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The Characterization and Regulatory Properties of Pyruvate Kinase from Cotton Seeds[1]

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Pyruvate kinase (ATP:pyruvate phosphotransferase, EC 2.7.1.40) was partially purified from cotton seeds. The enzyme shows normal kinetics toward phosphoenolpyruvate, ADP, and magnesium or manganese. Of nearly 50 metabolites tested, the enzyme is inhibited only by ATP, UTP, citrate, and malate, and activated by AMP, GMP, and fumarate. The inhibition by citrate and ATP is not due to metal chelation; both compounds appear to directly affect the enzyme. The kinetics of the activations by AMP and by fumarate suggest the existence of separate activator sites for the two compounds.

It is suggested that cotton seed pyruvate kinase is a regulatory enzyme, although it differs markedly from the regulatory pyruvate kinases which have been described in animals and in microorganisms. This is the first instance in which regulatory properties have been reported for a pyruvate kinase from a higher plant.

The regulatory properties of pyruvate kinase (ATP: pyruvate phosphotransferase, EC 2.7.1.40) from liver (1–6), erythrocytes (7–12), yeast (1, 13–16), and a variety of bacteria (17–24) have been extensively described. Characteristically, these properties include a sigmoid dependence of reaction rate on PEP[3] concentration, and activation by FDP in eucaryotes, and by AMP in procaryotes.

Most of the work on the higher plant enzyme (25–29) has been concerned with the effect of potassium in relation to plant nutrition. The plant enzyme, like that from other eucaryotes but unlike that from most bacteria, shows an absolute dependence on monovalent cations. The available data from these studies (26, 28) indicate that the higher plant enzyme has normal kinetics toward PEP, but these studies were not designed to detect regulatory properties. Ohmann (30) has reported that the enzyme from the alga *Euglena gracilis* shows regulatory properties, while that from the higher plant *Lemna minor* does not. However, it has been pointed out (31, 32) that regulatory properties are usually associated with the enzyme from gluconeogenic tissues, and the choice of *Lemna* may have been inappropriate.

For these reasons, it was decided to undertake a study of pyruvate kinase from a fat-storing seed, since such seeds are known to be gluconeogenic during the early stages of germination (33).

MATERIALS AND METHODS

Cotton (*Gossypium sp.* L.) seeds were obtained from Carolina Biological Supply Co. Biochemicals, enzymes, and buffers were obtained from Sigma

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[3] The following abbreviations are used: PEP, phosphoenolpyruvate; FDP, fructose-1,6-diphosphate; TES, *N*-tris-[hydroxymethyl]methyl-2-ethane sulfonic acid; XMP, xanthosine-5′-monophosphate.

Chemical Co.; other chemicals were of the highest grade commercially available.

Assay of pyruvate kinase. Pyruvate kinase was usually assayed by coupling pyruvate production to NADH oxidation with lactic dehydrogenase (EC 1.1.1.27), and following the decrease in absorbance at 340 nm, using a Gilford-modified Beckman DU spectrophotometer. Unless otherwise indicated, the assay contained 0.1 mM Tris ADP, 0.05 mM cyclohexylamine PEP, 10 mM magnesium chloride, 45 mM potassium chloride, 0.1 mM NADH, 8 units[4] of rabbit muscle lactic dehydrogenase (Sigma type II), and 50 mM TES, adjusted to pH 7.50 with Tris base, in a final volume of 3 ml. Reactions were carried out at 25°C and initiated by the addition of pyruvate kinase. Rates are expressed as the change in absorbance per minute, and are corrected for the slow change in absorbance in the absence of added ADP, which is due to a phosphatase (26–28).

Extraction and partial purification of pyruvate kinase. Dry cotton seeds were fragmented with a hammer, the kernels removed manually, and crushed to a fine powder with a pestle and mortar. All subsequent operations were performed at 0–4°C. All buffers used in purification were made up in 50% (v/v) glycerol. The powdered kernels were homogenized with a Servall Omnimixer in 100 mM Tris–chloride buffer (pH 7.5) using 10 ml per g of powder. The homogenate was filtered through several layers of muslin, and centrifuged at 34,000*g* for 15 min. The plug of lipid was punctured, and 10–20 ml of the cloudy supernatant fluid applied to a 3.5 × 20-cm column of DEAE–cellulose which had previously been washed and equilibrated with 10 mM Tris–chloride buffer, pH 7.5. The enzyme was washed on to the column with an equal volume of this buffer, then eluted with 400 ml of a 0.0–0.5 M potassium chloride gradient. The most active fractions, eluting between 0.10 and 0.15 M potassium chloride, were pooled and used without further treatment. The pooled fractions usually contained 0.3 units of pyruvate kinase per milliliter, with a specific activity of 0.35 units per mg protein, 25–30 times greater than that of the crude extract. This preparation is fairly stable, losing half of its activity in 3 months. In the absence of 50% glycerol, the enzyme loses all activity over a period of 8 hr at 0°C.

RESULTS

It was found (R. G. Duggleby and D. T. Dennis, unpublished observations) that many plant tissue extracts give a substantial rate of reaction in assays where ADP is omitted. This activity is presumed to be a phosphatase which converts PEP to pyruvate, and interferes in the coupled assay for pyruvate kinase. Cotton seeds contain relatively little of this activity at the pH of the pyruvate kinase assay, and were used as a source of the enzyme. The purification procedure employed does not separate the two activities.

Preliminary studies. The partially purified enzyme shows hyperbolic kinetics toward both substrates (Fig. 1). The kinetic pattern obtained is consistent with the sequential mechanism which has been proposed for the enzyme from other sources (22, 34, 35). It is activated by several divalent metals (Table I), and shows hyperbolic kinetics toward both magnesium and manganese. A variety of metabolites were tested as possible activators or inhibitors of

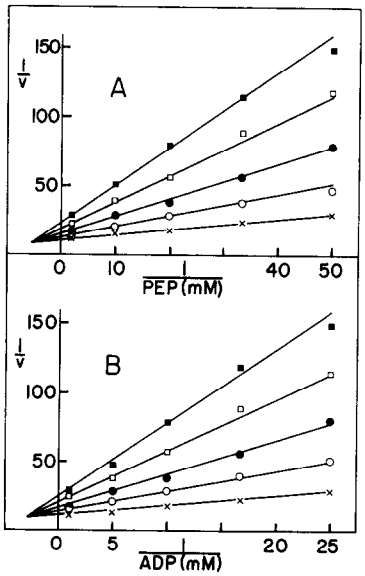

FIG. 1. Kinetics of cotton seed pyruvate kinase with respect to PEP and ADP. Assays were performed as described in Materials and Methods, using 0.2 ml of partially purified enzyme per assay. In A, PEP was varied as indicated, using fixed ADP concentrations of 1.0 mM (×), 0.2 mM (○), 0.1 mM (●), 0.067 mM (□), and 0.04 mM (■). In B, ADP was varied as indicated, using fixed PEP concentrations of 0.5 mM (×), 0.1 mM (○), 0.05 mM (●), 0.033 mM (□), and 0.02 mM (■). The data are plotted in double-reciprocal form.

[4] The unit of enzyme activity is defined as the amount which catalyzes the formation of 1 μmole of product per minute.

TABLE I

ACTIVATION OF COTTON SEED PYRUVATE KINASE BY DIVALENT METAL IONS[a]

Metal	$10^4 \times$ rate		
	Metal concentration (mM)		
	0.1	1.0	10.0
Ca^{2+}	2	0	—
Cd^{2+}	146	P	—
Co^{2+}	—	303	174
Cu^{2+}	0	9	P
Fe^{2+}	0	P	—
Mg^{2+}	—	597	968
Mn^{2+}	—	680	643
Ni^{2+}	8	15	24
Zn^{2+}	0	15	P

[a] The enzyme was assayed as described in Materials and Methods, except that the divalent metal ion was as indicated, and the substrate concentrations were 0.25 mM PEP and 0.50 mM ADP. Each assay contained 0.1 ml of partially purified enzyme. The concentrations not tested are indicated by a dash —, and in those cases where a precipitate formed in the assay, this is indicated by P. The enzyme is inactive in the absence of added divalent metals.

the enzyme.[5] Activation was observed with AMP (20%), GMP (12%), and fumarate (15%), and inhibition with ATP (46%), UTP (41%), citrate (46%), and malate (25%) FDP, the characteristic activator of a number of pyruvate kinases (1) has no effect on the cotton seed enzyme. No effect was noted on addition of asparagine, cysteine, glutamate, glutamine, glycine, phenylalanine, serine, shikimic acid, ribose-5-phosphate, 6-phosphogluconate, glucose-1-phosphate, glucose-6-phosphate, fructose-6-phosphate, dihydroxyacetone phosphate, glyceraldehyde-3-phosphate, 3-phosphoglyceric acid, acetyl CoA (0.4 mM), isocitrate, 2-ketoglutarate, succinate, propane-1,2,3-tricarboxylic acid, glyoxylate,[6] adenine, deoxy-AMP, guanine, guanosine, deoxyguanosine, (2′ + 3′)-GMP, uracil, UMP, deoxy-CMP, 5-methylcytosine, xanthine, xanthosine, XMP, kinetin riboside, NAD, NADP, NADH (0.02–0.40 mM), NADPH (0.5 mM). The effects of AMP, fumarate, ATP, and citrate were chosen for further study.

Citrate inhibition. The inhibition by citrate is shown in Fig. 2. Addition of either AMP or fumarate reduces the amount of inhibition by citrate (Fig. 2), but neither of these activators is capable of completely preventing citrate inhibition (Fig. 3).

Fumarate activation. The activation by fumarate is shown in Fig. 4. In the presence of low concentrations of citrate, the percentage of activation by fumarate is increased (Figs. 4 and 5A), but higher concentrations of citrate do not show this enhancement to the same extent (Fig. 5A). Low concentrations of AMP reduce the percentage of activation by fumarate (Fig. 4) and saturation with AMP completely prevents fumarate activation (Fig. 5B).

Since fumarate is not generally considered to be a regulatory signal, the activation was studied in detail to determine if it is artifactual. It is thought not to be artifactual for the following reasons: (a) no fumarate activation is shown by the commercial rabbit

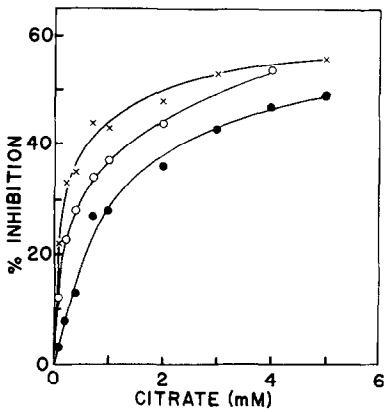

FIG. 2. Citrate inhibition of cotton seed pyruvate kinase. Assays were performed as described in Materials and Methods, using 0.2 ml of partially purified enzyme per assay. Citrate was varied as indicated, with the following additions: none (×), 3 mM fumarate (●), or 0.05 mM AMP (○). The data are expressed as the percentage of inhibition of controls without citrate.

[5] Each compound was tested at a concentration of 1 mM, unless indicated to the contrary. Assays contained 2.0 mM manganese sulfate in place of magnesium chloride.

[6] Assays performed by the method of Pon and Bondar (36), since NADH will reduce glyoxylate in the presence of lactic dehydrogenase.

muscle enzyme under the same conditions; (b) no activation is shown by succinate, maleate, isocitrate, propane-1,2,3-tricarboxylic acid, or acetate; (c) identical activation is observed if NADH and lactic dehydrogenase are omitted, and the enzyme assayed by following PEP disappearance at 230 nm (36); and, (d) fumarate does not appear to undergo any change under assay conditions, as judged by its absorbance at 250 nm. Thus, the activation appears to be a direct effect of fumarate on cotton seed pyruvate kinase.

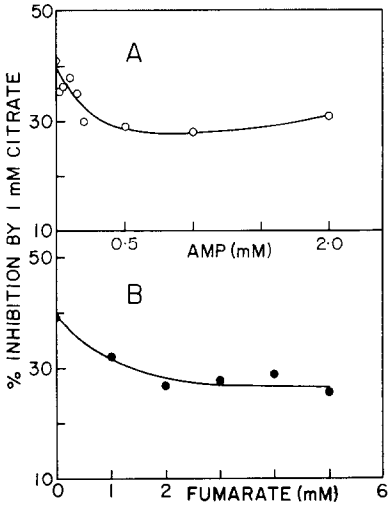

FIG. 3. Effect of AMP and fumarate concentration on the citrate inhibition of cotton seed pyruvate kinase. Assays were performed as described in Materials and Methods, using 0.2 ml of partially purified enzyme per assay. AMP (panel A) or fumarate (panel B) was varied as indicated, and assays were performed in the presence and absence of 1 mM citrate. The data are expressed as the percentage of inhibition by 1 mM citrate at each AMP or fumarate concentration.

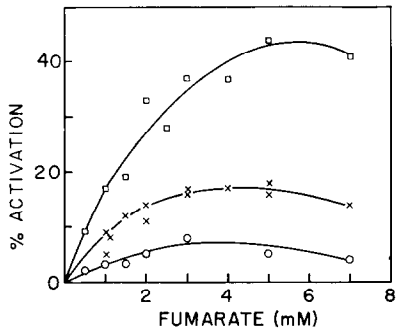

FIG. 4. Fumarate activation of cotton seed pyruvate kinase. Assays were performed as described in Materials and Methods, using 0.2 ml of partially purified enzyme per assay. Fumarate was varied as indicated, with the following additions: none (×), 1 mM citrate (□), or 0.05 mM AMP (○). The data are expressed as the percentage of activation of controls without fumarate.

AMP activation. The enzyme is activated by low concentrations of AMP. The percentage of activation is greater when the enzyme is inhibited by citrate, but is reduced when the enzyme is activated by fumarate (Fig. 6). When the enzyme is fully activated by fumarate, AMP is capable of eliciting further activation (Fig. 7A). The percentage of activation by a nearly saturating concentration of AMP is greatly increased by the addition of citrate, whereas citrate does not have this effect at low AMP concentrations (Fig. 7B).

ATP inhibition. The inhibition by ATP is shown in Fig. 8. The inhibition is not due to magnesium chelation, since the same

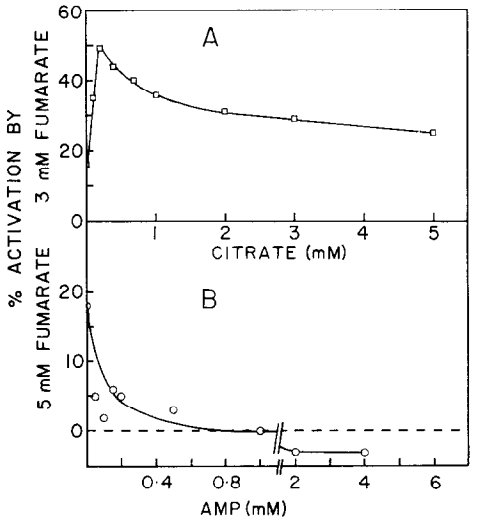

FIG. 5. Effect of citrate and AMP concentration on the fumarate activation of cotton seed pyruvate kinase. Assays were performed as described in Materials and Methods, using 0.2 ml of partially purified enzyme per assay. In A, citrate was varied as indicated, in the presence and absence of 3 mM fumarate. In B, AMP was varied as indicated, in the presence and absence of 5 mM fumarate. The data are expressed as the percentage of activation by fumarate at each citrate or AMP concentration.

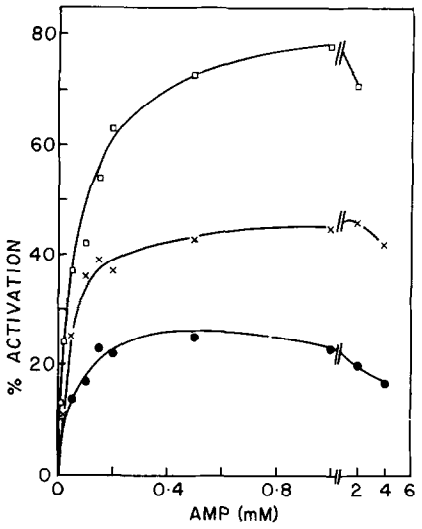

FIG. 6. AMP activation of cotton seed pyruvate kinase. Assays were performed as described in Materials and Methods, using 0.2 ml of partially purified enzyme per assay. AMP was varied as indicated, with the following additions: none (×), 1 mM citrate (□), or 5 mM fumarate (●). The data are expressed as the percentage of activation of controls without AMP.

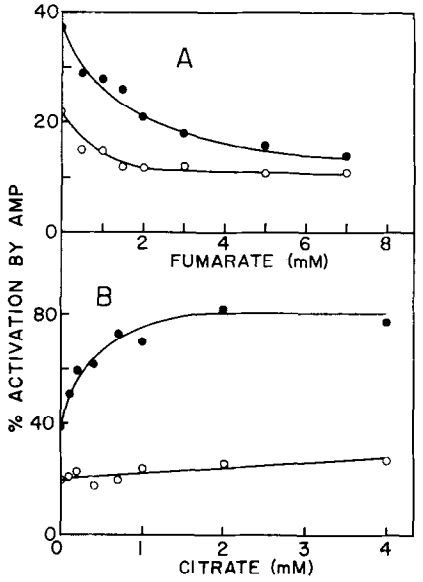

FIG. 7. Effect of fumarate and citrate concentration on the AMP activation of cotton seed pyruvate kinase. Assays were performed as described in Materials and Methods, using 0.2 ml of partially purified enzyme per assay. Fumarate (panel A) or citrate (panel B) was varied as indicated, and assays were performed in the presence and absence of 0.05 mM (○) or 1.0 mM (●) AMP. The data are expressed as the percentage of activation by these AMP concentrations at each fumarate or citrate concentration.

effect is seen when ATP is added together with an equimolar concentration of magnesium chloride. Furthermore, the shape of the curve is quite unlike that which would be expected if the inhibition were due to chelation. EDTA will inhibit the enzyme, presumably by magnesium chelation, and this compound gives an inhibition curve quite different from that seen with ATP (Fig. 8, inset).

Effect of magnesium chloride, PEP, and ADP. Citrate inhibition is virtually unaffected by a wide range of magnesium concentrations (Fig. 9). In contrast, the activation by AMP or by fumarate is completely prevented by high concentrations of magnesium chloride. In part, this may be an effect of the chloride ion, since the addition of 50 mM sodium chloride or Tris–chloride will reduce the amount of activation by 1 mM AMP to about 15%. These are not ionic-strength effects, since the addition of 50 mM sodium TES increases AMP activation. At low magnesium concentrations, AMP activation declines (Fig. 9), probably because the activating effect is being offset by magnesium chelation. In contrast, the percentage of activation by fumarate increases dramatically as the magnesium concentration is decreased (Fig. 9).

The effects of ATP, AMP, citrate, and fumarate on the kinetic parameters of cotton seed pyruvate kinase are illustrated in Table II. Fumarate appears to affect the affinity of the enzyme for both substrates, and saturation with either substrate eliminates fumarate activation. AMP activation is reduced by saturation with PEP and is virtually eliminated by saturation with ADP. ATP inhibition is prevented by saturation with PEP but not ADP, and citrate remains inhibitory when either substrate is increased to a saturating concentration. In all cases, saturation curves for PEP and ADP are hyperbolic.

DISCUSSION

Unlike most regulatory pyruvate kinases, the enzyme from cotton seeds shows normal

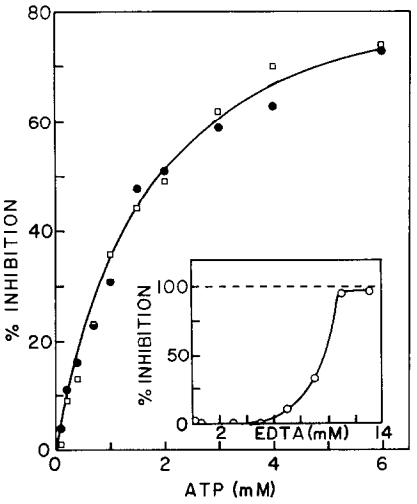

FIG. 8. ATP and EDTA inhibition of cotton seed pyruvate kinase. Assays were performed as described in Materials and Methods, using 0.2 ml of partially purified enzyme per assay. ATP was varied as indicated, in the presence (□) or absence (●) of an equimolar concentration of magnesium chloride, over and above that normally contained in the assay (10 mM). The inset shows the effect of EDTA concentration in the absence of additional magnesium chloride. The data are expressed as the percentage of inhibition of controls without ATP, EDTA, or additional magnesium chloride.

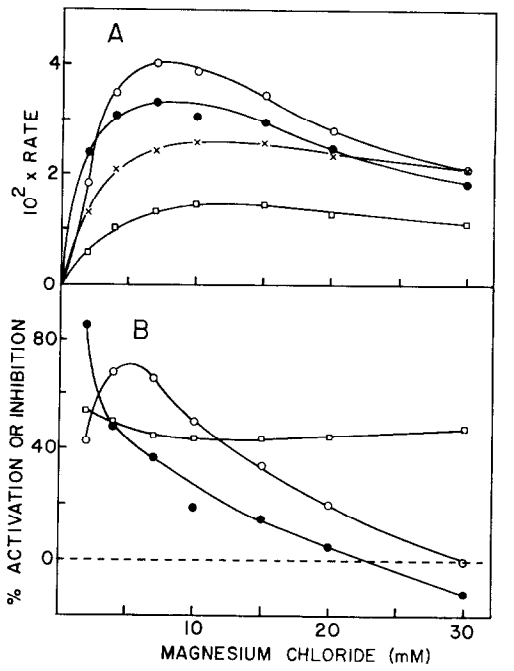

FIG. 9. Effect of magnesium concentration on cotton seed pyruvate kinase. Assays were performed as described in Materials and Methods, using 0.2 ml of partially purified enzyme per assay. In A, magnesium chloride was varied as indicated, with the following additions: none (×), 1 mM AMP (○), 3 mM citrate (□), or 4 mM fumarate (●). In B, the percentage of activation by fumarate or AMP, or the percentage of inhibition by citrate, at each magnesium concentration, is plotted against magnesium concentration.

kinetics toward both substrates (Fig. 1), and is not activated by FDP. AMP (and to a lesser extent GMP) was found to activate the cotton seed enzyme (Fig. 6), just as it does several bacterial pyruvate kinases (17, 19–23). ATP inhibition (Fig. 8) was not unexpected since this compound is a product. The effect of ATP could not be ascribed to metal chelation, as has occasionally been suggested for the enzyme from rabbit muscle (37, 38). UTP is also inhibitory, but since pyruvate kinases show a fairly low specificity for the phosphoryl acceptor (39–41), this inhibition is also to be expected.

Citrate has been reported to be effective in converting the nonregulatory form of the adipose tissue enzyme to its regulatory form (32, 42), and to inhibit pyruvate kinase from yeast (14) and from *Brevibacterium flavum* (19). Several other bacterial pyruvate kinases (21, 22, 24) are unaffected by citrate. Cotton seed pyruvate kinase is inhibited by this compound (Fig. 2), although several other carboxylic acids have no effect. Some inhibition is seen with malate, which may result from the structural similarity of citrate and malate. The citrate-inhibited enzyme shows normal kinetics toward both substrates, so this inhibition does not involve the conversion of the enzyme from a nonregulatory to a regulatory form. The amount of citrate inhibition is largely unaffected by the concentration of PEP, ADP (Table II), or magnesium (Fig. 9). The addition of either AMP or fumarate reduces the effect of citrate (Fig. 2), but neither is capable of completely preventing citrate inhibition (Fig. 3).

Fumarate activation of pyruvate kinase has not previously been reported. Cornish and Johnson (23) have observed fumarate inhibition of the enzyme from *Thiobacillus neapolitanus*, but most other pyruvate

TABLE II

EFFECTS OF ATP, AMP, CITRATE, AND FUMARATE ON THE KINETICS OF COTTON SEED PYRUVATE KINASE[a]

Addition	Relative kinetic constants			
	PEP		ADP	
	V	K_m	V	K_m
None	1.00	1.00	1.00	1.00
3 mM ATP	1.05	3.41	0.60	0.97
1 mM AMP	1.17	0.79	1.05	0.58
2 mM citrate	0.73	1.04	0.68	0.92
5 mM fumarate	1.00	0.80	1.04	0.86

[a] Saturation curves (6 points per curve) were prepared for PEP and for ADP, using the assay described in Materials and Methods, except for the concentration of the varied substrate, and the indicated additions. Each assay contained 0.2 ml of partially purified enzyme. The Michaelis constants and maximum velocities were estimated from a linear regression of the data plotted as v against v/[S]. The values of the kinetic parameters were then divided by the values obtained in the absence of any additions (V_{PEP} = 0.0755, K_{mPEP} = 0.085 mM, V_{ADP} = 0.0705, and K_{mADP} = 0.155 mM).

kinases have not been tested for fumarate effects. The fumarate activation of the cotton seed enzyme (Fig. 4) is not artifactual, and is difficult to explain without postulating a fumarate-specific site on the enzyme. This site does not appear to be the same as the AMP site, since saturation with fumarate does not prevent AMP activation, although it does reduce it somewhat (Fig. 7).

The effects of AMP, ATP, and citrate all suggest that cotton seed pyruvate kinase is a regulatory enzyme. The effect of each might be expected to combine to decrease the flux through pyruvate kinase when energy supply is high, and the tricarboxylic acid cycle is inhibited. It is under just these conditions that we might expect PEP to be diverted away from pyruvate formation.

The interpretation of the significance of the activation by fumarate is difficult, as it is hard to conceive of a role that this activation could fulfill *in vivo*. It should be remembered that malate is inhibitory, and the effect of fumarate might well be balanced by this inhibition. Furthermore, animal mitochondria appear to be relatively impermeable to fumarate (43), and the cytoplasmic concentration of fumarate would be a poor indicator of the activity of the tricarboxylic acid cycle if plant mitochondria show the same impermeability. While there are other metabolic sources of fumarate (e.g., from the catabolism of aspartate and aromatic amino acids), the relationship between these metabolic pathways and PEP is remote Nevertheless, the activation is clearly a real effect, and should not be dismissed simply because one is unable to postulate a reason for it.

We conclude that cotton seed pyruvate kinase is a regulatory enzyme, being activated by AMP, and inhibited by ATP and citrate. The enzyme is also activated by fumarate, but the significance of this observation remains to be elucidated.

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