Fig. 7. A. The effect of hydroxypyruvate on the activity of the pyruvate dehydrogenase complex from cauliflower as a function of the pyruvate concentration. The assays were initiated by 3.6 μg of enzyme (specific activity = 3.3). The NAD^+ concentration was 2.4 mM and the CoASH 0.12 mM with (○) no hydroxypyruvate; (■) 0.25 mM hydroxypyruvate; (▲) 0.5 mM hydroxypyruvate; (●) 1.0 mM hydroxypyruvate. B. The effect of glyoxylate on the pyruvate dehydrogenase complex activity as a function of the pyruvate concentration. The NAD^+ concentration was 2.4 mM and the CoASH was 0.12 mM with (●) no glyoxylate; (■) 0.5 mM, glyoxylate; (▲) 1.0 mM glyoxylate.

Fig. 8. The time-dependent ATP inactivation of pyruvate dehydrogenase complex and ^{32}P incorporation. The reaction mixture contained 68 μg pyruvate dehydrogenase complex purified through the polyethylene glycol fractionation step, 20 mM TES, pH 7.5 and either 1 mM ATP or 1 mM $\gamma^{32}P$ ATP (specific activity 28 000 cpm/nmol) in 0.5 ml final volume. At indicated times pyruvate dehydrogenase complex activity was determined with the standard assay using a 20-μl aliquot of the above incubation mixture. ^{32}P incorporation was determined by placing 50 μl aliquots of the above reaction mixture on filter papers at indicated times as described in Methods. Corrections were made for non-specific ^{32}P binding by subtracting the 1520 cpm determined in a parallel time-course experiment using boiled enzyme.

However, application of the inactivation mixture to a G-25 Sephadex column during the incubation period prior to complete inactivation curtailed the inactivation. Partially purified pyruvate dehydrogenase complex phosphatase from bovine heart [17] slowly reactivated the inactivated, phosphorylated cauliflower pyruvate dehydrogenase complex (data not shown).

Discussion

The pyruvate dehydrogenase complex in crude extracts of mitochondria was unstable. Enzyme activity was lost if the preparation was left as a crude extract for several hours. This loss of pyruvate dehydrogenase complex activity was accelerated if the freeze-thawed mitochondria were extracted by treatment with

detergents or by pressure bomb, both of which helped to increase the enzyme yield. The enzyme was also found to be labile at protein concentrations below 2 mg per ml. Therefore, the complex was purified and concentrated as quickly as possible. The purification procedure for the cauliflower takes advantage of the large molecular weight of the complex. Approximately 100% of the enzyme can be pelleted by a 2.5-h, 140 000 × *g* centrifugation indicating the cauliflower complex probably has a molecular weight in the millions, similar to the pyruvate dehydrogenase complex in other tissues [9]. The ultracentrifugation step provided a rapid method to concentrate and initially purify the enzyme.

The purified complex was stable at a protein concentration greater than 5 mg per ml. The addition of pig heart dihydrolipoyl dehydrogenase (flavoprotein) would occasionally increase pyruvate dehydrogenase complex activity. Poulsen and Wedding [28] found it necessary to include pig heart dihydrolipoyl dehydrogenase in all experiments on cauliflower 2-oxoglutarate dehydrogenase. The fact that the pyruvate dehydrogenase complex did not respond to added flavoprotein does not prove that the complex was not dissociating, especially on the glycerol gradients where such a large amount of activity was unexpectedly lost. The cauliflower pyruvate dehydrogenase complex was purified to specific activities of 5—6.5 μmol NADH/min per mg protein. This activity is comparable to pyruvate dehydrogenase complex from other tissues [6—8,29—31], with specific activities of 1—20 μmol per min per mg protein. The purified cauliflower pyruvate dehydrogenase complex was free of detectable NADH oxidase ATPase, fumarase and malate dehydrogenase activities.

The substrate and cofactor requirements (Table II) indicate that the usual three enzyme · pyruvate dehydrogenase complex was present even though we have not resolved the cauliflower complex into its components. No enzyme activity was observed with 2-oxoglutarate after Step II in the purification procedure, however the low rate of oxidation activity with 2-oxobutyrate remains with the complex throughout the purification. A similar level of activity with 2-oxobutyrate was seen with purified *Neurospora crassa* complex [7]. The cauliflower enzyme was specific for NAD^+. NAD^+ reduction did not occur in the absence of CoASH. Although some thiamine pyrophosphate and Mg^{2+} tended to copurify with the complex, passage of the enzyme through G-25 Sephadex or the presence of EDTA established that these factors were also required in the overall pyruvate dehydrogenase complex reaction in cauliflower (Table II). Ca^{2+} or Mn^{2+} would not substitute for the Mg^{2+} requirement as they do with the broccoli enzyme [22]. Reports differ on the divalent cation requirement for pyruvate dehydrogenase complex in various tissues [7,8,31,32]. Since the thiamine pyrophosphate was found to be required for activity the purified complex was maintained in the presence of at least 10 μM thiamine pyrophosphate. However, the binding of this cofactor to the complex appears to have rather involved kinetics (data not shown) that have not been fully established yet. Wedding and Colleagues [8,29,33] observed a similar phenomenon with cauliflower 2-oxoglutarate dehydrogenase complex.

The substrate K_m values (apparent) for the cauliflower enzyme are similar to those reported in other tissues [9—11,15,20,34]. The K_m values reported are only apparent since the initial velocity experiments were performed at only one concentration of the third substrate in each case. NAD^+ was present at 2.4 mM

or approximately saturating (20 times the K_m) when it was the third substrate, at a constant concentration. CoASH as the third substrate was also present at near saturation (0.12 mM or 20 times the K_m) and pyruvate as a third substrate was present at 7.5 times the K_m or less than 90% of saturation. The initial velocity patterns of the purified complex gave a series of parallel lines for all three combinations of substrates. These results are in agreement with the pattern predicted from the rate equation for a multisite ping-pong mechanism described by Cleland [35] and are identical to those of Tsai et al. [11] and Hamada et al. [34] for the mammalian complex from bovine kidney and porcine heart.

NADH and acetyl-CoA exhibited competitive inhibition (Fig. 4A and B) with their respective substrates as predicted by the multisite ping-pong mechanism [35] and as reported for the complex in other tissues [11,15,20,34]. This multisite ping-pong mechanism predicts uncompetitive inhibition by NADH and acetyl-CoA against pyruvate and this was observed with the cauliflower complex, as seen in Fig. 5A and B. Such inhibition patterns would support the conclusion that there is little interaction between the NAD^+ (NADH) and CoASH (acetyl-CoA) binding sites and the binding site for pyruvate. Furthermore, the multisite ping-pong mechanism predicts that acetyl-CoA should also be uncompetitive versus NAD^+ and likewise NADH uncompetitive versus CoASH. However, both yielded non-competitive inhibition patterns. Tsai et al. [11] and Hamada et al. [34] observed the same results with pyruvate dehydrogenase complex from mammalian tissue. Tsai et al. [11] suggested that ligand-induced conformational changes could create some steric interference by the lipoyltransacetylase · ligand complex on the dihydrolipoyl dehydrogenase and vice versa.

Crompton and Laties [20], Bremer [15], and Atkinson et al. [36] concluded that small variations in the mole fractions, [S]/[S] + [I]), can effectively regulate enzyme activities by product inhibition (S is substrate and I is product inhibitor). Their discussion predicted little or no regulation of enzyme activity with $K_i \simeq K_m$. However, if the K_i is significantly less than or greater than the K_m, small variations in the mole fractions effectively alter enzyme activity. The cauliflower pyruvate dehydrogenase complex appears to be more sensitive to control through NADH inhibition than acetyl-CoA feedback as found by Crompton and Laties [20]. The apparent K_i of acetyl-CoA versus CoASH is about 13 μM versus an apparent K_i vs. NAD^+ of 125 μM. Fig. 9 shows the plots of enzyme activity as a function of mole fractions. The concave nature of the $NAD^+/(NAD^+ + NADH)$ plot supports the previous conclusion [13,20] that the pyruvate dehydrogenase is more sensitive to the $NADH/NAD^+$ ratio which has been reported to be much more likely to change than the acetyl-CoA/CoASH ratio [15]. However, we have not measured these concentrations or fluctuations in cauliflower buds.

Glyoxylate and hydroxypyruvate are common plant metabolites in photosynthetic tissues where they are involved in photorespiration. Glyoxylate and hydroxypyruvate are formed in leaf peroxisomes and glycoxylate is metabolized to serine in the mitochondria [38,39]. Since both are analogs of pyruvate one would predict competitive inhibition, however an unexpected noncompetitive inhibition was observed. Glyoxylate was previously reported to be a com-

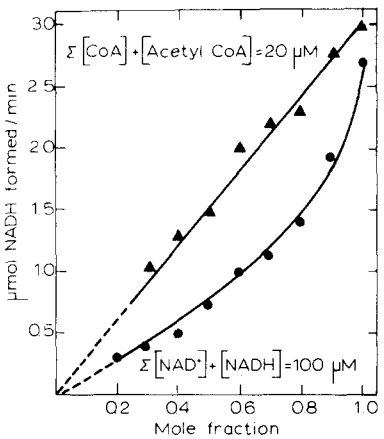

Fig. 9. Regulation of pyruvate dehydrogenase complex by substrate-product ratios. The pyruvate dehydrogenase complex activity is shown as a function of CoASH/(CoASH + Acetyl-CoA) (▲) and (NAD^+/(NAD^+ + NADH) (●). The total concentration of CoASH and acetyl-CoA was 20 μM and NADH and NAD^+ was 100 μM. The substrates were present at standard assay concentration.

petitive inhibitor versus pyruvate [19,20], however both reports used crude enzyme preparations. Whether or not glyoxylate or hydroxypyruvate have in vivo or physiological importance as regulators of the complex will have to be established. At present the high concentrations needed for significant inhibition of the complex do not suggest very sensitive control by either metabolite.

The ATP-dependent phosphorylation and inactivation of the cauliflower complex is similar to that catalyzed by the pyruvate dehydrogenase kinase described by Reed et al. [9]. The effect of temperature, and results of treating the inactivated pyruvate dehydrogenase complex by dialysis, G-25 Sephadex and the bovine heart pyruvate dehydrogenase phosphatase are all supportive of a kinase for the cauliflower complex. Potato acid phosphatase which dephosphorylates casein, phosvitin and phosphorylase [27] does not reactivate the inactivated and phosphorylated cauliflower pyruvate dehydrogenase complex. We have observed phosphorylation and inactivation of pyruvate dehydrogenase complex from broccoli [23], which could not be reactivated by in situ phosphatase action. The lack of reactivation could be due to lability of the phosphatase. The early attempts to observe 'kinase' activity with the cauliflower complex were also negative because of its sensitivity to the presence of sulphydryl reagents such as dithiothreitol. Considering the precedence of this mechanism in mammalian [9] and fungal tissues [40], we feel that it is logical to conclude that the phosphorylation-dephosphorylation mechanism for regulating the pyruvate dehydrogenase complex is also operating in plant tissue.

In conclusion, the cauliflower pyruvate dehydrogenase complex is quite similar in most properties to the complex from other tissues. The initial examination of its regulation continues to support the hypothesis that it occupies an important role in controlling carbon flow into the Krebs cycle, fatty acid metabolism and perhaps is a major control point for oxidative respiration in plants. Its role in the interaction between photosynthetic, photorespiration and mitochondrial metabolic events is currently under investigation.

Acknowledgements

We thank Ms. Rise' Femmer and Barbara Rapp for the excellent technical assistance and Drs. W.L. Zahler and L.J. Reed for helpful discussion. We thank Dr. W.W. Cleland for his generosity in supplying the computer programs necessary to process the kinetic data. This work was supported by the Missouri Agricultural Experiment Station (Journal Series 7728) and National Science Foundation Grant BMS 75-08107.

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