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Some Kinetic and Regulatory Properties of the Pea Mitochondrial Pyruvate Dehydrogenase Complex[1]

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ABSTRACT

The pyruvate dehydrogenase complex was isolated, partially purified, and characterized from green pea (*Pisum sativum L.*, cv Little Marvel) leaf mitochondria. The pH optimum for the overall reaction was 7.6. The divalent cation requirement was best satisfied by Mg^{2+}. Reaction velocity was maximal at 40°C. Pyruvate was a better substrate than 2-oxobutyrate; other 2-oxo-acids were not substrates. Michaelis constants for substrates were; pyruvate, 57 micromolar; NAD, 122 micromolar; Coenzyme-A, 5 micromolar; Mg^{2+}, 0.36 millimolar; Mg-thiamine pyrophosphate, 80 nanomolar. The products, NADH and acetyl-Coenzyme-A, were linear competitive inhibitors with respect to NAD and Coenzyme A. Inhibition constants were 18 and 10 micromolar, respectively. Glyoxylate inhibited complex activity only in the absence of thiol reagents. Glyoxylate inhibition was competitive with respect to pyruvate with an inhibition constant of 51 micromolar. Among mitochondrial metabolites examined as potential effectors, only ADP with an inhibition constant of 0.57 millimolar could be of physiological significance.

The pyruvate dehydrogenase complex (PDC)[3] is a very large multienzyme complex that catalyzes the oxidative decarboxylation of pyruvate, esterification of the resulting two-carbon fragment to CoA, and reduction of NAD. Several cofactors including, divalent cations, TPP, lipoic acid, and flavin adenine dinucleotide, are required for overall complex activity. Pyruvate dehydrogenase (EC 1.2.4.1), dihydrolipoyl acetyl-transferase (EC 1.6.4.3), and dihydrolipoyl dehydrogenase (EC 1.6.4.3) are components of all PDCs. Mitochondrial PDCs contain two additional enzymic components, PDH-kinase (EC 2.7.1.99), and phosphopyruvate dehydrogenase phosphatase (EC 3.1.3.43).

The PDC is one prominent entry point for carbon into mitochondrial metabolism, and it occupies a key regulatory position. In all instances thus far examined the PDC reaction was displaced substantially from equilibrium. Demonstrated regulatory mechanisms for mitochondrial PDC include product inhibition (19, 22), allosteric regulation (1, 12), and reversible phosphorylation (23, 33).

The importance of PDC to an overall understanding of mammalian (6, 8, 13, 19, 23), and microbial (3, 31, 32), metabolism has been adequately perceived, and the complex has been extensively studied. Unfortunately, there has been much less study of plant PDCs. We recently reviewed the available knowledge of plant PDCs (14). Most reports have concentrated upon the subcellular localization of the complex in plant tissues (9, 21, 24, 25), the kinetic mechanism (26, 29), and regulation of the mitochondrial complex by reversible phosphorylation (16–18, 20, 27).

Some characteristics of pea leaf mitochondrial PDC have been reported. It has been shown that like all mitochondrial PDCs thus far examined, the pea leaf complex is regulated in part by covalent modification (18), and the PDC from etiolated pea tissues exhibits product inhibition (29). While the pea mitochondrial PDC is the most thoroughly studied plant PDC, there are some inconsistencies in the reported results. We have reexamined several properties of mitochondrial PDC from green pea leaves, and further extended the characterization.

MATERIALS AND METHODS

Plant Material. Peas (*Pisum sativum* L., cv Little Marvel) were from the Hummert Seed Co., St. Louis. Germination and seedling growth were described by Camp and Randall (4).

Chemicals. Buffers were from Research Organics, Inc.; P L-Biochemicals supplied the NAD, CoA, acetyl-CoA, and succinyl-CoA. Other biochemicals and $LaCl_3$ were from Sigma Chemical Co. Other inorganics and ethylene glycol were obtained from J. T. Baker.

Preparation of Mitochondria and Complex. Pea shoots were homogenized by six 1 s bursts with a Braun homogenizer adapted to use single edged razor blades, in 2.5 volumes of organelle isolation medium: 50 mM imidazole-HCl (pH 6.9), 500 mM sucrose. The homogenate was filtered through four layers of premoistened cheesecloth plus one layer of Miracloth followed by centrifugation at 5,000*g* for 5 min using a Sorvall GSA rotor in a RC-5 centrifuge. The resultant supernatant was centrifuged at 17,300*g* in a SS-34 rotor for 30 min. The 17,300*g* pellet was resuspended in isolation medium by gentle swirling with a fine paint brush, followed by repelleting to yield a crude mitchondrial fraction. In some instances the mitochondria were further purified by rate-zonal sedimentation on discontinuous sucrose gradients as described by Dennis and Green (7). The mitochondrial band was slowly diluted with ice-cold isolation medium and the mitochondria collected by pelleting as described above. Mitochondrial pellets could be used immediately or stored at −20°C for several months without loss of activity.

For isolation of PDC the mitochondrial pellet was suspended in five volumes of 100 mM Tes (pH 7.5), 2 mM DTT, and 10 μM leupeptin, then homogenized by two 15 s bursts with a Brinkman Polytron at a setting of six. Membranous material was removed by centrifugation at 16,500*g* for 20 min, and the tan colored supernatant was dialyzed overnight against 500 volumes of 25 mM Tes (pH 7.5), 2 mM DTT, 1 μM leupeptin, and 20% (v/v)

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[3] Abbreviations: PDC, pyruvate dehydrogenase complex; PDH, pyruvate dehydrogenase (EC 1.2.4.1); TPP, thiamine pyrophosphate.

ethylene glycol. The PDC was further purified by PEG precipitation and ultracentrifugation as previously described (26). The partially purified preparations of PDC ranged in specific activity from 0.60 to 0.81 μmol min^{-1} mg^{-1} protein.

Enzyme Assays. Activity of PDC was measured essentially as described by Randall *et al.* (18), except that the Mg concentration was increased to 2.5 mM. While dihydrolipoyl dehydrogenase may dissociate from the PDC during purification and become limiting to overall complex activity, this did not appear to be a problem in the present studies. Addition of purified dihydrolipoyl dehydrogenase (pig heart, *E. coli*, or pea) to the PDC preparations had no effect upon overall activity. For studies on the pH dependence of the reaction the standard Mops-glycylglycine buffer was replaced by 80 mM Mes, Tes, or bicine adjusted to the indicated pH values at room temperature. When examining metabolite effects, PDC plus metabolites were preincubated at room temperature for 2 min in assay buffer minus pyruvate and CoA.

Data Analysis. Kinetic data were analyzed as described in Garland and Dennis (10), using iterative curve-fitting by nonlinear regression.

RESULTS AND DISCUSSION

Most studies of the mitochondrial PDC from plant sources have dealt with the kinetic mechanism or with phosphorylation/dephosphorylation and both of these aspects have been the subject of previous reports on pea mitochondrial PDC (18, 29). There has, however, been little basic information available on the enzymic characteristics of the complex from green leaf tissue. As a preliminary to a thorough study of the control of pea mitochondrial PDC, including detailed characterization of the kinase and phosphatase, we now report some enzymic and regulatory properties of complex itself.

Examination of pea mitochondrial PDC activity as a function of pH over the range of 6.0 to 9.0 revealed a peak at pH 7.6 with 50% maximal activity at 6.8 and 8.5 (Fig. 1). While Reid *et al.* (25) reported an optimal pH of 6.5 to 7.5 for mitochondrial PDC from etiolated pea shoots, the shape of their curve differed substantially from ours with a precipitous drop at the more alkaline pH values. We found that pea mitochondrial PDC was quite sensitive to the buffer used at alkaline pH values, that glycylglycine buffers for example resulted in relatively low activity. The use of Tricine (JA Miernyk, unpublished data) or bicine (Fig. 1) buffers resulted in a more typical gradual decline in activity with change in pH. In contrast to Williams and Randall (35), the pH-activity relationship seemed unaffected by buffer concentration between 50 to 250 mM.

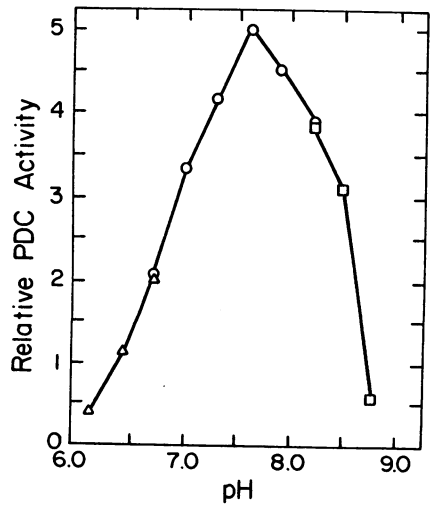

FIG. 1. Activity of the pea mitochondrial pyruvate complex as a function of pH. Buffers used were Mes (△), Tes (○), and bicine (□).

There is an absolute requirement for a divalent cation, typically Mg^{2+}, for PDC activity (4, 26, 30). While pea mitochondrial PDC was most active with Mg^{2+} (Table I) there was substantial activity with Mn^{2+} and Ca^{2+} as well. Relatively high activity in the presence of Ni^{2+} was unexpected but was reproducibly observed. Complex activity was not activated by monovalent cations, other divalent, or trivalent cations (Table I). Examination of PDC activity as a function of Mg^{2+} concentration allowed determination of a K_m Mg^{2+} of 0.36 ± 0.05 mM (Fig. 2).

It has been suggested that the role of divalent cations in PDC activity is for binding of the obligate cofactor, TPP (14). As the requirement of plant PDCs for TPP has not been examined in detail, we measured the activity of pea mitochondrial PDC as a function of TPP concentration. Initial results using equal molar $MgCl_2$ and TPP resulted in sigmoidal kinetics (JA Miernyk, unpublished data). Subsequent use of the Mg-PPi stability constant (15), as corrected for pH, allowed us to more realistically examine activity as a function of Mg-TPP concentration, and resulted in hyperbolic kinetics (Fig. 2). The K_m Mg-TPP of 79.6 ± 8.0 nM is in the same range as the value reported for mammalian PDCs (34). Our initial results suggest that free Mg^{2+} is an inhibitor of TPP binding and resulted in nonhyperbolic kinetics. That the K_m Mg^{2+} is more than three orders of magnitude greater than the K_m Mg-TPP suggests a role for Mg^{2+} in addition to that in TPP binding.

Table I. *Divalent Cation Specificity of the Pea Mitochondrial Pyruvate Dehydrogenase Complex*

The following cations did not activate the reaction: K^+, Na^+ NH_4^+, Cu^{2+}, Fe^{2+}, Sn^{2+}, Sr^{2+}, Zn^{2+}, La^{3+}, Al^{3+}. All were examined at a final concentration of 2.5 mM. All cations were tested as chloride salts except $CuSO_4$ and $AlK(SO_4)_2$.

Relative Activity					
None	Mg^{2+}	Mn^{2+}	Ca^{2+}	Ni^{2+}	Co^{2+}
		%			
0	100	89	81	62	4

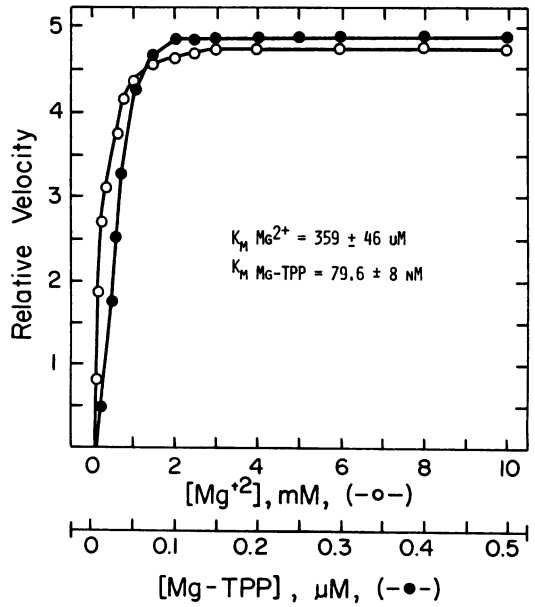

FIG. 2. Partially purified and dialyzed pea mitochondrial pyruvate dehydrogenase complex activity as a function of Mg concentration (○), or Mg-TPP concentration (●).

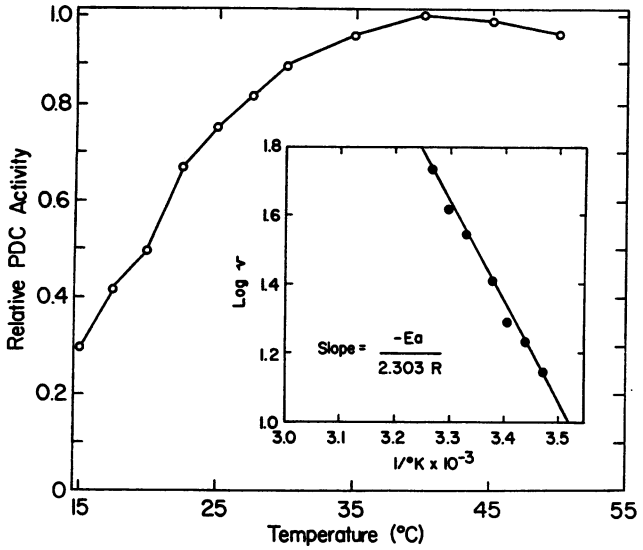

FIG. 3. Activity of the pea mitochondrial PDC as a function of assay temperature. Assays were conducted using a Gilford Response spectrophotometer with a temperature controlled cuvette holder. Reaction mixtures were allowed to equilibrate at the indicated temperatures before initiation of the assays with CoA. Inset is a replot of the data from 25 to 50°C according to the Arrhenius equation.

Table II. *Kinetic Constants of the Pea Mitochondrial Pyruvate Dehydrogenase Complex*

Substrate	K_m	K_i
	μM	
Pyruvate	56.7 ± 5.2	
NAD	121.9 ± 8.4	
CoA	4.6 ± 0.3	
NADH		18.1 ± 0.9
Acetyl-CoA		9.8 ± 0.4

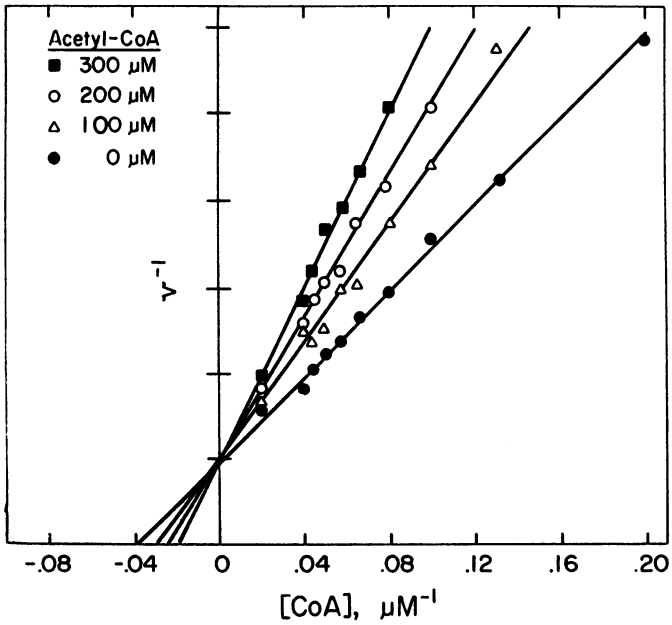

FIG. 4. Kinetic analysis of pea mitochondrial PDC. Activity was measured as a function of CoA-concentration in the presence of zero (●), 100 μM (Δ), 200 μM (○), and 300 μM acetyl-CoA (■). Pyruvate and NAD concentrations were 1.5 and 2.3 mM, respectively.

Table III. *2-Oxo-Acid Specificity of the Pea Mitochondrial Pyruvate Dehydrogenase Complex*

Relative activity was determined at a fixed final 2-oxo-acid concentration of 2.5 mM.

2-oxo-acid	Relative Activity	K_m
		μM
Pyruvate	1.0	57
2-Oxo-butyrate	0.2	1350
2-Oxo-glutarate	0	
2-Oxo-isovalarate	0	
2-Oxo-isocaproate	0	
Glyoxylate	0	

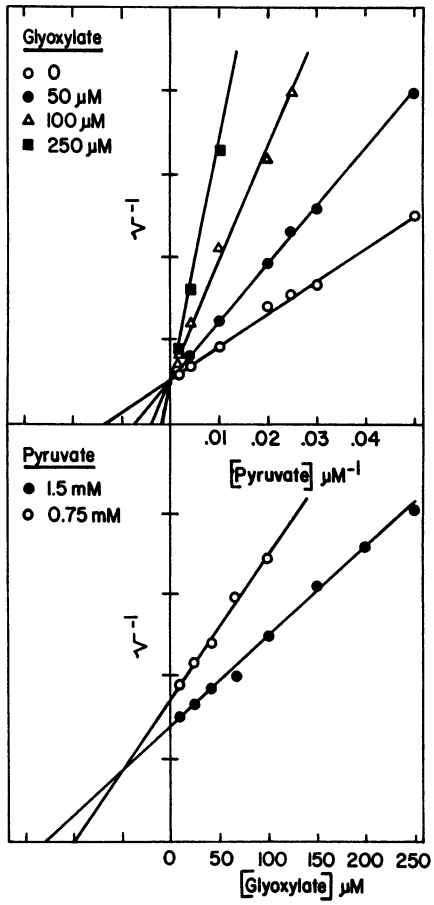

FIG. 5. Inhibition of pea mitochondrial PDC activity by glyoxylate, at a final DTT concentration of 0.02 mM. Upper panel is a double reciprocal plot of reaction velocity as a function of pyruvate concentration, at fixed variable concentrations of glyoxylate. Lower panel, a Dixon plot of glyoxylate inhibition giving a K_i of 51.3 ± 0.9 mM.

Some reports of PDC have proposed a regulatory role for TPP. Reid *et al.* (25) found that *Ricinus* plastid PDC was only partially dependent upon added TPP, and that concentrations of TPP in excess of 1 mM were inhibitory. It may be that an excess of TPP tends to bind required Mg, thus inhibiting complex activity. This would be exacerbated in the cases of plastid PDCs which have a higher divalent cation requirement than the mitochondrial counterparts (4, 30). Rubin *et al.* (28) reported that broccoli mitochondrial PDC was wholly dependent upon added TPP and that Mg-TPP easily dissociated from complex. They proposed that the dissociation for TPP and resultant loss of activity might be of regulatory significance. The binding of TPP to pea mitochondrial PDC seemed quite tight. It was necessary to dialyze complex overnight at pH 6.0 in the presence of 2 mM EDTA in order to

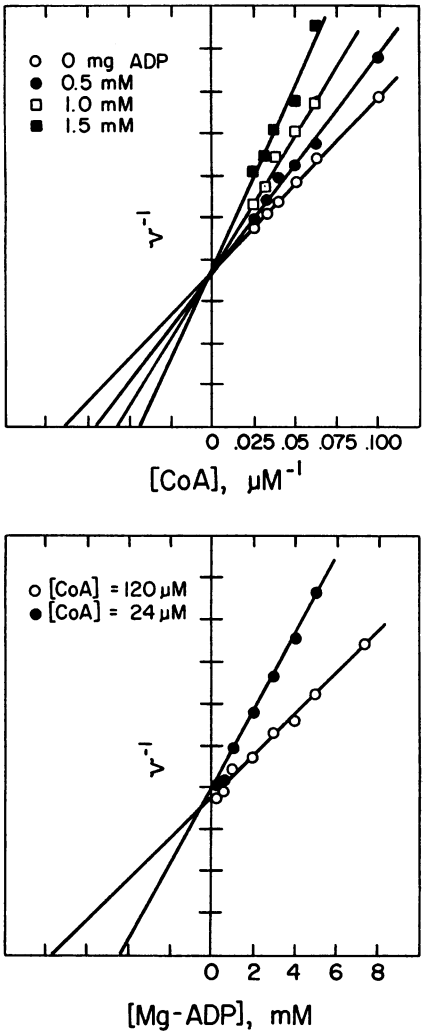

FIG. 6. Inhibition of pea mitochondrial PDC activity by ADP. Upper panel is double reciprocal plot of reaction velocity as function of CoA concentration at fixed variable concentration of Mg-ADP. Lower panel is a Dixon plot of Mg-ADP giving a K_i of 0.57 ± 0.04 mM.

observe a complete dependence of activity upon added TPP. It does not appear that association-dissociation of TPP is a regulatory mechanism for pea leaf mitochondrial PDC.

Activity of the pea mitochondrial PDC increased as a function of assay temperature from 15 to 35°C then remained relatively constant from 35 to 50°C (Fig. 3). Assay temperatures above 50°C resulted in a rapid, irreversible loss of activity (data not shown). Replotting of the activity/temperature relationship according to the Arrhenius equation gave an E_a of −12.96 kcal/mol (inset, Fig. 3).

Under optimal conditions of pH, divalent cations, and cofactors, the kinetic constants for mitochondrial PDC from green pea leaves were determined (Table II). The values obtained were in the range of those previously reported for PDC from etiolated pea shoots (29) and mitochondrial PDCs from other plant sources (5, 17, 28). The K_i value for NADH is substantially lower than the K_m NAD suggesting that product inhibition is of regulatory significance.

Kinetic analyses of PDC from various sources have resulted in a consensus multisite hexa-uni, ping-pong mechanism and nearly all experimentally determined kinetic patterns are in agreement. A competitive inhibition pattern was observed when varying CoA concentrations in the presence of fixed varying acetyl-CoA, from bovine heart or kidney mitochondria and *Ricinus* endosperm plastids (29), as well as for *Ricinus* mitochondrial PDC (BJ Rapp, DD Randall, unpublished data) and pea chloroplast PDC (PJ Camp, DD Randall, unpublished data). The only exception is with pea mitochondrial PDC, where Thompson *et al.* (29) reported noncompetitive inhibition. Reexamination of this interaction using an identical computer analysis program resulted in a competitive inhibition pattern (Fig. 4). The F value determined from the residual sum of squares was nearly the same as the F value (0.99) from statistical tables, requiring acceptance of the null hypothesis that acetyl-CoA best fits the pattern of a competitive inhibitor. We have found (JA Miernyk, DD Randall, unpublished data) that varying the concentrations of the other assay components can alter slightly the slopes of lines, and this may have contributed to the conclusion of Thompson *et al.* (29).

Beatty and Hamilton (2) recently reported that, in the absence of thiol reagents, glyoxylate was a strong inhibitor of rat epididymal fat tissue PDC competitive with pyruvate. They suggested that the previous low levels of inhibition of mammalian, fungal, and plant mitochondrial PDCs by glyoxylate were due to formation of thiohemiacetal-adducts with thiol reagents present in the assay mixtures. As Beatty and Hamilton (2) noted, the high concentration of cysteine (2.5 mM) used in the assay of broccoli mitochondrial PDC by Rubin *et al.* (28) may have contributed to the relatively high K_i value (3.3 mM) that was observed. In light of these observations we examined the pea mitochondrial PDC for inhibition by glyoxylate. With a low final thiol concentration (0.02 mM DTT) in the assays glyoxylate was inhibitory. While glyoxylate was not a substrate for pea mitochondrial PDC (Table III), inhibition was competitive with respect to pyruvate (Fig. 5). The K_i value for glyoxylate in the presence of 0.02 mM DTT was 51.3 ± 0.9 μM while in the presence of 2.5 mM cysteine the K_i value was 3.17 mM (data not shown), very close to the value previously reported by Rubin and Randall (26). Our results are in complete agreement with Beatty and Hamilton (2) and support the suggestion that glyoxylate-adduct formation must be taken into account when considering inhibition.

A number of mitochondrial matrix metabolites were examined as potential effectors of PDC activity (Table IV). In addition to NADH and acetyl-CoA, ADP appeared to be of possible regulatory importance and was studied in more detail. The inhibition by ADP was linear competitive with respect to CoA (Fig. 6) and the K_i value of 0.57 ± 0.04 mM is within the range of *in vivo* ADP concentrations reported for the plant mitochondrial matrix (11). Inhibition by AMP was similar to that of ADP, but the K_i value of 5.9 mM is far in excess of the mitochondrial matrix concentration. Intermediates of the tricarboxylic acid cycle, other nucleotides, amino acids, and polyamines had no effect on the *in vitro* activity of pea mitochondrial PDC (Table IV). Even in the presence of saturating levels of Mg^{2+} several divalent cations strongly inhibited PDC activity, but this would seem to be of mechanistic rather than regulatory importance.

The pea mitochondrial PDC is the most throughly studied of plant PDCs, although its properties are still poorly understood relative to bacterial or mammalian mitochondrial PDCs. The studies reported herein further extend our knowledge of this important regulatory system with regard to divalent cation and 2-oxo-acid specificity, Mg^{2+} and Mg-TPP kinetics, *in vitro* temperature optimum, and energy of activation. Additionally, several previously reported inconsistencies (*e.g.* pH optimum, acetyl-CoA inhibition, glyoxylate inhibition) have been clarified. The potential of metabolite regulation of complex activity by ADP was observed. This inhibition could contribute to fine control of PDC activity *in vivo*. Pea mitochondrial PDC activity is regulated in part by product inhibition (Table II; [30]) and reversible phosphorylation (14, 17). The different layers of regulatory mechanisms should allow a very fine control over PDC activity *in vivo*, and it is likely that steady state activity reflects

Table IV. *Potential Effectors of Pea Mitochondrial Pyruvate Dehydrogenase Complex Activity*

Krebs cycle intermediates tested were: citrate, *cis*-aconitate, isocitrate, 2-oxoglutarate, succinate, fumarate, malate, and succinyl-CoA (0.2 mM). Amino acids tested were: Glu, aspartate, and Gly. Polyamines tested were: spermine, spermidine, and putrescine. Phospho-amino acids tested were: P-serine, P-threonine, and P-tyrosine. Monovalent cations tested were: NaCl, KCl, and NH_4Cl. All metabolites were examined as Mg-chelates, adjusted to pH 7.5.

Compound		Inhibition	No Effect	Stimulation
NADH	10 μM	+++	−	−
Acetyl-CoA	10 μM	++	−	−
ADP	2 mM	++	−	−
AMP	2 mM	+	−	−
PPi	2 mM	+	−	−
Pi	2 mM	+	−	−
Krebs cycle intermed.	2 mM	−	+	−
Amino acids	2 mM	−	+	−
2-Oxo-isocaproate	2 mM	−	+	−
Carbamyl-P	2 mM	−	+	−
Polyamines	2 mM	−	+	−
Phospho-amino acids	2 mM	−	+	−
$ZnCl_2$	2 mM	+++	−	−
$CuSO_4$	2 mM	+++	−	−
$SnCl_2$	2 mM	+++	−	−
$CaCl_2$	2 mM	−	+	−
$NiCl_2$	2 mM	−	+	−
Monovalent cations	2 mM	−	+	−

the contribution of each. Detailed reports on the phosphorylation/dephosphorylation, and the properties of the kinase and phosphatase are in preparation. Experiments are in progress attempting to integrate these data into a model which will explain the *in organello* regulation of plant mitochondrial PDCs.

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