Biochimica et Biophysica Acta, 614 (1980) 151—162

BBA 69035

KINETIC ANALYSIS OF EFFECTORS OF PHOSPHO*ENOL*PYRUVATE CARBOXYLASE FROM *BRYOPHYLLUM FEDTSCHENKOI*

ANNETTE G.G. PAYS *, ROBERT JONES **, MALCOLM B. WILKINS, CHARLES A. FEWSON [x] and ALAN D.B. MALCOLM ***

Departments of Botany and Biochemistry, University of Glasgow, Glasgow G12 8QQ (U.K.)

(Received July 16th, 1979)
(Revised manuscript received February 20th, 1980)

Key words: Phosphoenolpyruvate carboxylase; Crassulacean acid metabolism; Circadian rhythm; Plant enzyme regulation; Malate inhibition; (Bryophyllum fedtschenkoi)

Summary

The activity of phospho*enol*pyruvate carboxylase (orthophosphate:oxaloacetate carboxy-lyase (phosphorylating) EC 4.1.1.31) purified from *Bryophyllum fedtschenkoi* has been measured in the presence of various concentrations of phospho*enol*pyruvate, L-malate and glucose 6-phosphate. At high pH, the enzyme is competitively inhibited by L-malate and activated by glucose 6-phosphate. A reaction scheme describing the interaction of enzyme, substrate and effectors is proposed. Values for the appropriate equilibrium constants have been calculated for the enzyme acting at pH 7.8, which is one of its two pH optima. The kinetics are more complicated at low pH, partly because of non-linear reaction rates and partly because inhibition by L-malate is not competitive. Activation by glucose 6-phosphate is similar at high and low pH values. The behaviour of a wide range of other possible effectors is described briefly.

Introduction

Phospho*enol*pyruvate carboxylase (orthophosphate:oxaloacetate carboxy-lyase (phosphorylating) EC 4.1.1.31) is found in a wide range of plants and

* Present address: Departement de Biologie Moleculaire, Université Libre de Bruxelles, Rue des Chevaux 67, 1640 Rhose-St. Genêse, Belgium.
** Present address: Department of Physics, University of Lancaster, Lancaster LA1 4YB, U.K.
*** Present address: Department of Biochemistry, St. Mary's Hospital Medical School, Paddington, London W2 1PG, U.K.
[x] To whom correspondence should be addressed.
Abbreviation: Mes, 2-(*N*-morpholino)ethanesulphonic acid.

microorganisms. It has a variety of metabolic roles and the enzymes from different organisms have correspondingly varied regulatory properties [1,2]. Phospho*enol*pyruvate carboxylase plays a particularly important part in the circadian and diurnal rhythms of plants with crassulacean acid metabolism [3,4]. For instance, leaves of *Bryophyllum fedtschenkoi* display an endogenous circadian rhythm of CO_2 output when maintained in continuous darkness [5] and this appears to be caused by periodic activity of phospho*enol*pyruvate carboxylase resulting in refixation of respiratory CO_2 into malate. Alterations of enzyme capacity (i.e. total extractable activity assayed under non-limiting conditions), differential formation of isoenzymes and interconversion of enzyme forms may all be important in the regulation of crassulacean acid metabolism [4,6—8] although some of the results with crude extracts may have been complicated by changes in concentration of inhibitors and activators. There is little doubt that control of enzyme activity by metabolites is also important in controlling phospho*enol*pyruvate carboxylase activity in plants with crassulacean acid metabolism [4,6]; for instance malate is a powerful inhibitor of the enzyme [9] while glucose 6-phosphate is an activator [10,11]. So far, however, all experiments of this type have been done with crude extracts or partially purified enzymes. We have recently purified to homogeneity the phospho*enol*pyruvate carboxylase from *B. fedtschenkoi* [12]. The present paper describes a detailed kinetic analysis of inhibition by L-malate and activation by glucose 6-phosphate at pH 7.8. Equations are derived for the situation where both effectors are present simultaneously, and the values for the appropriate equilibrium constants are calculated. Results are also reported at pH 5.8 because we have previously shown that the enzyme has two pH optima [12]. This paper also describes the effects of a wide range of metabolites and related compounds on the activity of phospho*enol*pyruvate carboxylase and their interactions with L-malate and glucose 6-phosphate.

Methods

Materials

Phospho*enol*pyruvate carboxylase was purified from *Bryophyllum fedtschenkoi* Hamet et Perrier and stored at 4 °C as a suspension (approx. 2 mg protein · ml^{-1}) in 0.2 M KH_2PO_4 (pH 6.5)/$(NH_4)_2SO_4$ (300 g · l^{-1})/2 mM EDTA/2 mM dithiothreitol exactly as described previously [12]. Mes, dithiothreitol, L(—)- and D(+)malic acids, 6-phosphogluconic acid (trisodium salt), D-fructose 1,6-bisphosphate (tetrasodium salt), 2-deoxy-D-glucose 6-phosphate (sodium salt), D-galactose 6-phosphate (disodium salt), D-fructose 6-phosphate (disodium salt), glucose 6-sulphate (potassium salt) glucosamine 6-phosphate (sodium salt) and D-ribose 5-phosphate (disodium salt) were from Sigma (London) Chemical Co. (Fancy Road, Poole, Dorset, BH17 7NH, U.K.); *S*-acetyl coenzyme A (trilithium salt) and 3-phosphoglyceric acid (sodium salt) were from Boehringer Corporation (London) Ltd. (Bell Lane, Lewes, East Sussex, BN7 1LG, U.K.); L-aspartic acid was from T.J. Sas and Son Ltd. (Victoria House, Vernon Place, London, WC1, U.K.); other compounds were from B.D.H. Chemicals Ltd. (Poole, Dorset, BH12 4NN, U.K.) or were as described previously [12].

Enzyme assays

Phospho*enol*pyruvate carboxylase activity was routinely measured at 27°C in a coupled spectrophotometric assay [12] by following the oxidation of NADH (ϵ = 6200 l · mol^{-1} · cm^{-1}) at 340 nm in 1-cm pathlength cuvettes in a Unicam SP. 800 spectrophotometer connected to a Servoscribe chart recorder. Each run contained four cuvettes. The amount of enzyme was chosen so that the rate of ΔA_{340} min^{-1} was usually in the range —0.01 to —0.2 and the reaction was followed for 7 min. The pH values of individual reaction mixtures were always checked after assay; for all the results given in this paper the pH values were within 0.1 unit of the quoted value.

The stock suspension of phospho*enol*pyruvate carboxylase was diluted (usually 1 in 100) in 50 mM Tris-HCl (pH 7.8), containing 1 mM-dithiothreitol, kept at 4°C and the assayed between 30 min and 4 h after dilution. The diluted enzyme had constant activity during this period. Phospho*enol*pyruvate, glucose 6-phosphate and the compounds tested as possible effectors were dissolved in 50 mM Tris-HCl or 50 mM Mes buffer, final pH values 7.7 and 5.5, respectively, depending on the pH at which the enzyme was to be assayed. L-Malic acid was dissolved in water and neutralized with 5 M NaOH. Dilutions of all compounds were made in 50 mM Tris-HCl or Mes buffer as appropriate.

For assays at pH 7.8, a stock solution A containing 50 mM Tris-HCl buffer (pH 7.8)/20 mM $NaHCO_3$/10 mM $MgCl_2$/0.2 mM NADH/2 mM dithiothreitol/ 10 μg malate dehydrogenase per ml was prepared at the start of each day. Before each assay, 5 ml solution A and 100 μl diluted enzyme were mixed and 1 ml portions added to cuvettes already containing appropriate concentrations of effectors in 1 ml of 50 mM Tris-HCl (pH 7.8). After mixing by inversion, the solutions were incubated at 27°C for 10 min and then the reaction initiated by addition of phospho*enol*pyruvate (usually in 20 μl).

For assays at pH 5.8, stock solution B containing 100 mM Mes buffer (pH 5.5)/40 mM $MgCl_2$/2 mM dithiothreitol, and stock solution C containing 0.5 M $NaHCO_3$/5 mM NADH/250 μg malate dehydrogenase per ml were prepared at the start of each day. Immediately before each assay, 5 ml solution B, 210 μl solution C and 100 μl diluted enzyme were mixed and 1 ml samples added to cuvettes already containing appropriate concentrations of effectors in 1 ml of 50 mM Mes buffer (pH 5.5). After mixing by inversion, the solutions were incubated at 27°C for 10 min and then the reaction initiated by addition of phospho*enol*pyruvate (usually in 20 μl).

The 10 min incubation was chosen for convenience but in preliminary experiments with various concentrations of phospho*enol*pyruvate, L-malate and glucose 6-phosphate incubations between 2 and 20 min gave the same results. There is no evidence that changes in ionic strength as a result of the addition of effectors in the concentration range we used here have any effect on enzyme activity.

Unless otherwise stated, rates were calculated from slopes of progress curves between 0.5 and 3 min after addition of substrate. A unit of enzyme activity (U) is defined as the formation of 1 μmol product per min. Mean results are given ±S.D., with the number of determinations in parentheses.

Results

Inhibition by L-malate and activation by glucose 6-phosphate

The maximum velocity for phospho*enol*pyruvate carboxylase has two pH optima, at pH 5.8 and pH 7.8, although the variation between pH 5.5 and 8.5 is surprisingly small (Ref. 12, Bentley, A.J., Fewson, C.A. and Wilkins, M.B., unpublished results). We have therefore studied the activation of phospho*enol*-pyruvate carboxylase by glucose 6-phosphate and its inhibition by L-malate at pH 7.8 (Figs. 1, 2, 4, 5) and at pH 5.8 (Figs. 1—3).

Reaction progress curves at pH 7.8 were always linear for at least 7 min and rate measurements were threfore easy. At pH 5.8, however, the slope of the progress curves varied by up to 20% although this could be decreased by suitable preincubation.

Glucose 6-phosphate activated phospho*enol*pyruvate carboxylase over the entire pH range tested (5.3—9.0). The degree of activation was approximately the same at pH 5.8 and 7.8 (Figs. 1 and 2). This contrasted with inhibition by L-malate which was much more severe at low pH values (Figs. 1 and 2) [12].

Results were obtained with several batches of purified enzyme. A few of the most important experiments were repeated using an assay based on direct measurement of oxaloacetate formation [12] and they all gave results identical to those using the standard coupled assay.

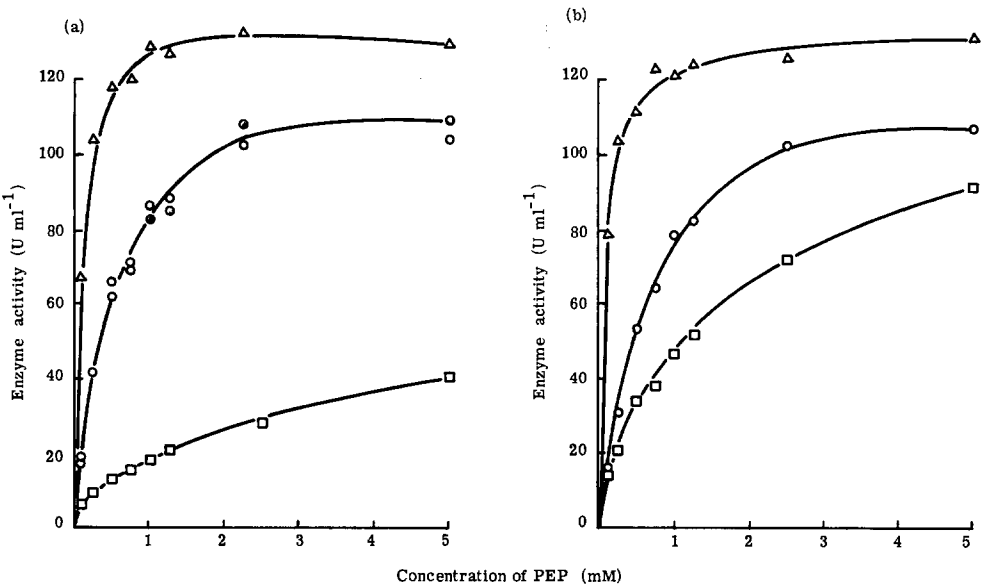


Fig. 1. Effects of glucose 6-phosphate and L-malate on phospho*enol*pyruvate carboxylase activity in the presence of various concentrations of phospho*enol*pyruvate (PEP) at (a) pH 5.8 and (b) pH 7.8. Control rates, ○———○; with 20 mM glucose 6-phosphate, △———△; with 5 mM L-malate, □———□.

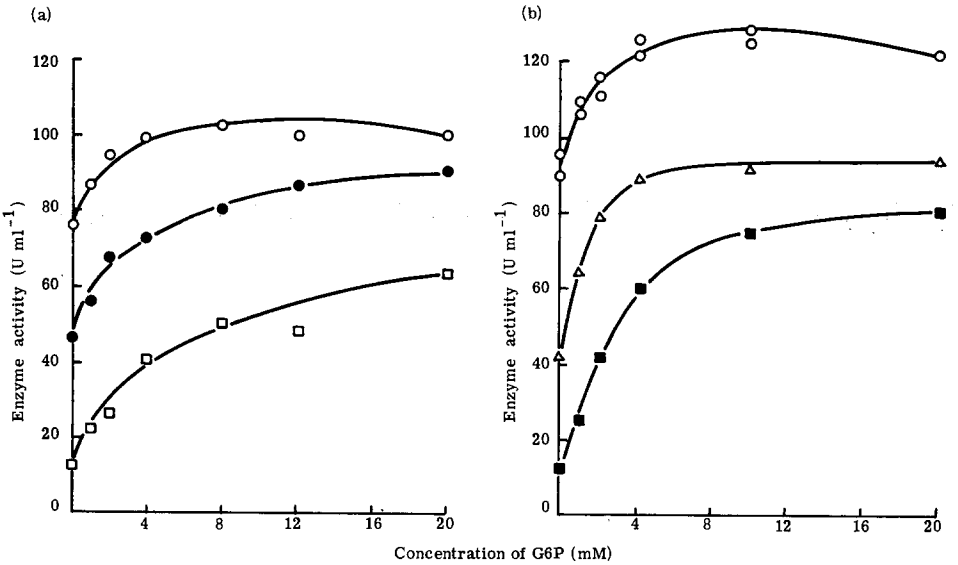


Fig. 2. Effects of malate on phospho*enol*pyruvate carboxylase activity in the presence of various concentrations of glucose 6-phosphate (G6P) at (a) pH 5.8 and (b) pH 7.8. Reaction mixtures contained 2 mM phospho*enol*pyruvate alone (○———○) or 2 mM phospho*enol*pyruvate + 1 mM (●———●), 5 mM (□———□), 10 mM (△———△) or 30 mM (■———■) L-malate.

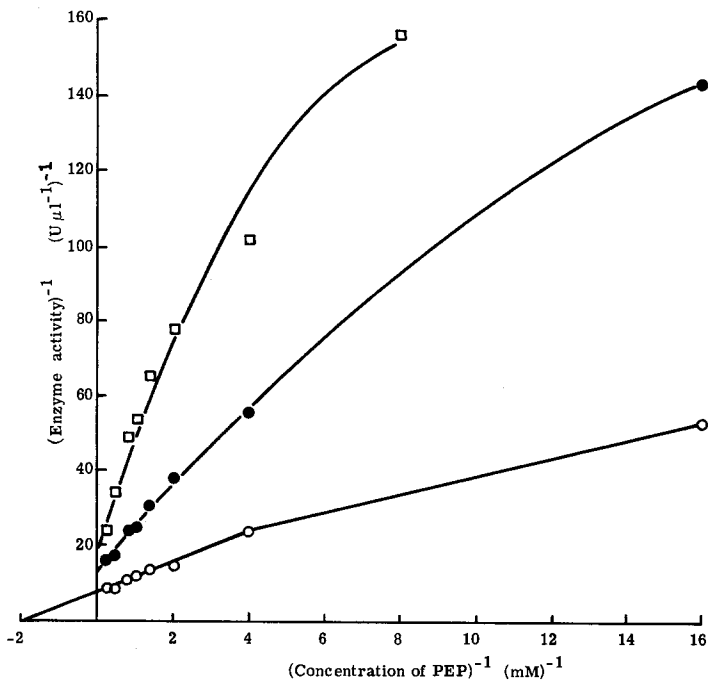


Fig. 3. Double reciprocal plot showing the effects of L-malate (○———○, none added; ●———●, 1 mM; □———□, 5 mM) on the saturation of phospho*enol*pyruvate carboxylase with phospho*enol*pyruvate (PEP) at pH 5.8.

TABLE I

EFFECTS OF VARIOUS COMPOUNDS ON THE ACTIVITY OF PHOSPHO*ENOL*PYRUVATE CARBOXYLASE AT pH 7.8

The enzyme was incubated at 27°C for 10 min with the appropriate compound (final concn. 5 mM), with or without L-malate or glucose 6-phosphate. The reaction was then initiated by addition of phospho*enol*pyruvate and the results refer to rates measured between 0.5 and 3 min. Each compound was tested in between 2 and 4 experiments, each with suitable controls. The average results are expressed as percentage of the corresponding rate in the absence of the compound under test.

Compound under test	Phospho*enol*pyruvate carboxylase activity (% of the rate in the absence of the compound under test) *			
	0.5 mM phospho*enol*pyruvate		2 mM phospho*enol*pyruvate	
	No further addition	+ 5 mM glucose 6-phosphate	No further addition	+ 5 mM L-malate
Glucose 6-phosphate	181	—	125	153
L-Malate	58	68	70	—
Glucose 1-phosphate	139	100	115	111
Glucosamine 6-phosphate	108	93	95	78
Galactose 6-phosphate	158	103	91	118
Fructose 6-phosphate	129	88	101	98
Fructose 1,6-bisphosphate	100	92	94	78
3-Phosphoglycerate	83	67	92	95
Malonate	67	62	60	63
* Average enzyme rate in the absence of the compound under test (U/ml)	63	114	101	68

Results at pH 5.8

There are several reasons why it was more difficult to interpret the kinetics at pH 5.8:

(a) Progress curves were frequently non-linear at pH 5.8.

(b) Phospho*enol*pyruvate saturation did not show Michaelis-Menten kinetics at very low concentrations (Fig. 3).

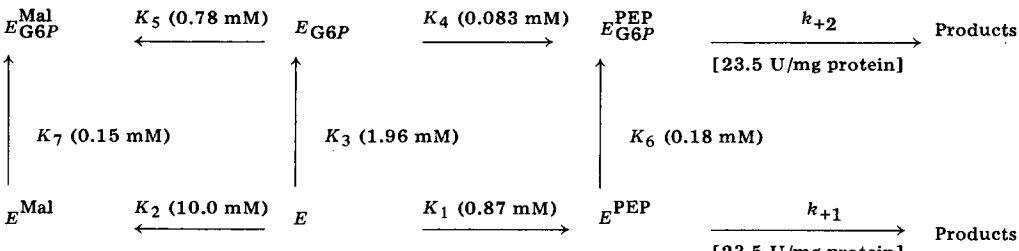
(c) Inhibition by L-malate was not competitive (compare Fig. 3 with Fig. 8 in Ref. 12).

Other effectors

A wide range of metabolites and related compounds were screened as possible effectors of phospho*enol*pyruvate carboxylase at pH 7.8 (Table I). Interactions with L-malate and glucose 6-phosphate were sought under conditions where these two compounds had their most striking effects. The following were without significant effect on enzyme activity when tested at a final concentration of 5 mM: glucose, glucose 6-sulphate, 2-deoxyglucose 6-phosphate, ribose 5-phosphate, L-aspartate, glycine, acetyl-CoA (0.5 mM), citrate, D-malate, KCl, KH_2PO_4, NH_4Cl, NAD^+, $NADP^+$, ATP. Fructose 1,6-bisphosphate had little effect at pH 7.8 (Table I), but at pH 5.8 was a slightly more powerful activator than was glucose 6-phosphate. Malonate was unusual in that it was an inhibitor at pH 7.8 (Table I) but an activator at pH 5.8.

Discussion

The variation of rate with phospho*enol*pyruvate concentration at pH 7.8 (Fig. 1b) may be treated as hyperbolic in the presence of either glucose 6-phosphate or malate (Figs. 1b, 4 and Fig. 8 of Ref. 12). L-Malate behaves as a competitive inhibitor (Fig. 8 of Ref. 12). Hyperbolic kinetics are maintained when the concentration of the activator glucose 6-phosphate is varied (Fig. 2b, 4). The simplest reaction scheme consistent with these findings is shown in Scheme I. The kinetic constants will be treated as equilibrium constants of



SCHEME I

Reaction scheme for phospho*enol*pyruvate carboxylase, its substrate and effectors. E = enzyme; Mal = malate; G6*P* = glucose 6-phosphate, K_1, K_2, K_3, K_4, K_5, K_6 and K_7 are equilibrium dissociation constants; k_{+1} and k_{+2} are rate constants. Values obtained for the constants at pH 7.8 are shown in parentheses.

Michaelis and Menten. Since such an assumption is being made, the dissociation constants K_6 and K_7 are given by:

$$K_6 = \frac{K_3 K_4}{K_1} \tag{1}$$

and

$$K_7 = \frac{K_3 K_5}{K_2} \tag{2}$$

It is recognised that this treatment may be an oversimplification, but it is pointless to carry out a more refined kinetic analysis until the exact nature of the second substrate (HCO_3^- or CO_2) has been determined.

The velocity of the reaction, v, is given by:

$$v = k_{+1} \cdot E^{PEP} + k_{+2} \cdot E^{PEP}_{G6P} \tag{3}$$

where E^X_Y represents the concentration of enzyme with ligands X and Y bound. By conventional algebra it can be shown that this is equal to:

$$v = \frac{k_{+1} \cdot E_0 \left(1 + \frac{k_{+2}}{k_{+1}} \cdot \frac{G6P}{K_6}\right) PEP}{K_1 \left(1 + \frac{Mal}{K_2}\right) + \left(1 + \frac{G6P}{K_6}\right) PEP + \frac{K_1}{K_3}\left(1 + \frac{Mal}{K_5}\right) G6P} \tag{4}$$

where E_0 represents total enzyme concentration and *PEP*, *G6P* and *Mal* represent concentrations of phospho*enol*pyruvate, glucose 6-phosphate and malate respectively.

In the absence of effectors, Eqn. 4 reduces to the Michaelis-Menten equation:

$$v = \frac{k_{+1} \cdot E_0 \cdot PEP}{K_1 + PEP} \tag{5}$$

or, in double reciprocal form:

$$\frac{1}{v} = \frac{K_1}{k_{+1} \cdot E_0} \cdot \frac{1}{PEP} + \frac{1}{k_{+1} \cdot E_0} \tag{6}$$

Conventional least-squares fits carried out on eight separate sets of data gave $K_1 = 0.87 \pm 0.14$ mM.

Experiments similar to those described previously [12] gave $K_2 = 10.0 \pm 2.7$ mM [6].

In the absence of malate, Eqn. 4 reduces to:

$$v = \frac{k_{+1} \cdot E_0 \left(1 + \frac{k_{+2}}{k_{+1}} \cdot \frac{G6P}{K_6}\right) PEP}{K_1 \left(1 + \frac{G6P}{K_3}\right) + \left(1 + \frac{G6P}{K_6}\right) PEP} \tag{7}$$

or, in double reciprocal form:

$$\frac{1}{v} = \frac{K_1}{k_{+1} \cdot E_0} \left(\frac{1 + \frac{G6P}{K_3}}{1 + \frac{k_{+2}}{k_{+1}} \cdot \frac{G6P}{K_6}} \right) \frac{1}{PEP} + \frac{1}{k_{+1} \cdot E_0} \left(\frac{1 + \frac{G6P}{K_6}}{1 + \frac{k_{+2}}{k_{+1}} \cdot \frac{G6P}{K_6}} \right) \tag{8}$$

Fig. 4 shows a plot of $1/v$ against $1/PEP$ at three different concentrations of glucose 6-phosphate; good straight lines (correlation coefficients greater than 0.98) were obtained in all graphs of this sort. Since the intercept on the ordinate is constant, it follows that $k_{+2} = k_{+1}$. Glucose 6-phosphate is therefore an activator because $K_4 < K_1$, and from Eqn. 1 it follows that $K_6 < K_3$.

When the concentration of glucose 6-phosphate greatly exceeds K_6, Eqn. 7 becomes:

$$v = \frac{k_{+2} \cdot E_0 \cdot PEP}{K_4 + PEP} \tag{9}$$

or, in double reciprocal form:

$$\frac{1}{v} = \frac{K_4}{k_{+2} \cdot E_0} \cdot \frac{1}{PEP} + \frac{1}{k_{+2} \cdot E_0} \tag{10}$$

In Fig. 4 the condition that concentration of glucose 6-phosphate greatly exceeds K_6 is best satisfied by the data for 20 mM glucose 6-phosphate. The ratio of slope/intercept for this line gives $K_4 = 0.083$ mM.

For the conditions that the concentration of L-malate = 0, $k_{+1} = k_{+2}$ and the concentration of glucose 6-phosphate much greater than K_6, Eqn. 4 can be rewritten to give:

$$\frac{1}{v} = \frac{K_4 + PEP}{k_{+1} \cdot E_0 \cdot PEP} + \frac{K_6(K_1 + PEP)}{k_{+1} \cdot E_0 \cdot PEP} \cdot \frac{1}{G6P} \tag{11}$$

Plots of $1/v$ against $1/G6P$ at fixed concentrations of phospho*enol*pyruvate

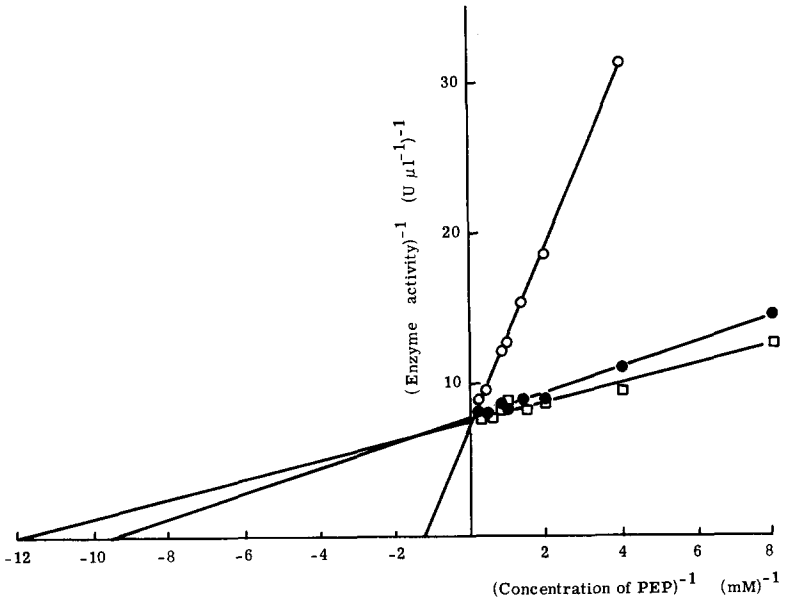


Fig. 4. Double reciprocal plot showing the effects of glucose 6-phosphate (○———○, none added; ●———●, 2 mM; □———□, 20 mM) on the saturation of phospho*enol*pyruvate carboxylase with phospho*enol*pyruvate (PEP) at pH 7.8. The lines of best fit were calculated by the method of least-squares.

allow K_6 and K_3 to be determined. Five separate sets of data at three concentrations of phospho*enol*pyruvate (0.34, 1.4 and 2.0 mM) have been analysed and gave K_6 = 0.18 mM. K_3 can then be calculated from Eqn. 1 to be 1.96 mM.

When L-malate is present, provided that the concentration of glucose 6-phosphate is much greater than K_6, Eqn. 4 may be rearranged to give, in reciprocal form:

$$\frac{1}{v} = \frac{1}{k_{+1} \cdot E_0} + \frac{K_3 K_4}{k_{+1} \cdot E_0 \cdot G6P \cdot PEP} + Mal\left(\frac{K_3 K_4}{K_2 \cdot k_{+1} \cdot E_0 \cdot G6P \cdot PEP} + \frac{K_4}{K_5 \cdot k_{+1} \cdot E_0 \cdot PEP}\right) + \frac{K_6}{k_{+1} \cdot E_0 \cdot PEP} + \frac{K_4}{k_{+1} \cdot E_0 \cdot PEP} \qquad (12)$$

Fig. 5 shows plots of $1/v$ against malate concentration at a fixed concentration of phospho*enol*pyruvate and with various concentrations of glucose 6-phosphate. From four experiments of this sort, K_5 was calculated to be 0.78 ± 0.27 mM. From Eqn. 2 K_7 was then calculated to be 0.15 mM. Experiments with 10 and 20 mM glucose 6-phosphate did not give such good straight lines in graphs of this sort (correlation coefficients no greater than 0.95), but the reasons for this are not yet clear.

It has been known for many years [4,6] that L-malate inhibits phospho*enol*pyruvate carboxylase activity of crude extracts and partially purified preparations from plants with crassulacean acid metabolism. However, this inhibition has seldom been studied systematically, for instance at different pH values, and this may account for the varied reports about the kinetics of inhibition [9,12,13]. Glucose 6-phosphate was mentioned as an activator of phospho*enol*pyruvate carboxylase by Pan and Waygood [10]; later, Ting and Osmond [11]

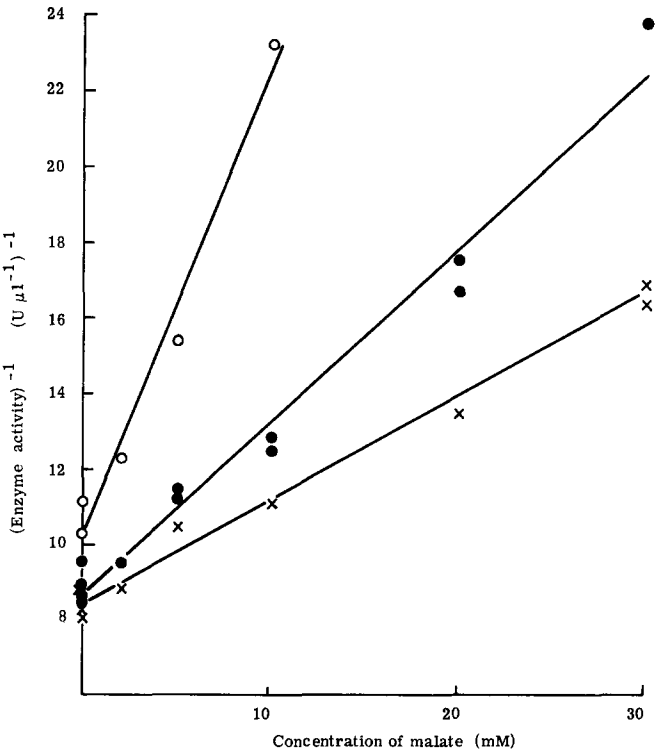


Fig. 5. Effects of glucose 6-phosphate on the activity of phospho*enol*pyruvate carboxylase in the presence of various concentrations of L-malate at pH 7.8. Reaction mixtures contained 2 mM phospho*enol*-pyruvate, L-malate and 0 (○———○), 2 (●———●) or 4 (X———X) mM glucose 6-phosphate. The lines of best fit were calculated by the method of least-squares.

showed that its chief effect on phospho*enol*pyruvate carboxylase activity in partially purified preparations of the crassulacean acid metabolism plant *Kalanchoe daigremontiana* was to lower the apparent K_m for phospho*enol*-pyruvate rather than to raise the V. Since L-malate and glucose 6-phosphate are probably important regulators of the enzyme in vivo, it is essential to understand their effects in detail and this became possible only after we had obtained a homogeneous preparation of the enzyme [12]. The molecular basis of the double pH optima of the enzyme [12] is not clear at present, but is probably physiologically important in view of the variety of observations of the complex pH-dependence of phospho*enol*pyruvate carboxylase in plants with crassulacean acid metabolism [7,8]. The experiments described in the present paper were therefore done at both pH optima.

The reaction scheme proposed in Scheme I appears to be in accord with all the result we have obtained at pH 7.8. The effects of glucose 6-phosphate are in general agreement with the results for the enzyme from *K. daigremontiana* [11] and our reaction scheme explains why glucose 6-phosphate is so effective at reversing inhibition by L-malate (Fig. 2) [11]. The reaction scheme does not invoke any co-operative effects.

The contrasting results we have obtained for L-malate inhibition at pH 5.8 and 7.8 (Fig. 3 compared with Fig. 8 of Ref. 12) emphasise the importance of doing these experiments under different conditions and may reconcile the results of Wilkinson and Smith [13], who reported non-competitive inhibition at pH 7.4 for partially purified enzyme from *B. fedtschenkoi*, with those of Kluge and Osmond [9] who showed mixed inhibition for preparations from *B. tubiflorum* at pH 6.25.

It is clear from our results and those of other workers [9,10,13] that glucose 6-phosphate and L-malate are particularly potent effectors. Nevertheless, there are other inhibitions, activations and interactions which may be of physiological significance or may be valuable in future experiments on the molecular properties of the enzyme.

Galactose 6-phosphate, glucose 1-phosphate and fructose 6-phosphate activate the enzyme at pH 7.8 (Table I) and may bind to the same site as glucose 6-phosphate since they do not have an additive effect in the presence of glucose 6-phosphate. Of all the metabolites tested, only fructose 1,6-bisphosphate (at pH 5.8, but not at pH 7.8) was more effective than glucose 6-phosphate in activating the enzyme. In view of the interdependence of glycolysis and dicarboxylic acid metabolism in crassulacean acid metabolism [14], it would not be surprising if fructose 1,6-bisphosphate exerted a regulatory influence on the metabolism of oxaloacetate and malate. It is strange, however, that fructose 1,6-bisphosphate should have an effect at pH 5.8 but not at pH 7.8. Wong and Davies [15] showed that phospho*enol*pyruvate carboxylase from *Zea mays* was activated by fructose 1,6-bisphosphate to a much greater extent at pH 7.0 than at pH 7.8, but stated that activation by other phosphate esters showed the same pH-dependence, so this is not comparable with the enzyme from *B. fedtschenkoi*.

3-Phosphoglycerate behaves as though it prevents binding (and hence activation) by glucose 6-phosphate. Both glucose and glucose 6-sulphate were virtually without effect on the enzyme activity, demonstrating the importance of the phosphate group. Malate inhibition is stereospecific since D-malate had little effect on enzyme activity and did not greatly alter the inhibition by L-malate.

The relative ineffectiveness of acetyl-CoA, L-aspartate and glycine, which are all potent effectors of phospho*enol*pyruvate carboxylases from other sources [1,2,16—18], emphasises the peculiarities of the enzyme from plants with crassulacean acid metabolism.

Malonate blocks the formation of malate in some plants with crassulacean acid metabolism and was found to be a competitive inhibitor of partially purified phospho*enol*pyruvate carboxylase from *Setaria italica* (a C_4 plant) when tested at pH 8.0 [19], but had no effect on the enzyme from *Zea mays* at either pH 7.0 or 7.5 [15]. Malonate inhibited the pure phospho*enol*pyruvate carboxylase from *B. fedtschenkoi* (Table I) at pH 7.8, possible competitively; curiously, however, it increased the activity at pH 5.8.

This paper has not tackled the problem of whether the second substrate is CO_2 or HCO_3^- [20] nor whether the mechanism follows an ordered or a random pathway [21]. It does, however, provide data and an analysis sufficient to predict the velocity of the phospho*enol*pyruvate carboxylase-catalysed reaction

over a wide range of substrate and effector concentrations. It is now necessary to measure variations in the cytoplasmic concentrations of these metabolites as the next step towards understanding the circadian and diurnal rhythms of CO_2 metabolism.

Acknowledgement

This work was supported by Science Research Council Grant No. B/RG/7670.3.

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