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Bryophyllum fedtschenkoi protein phosphatase type 2A can dephosphorylate phosphoenolpyruvate carboxylase

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Phosphoenolpyruvate carboxylase, which catalyses the nocturnal fixation of CO_2 in Crassulacean acid metabolism (CAM) plants, is regulated by reversible phosphorylation. The phosphorylated 'night' form of the enzyme is ten-fold less sensitive to inhibition by malate than is the dephosphorylated 'day' form. The phosphoenolpyruvate carboxylase of the CAM plant *Bryophyllum fedtschenkoi* can be dephosphorylated by rabbit muscle protein phosphatase type 2A but not by type 1. *B. fedtschenkoi* leaves contain protein phosphatase activity that can dephosphorylate phosphoenolpyruvate carboxylase. Inhibitor studies show that this enzyme is a type 2A protein phosphatase.

Phosphoenolpyruvate carboxylase; Crassulacean acid metabolism; Phosphorylation; Protein phosphatase; Malate inhibition; (*Bryophyllum fedtschenkoi*)

1. INTRODUCTION

Phosphoenolpyruvate carboxylase (EC 4.1.3.1) plays an important role in the regulation of Crassulacean acid metabolism (CAM). The enzyme catalyses the first committed step in the nocturnal fixation of atmospheric CO_2. The product oxaloacetate is reduced to malate which is stored in the vacuoles during the night and is then decarboxylated during the day to form CO_2 which is refixed photosynthetically via ribulose-1,5-bisphosphate carboxylase. Phosphoenolpyruvate carboxylase must be inactive during the day in order to avoid futile cycling. The enzyme is subject to feedback inhibition by malate and it is widely thought to be regulated by periodic accumulation of malate in the cytoplasm (for a review, see [1]). However, the malate sensitivity of phosphoenolpyruvate carboxylase from CAM plants can itself be affected by reversible phosphorylation [2–6]. The enzyme from the CAM plant *Bryophyllum fedtschenkoi* has been purified in two forms. The 'night' form is phosphorylated on one or more serine residues and has a relatively high K_i for malate of about 3 mM, whereas the 'day' form is not phosphorylated and is ten-fold more sensitive to inhibition by malate [3]. The interconversions between these forms are controlled by an endogenous rhythm rather than directly by light or darkness [2,4]. However, very little is known about the protein kinase(s) and protein phosphatase(s) that catalyse these interconversions.

Mammalian tissues contain several different protein phosphatases and there are four major types of catalytic subunits (for a review, see [7]). These are termed types 1, 2A, 2B and 2C. Recently it was shown that plant extracts contain activities very similar to the mammalian protein phosphatases types 1 and 2A, but no activities corresponding to types 2B or 2C were found [8]. Type 1 protein phosphatases are selectively inhibited by two heat-stable proteins, termed inhibitor-1 and inhibitor-2. Both type 1 and type 2A protein phosphatases are inhibited by the tumour promotor okadaic acid. The type 2A protein phosphatase is much more sensitive to okadaic acid than is the type 1 enzyme [8–11]. The concentration of oxadaic acid required to give 50% inhibition of the type 2A enzyme depends on the enzyme concentration itself [10] and can be 10- to 100-fold lower than that for the type 1 enzyme [8,10]. In the present paper we show that leaves of the CAM plant *B. fedtschenkoi* contain a type 2A protein phosphatase that can dephosphorylate phosphoenolpyruvate carboxylase.

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2. EXPERIMENTAL

2.1. *Materials*

Bryophyllum (*Kalanchoë*) *fedtschenkoi* Hamet et Perrier plants were maintained and harvested as described in [2].

Okadaic acid, purified catalytic subunits of rabbit skeletal muscle type 1 and type 2A protein phosphatases [12], inhibitor-2 [13] and ^{32}P-labelled phosphorylase *a* [12] were all gifts from Dr C. MacKintosh and Prof. P. Cohen (Department of Biochemistry, University of Dundee, Dundee, Scotland). Antipain, leupeptin, chymostatin and L-malic acid were from Sigma Chemical, Poole, Dorset, UK. $^{32}P_i$ (carrier free) was obtained from Amersham International. ^{32}P-casein was prepared as described in [14]. The sources of other materials were as given in [2,3].

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2.2. *Preparation of ^{32}P-phosphorylated phosphoenolpyruvate carboxylase*

Four detached *B. fedtschenkoi* leaves were each labelled with 1.0 mCi of carrier-free $^{32}P_i$ as described in [2]. They were then pooled with 22 g of unlabelled leaves and the 'night' form of phosphoenolpyruvate carboxylase was purified as in [3].

2.3. *Enzyme assays*

Phosphoenolpyruvate carboxylase activity was measured as in [3]. The malate sensitivity of the enzyme was determined using a range of malate concentrations as described in [2].

Protein phosphatase activity was quantified by measuring the release of ^{32}P from ^{32}P-labelled substrates as in [12]. Assays contained either 10 μM phosphorylase *a* [12], 4 μM casein [14] or 0.5 μM purified phosphoenolpyruvate carboxylase (450 cpm per assay). Activities quoted in the paper refer to phosphorylase *a* as substrate unless stated otherwise. One unit of activity catalyses the dephosphorylation of 1 μmol of protein subunits per min.

Estimates of the activities of type 1 and 2A protein phosphatases in partially purified *B. fedtschenkoi* preparations were made on the basis of differential inhibition by okadaic acid and inhibitor-2 as described in [10].

2.4. *Partial purification of plant protein phosphatase*

B. fedtschenkoi leaves (60 g) were taken from plants during the day and homogenised at 4°C in 60 ml of 100 mM Tris/HCl, pH 8.0, containing 2% (w/v) poly(ethylene glycol) 20000, 2 mM EDTA, 1 mM benzamidine hydrochloride, 2% (w/v) insoluble polyvinylpyrrolidone and 3 g sodium bicarbonate with 2–3 drops of octan-1-ol to prevent foaming. The extract was filtered through two layers of muslin, its pH was adjusted to 7.5 and it was then centrifuged for 15 min at 15000 × *g*. The supernatant was brought to 75% saturation with $(NH_4)_2SO_4$, stirred for 30 min at 4°C and centrifuged for a further 15 min at 15000 × *g*. The precipitate was resuspended in 8 ml of 20 mM Tris-HCl, pH 7.5, 0.1 mM EGTA, 10% (v/v) glycerol and 0.1 mM benzamidine hydrochloride (buffer A). 10 vols of 95% ethanol containing 1 mM phenylmethylsulfonyl fluoride were added at room temperature and the mixture was centrifuged immediately at 15000 × *g* for 10 min. The soluble material was discarded and the precipitate was resuspended at 4°C in 4 ml of buffer A and recentrifuged for 10 min. The supernatant was collected and the precipitate was re-extracted with a further 4 ml of buffer A. The pooled supernatants were desalted into buffer A on a Sephadex G-25M column (1.5 × 3.0 cm (50 ml)). Active fractions were pooled and chromatographed at room temperature on a Mono Q column (0.5 × 5 cm) connected to a Pharmacia FPLC system. The column was washed in buffer A at 1 ml/min, then a linear 25 ml gradient of 100–400 mM NaCl in buffer A was applied and 1 ml fractions were collected. Peak protein phosphatase fractions, as assayed with phosphorylase *a*, were pooled and concentrated four-fold with a Centricon 30 microconcentrator. The protein phosphatase was stored at 4°C.

3. RESULTS AND DISCUSSION

3.1. *Effect of mammalian protein phosphatases on phosphoenolpyruvate carboxylase*

The phosphorylated 'night' form of phosphoenolpyruvate carboxylase from *B. fedtschenkoi* has a ten-fold higher apparent K_i for malate than does the dephosphorylated 'day' form [2,3]. In addition, ^{32}P-phosphorylated enzyme can be obtained from leaves that have taken up $^{32}P_i$, as described in section 2. Thus, the effect of protein phosphatases on the enzyme can be monitored by measuring either a decrease in its apparent K_i for malate or the release of ^{32}P.

Incubations of the phosphorylated form of phosphoenolpyruvate carboxylase with purified rabbit muscle protein phosphatase 2A (3 mU/ml) consistently caused a decrease in the apparent K_i of the enzyme for malate from 3–4 mM to 0.5–1 mM. However, incubations with purified rabbit muscle protein phosphatase 1 (3 or 30 mU/ml for 1–2 h) caused no change in malate sensitivity (data not shown).

Fig. 1 shows a representative time course for the release of ^{32}P from phosphoenolpyruvate carboxylase and the increase in the malate sensitivity of the enzyme catalysed by rabbit muscle protein phosphatase 2A. Analysis of the results from several similar experiments indicated that there is a direct correlation between the release of ^{32}P from the enzyme and the increase in its malate sensitivity. These results indicate that *B. fedtschenkoi* phosphoenolpyruvate carboxylase can be dephosphorylated by the mammalian protein phosphatase type 2A but not by the type 1 protein phosphatase.

3.2. *Comparison of the effects of mammalian and plant protein phosphatases on phosphorylated phosphoenolpyruvate carboxylase*

In attempts to identify activities in *B. fedtschenkoi* capable of dephosphorylating phosphoenolpyruvate carboxylase, the purified 'night' form of the carboxylase was incubated with leaf extracts. This resulted in an increase in the K_i of the enzyme for malate rather than a decrease. This increase is attributed to the presence of proteases in the extract, as *B. fedtschenkoi* phosphoenolpyruvate carboxylase is very sensitive to limited proteolysis, which causes a substantial, irreversible increase in the apparent K_i of the enzyme for malate [3].

We therefore partially purified protein phosphatase activities from *B. fedtschenkoi* leaves (see Table I) using a modification of the procedure for isolation of the catalytic subunits of the type 1 and 2A protein phosphatases from rabbit muscle [12]. The protein phosphatase specific activity in leaf extracts was 0.5 mU/mg. For comparison, values for *Zea mays* leaf extracts and *Brassica napis* seed extracts were 2 mU/mg (G.A.L. McNaughton, C.A.F. Fewson, M.B. Wilkins and H.G. Nimmo, unpublished) and 3.7 mU/mg [8], respectively. Both phosphorylase phosphatase and casein phosphatase activities were determined at each stage of the purification as shown in Table I.

Treatment of the $(NH_4)_2SO_4$ precipitate with ethanol at room temperature increased the protein phosphatase activity. Similar results have been obtained with mammalian protein phosphatases and are attributed to the dissociation of the catalytic subunits from regulatory subunits [7]. Analysis of the phosphorylase phosphatase activities in the pooled Mono Q fractions with inhibitor-2 and okadaic acid showed that 75% of the activity was attributable to type 1 protein

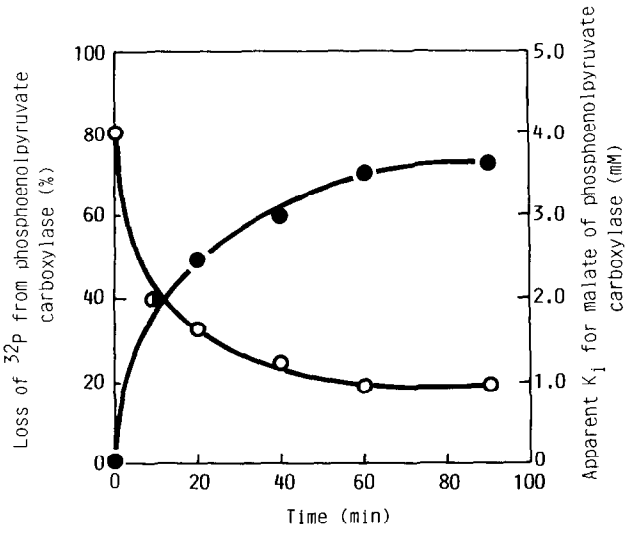

Fig. 1. Effect of mammalian protein phosphatase type 2A on phosphoenolpyruvate carboxylase. Phosphoenolpyruvate carboxylase was incubated with rabbit muscle protein phosphatase type 2A (3 mU/ml) under the protein phosphatase assay conditions given in [12]. Apparent K_i for malate (○); release of ^{32}P (●).

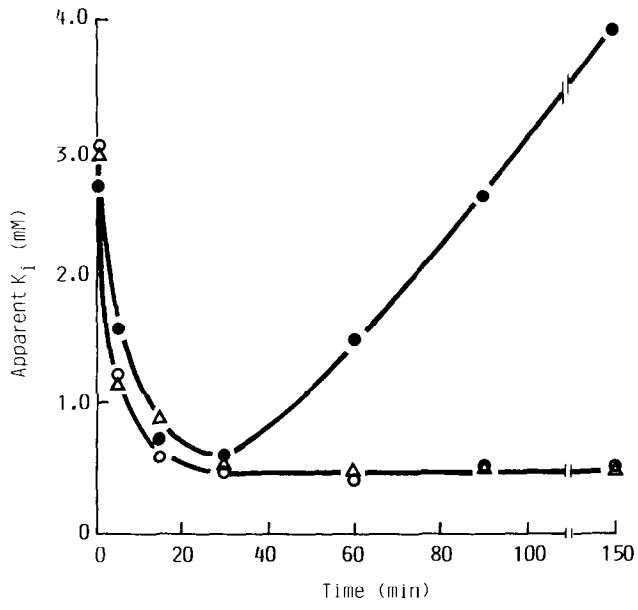

Fig. 2. Effect of plant protein phosphatase on phosphoenolpyruvate carboxylase. Phosphoenolpyruvate carboxylase was incubated with the partially purified plant protein phosphatase (3 mU/ml of type 2A activity) in the presence (○) or absence (●) of 1 mM L-malate, or in the presence of protease inhibitors (see text) (△).

phosphatase and 25% to type 2A protein phosphatase. Casein is a much better substrate for type 2A phosphatase activity than for the type 1 enzyme [8,15]. In agreement with this, the casein phosphatase activity was completely inhibited by 1 nM okadaic acid, indicating that it was specifically due to type 2A protein phosphatase. The partially purified protein phosphatase activities were stable for 2–3 weeks when stored at 4°C.

When the partially purified plant protein phosphatase fraction was incubated with the unlabelled, purified 'night' form of phosphoenolpyruvate carboxylase, there was an increase in the sensitivity of the enzyme to inhibition by malate within 30 min (Fig. 2). However, on prolonged incubation the malate sensitivity declined and the K_i for malate increased above the initial value of 2.7 mM. This increase was prevented by 1 mM L-malate, which probably acts by protecting the low K_i form of the enzyme from proteolysis, as was found in earlier studies [3]. This explanation was confirmed by the observation that incubation of the enzyme with the plant phosphatase fraction and a mixture of protease inhibitors (5 μg antipain/ml, 5 μg leupeptin/ml, 10 μg chymostatin/ml, 0.1 mM benzamidine hydrochloride) prevented the increase in K_i (Fig. 2). The K_i declined to 0.5 mM and remained at this value for over 2 h. In other experiments, 10 μM L-malate was found to protect the enzyme against the apparent proteolysis, although this is a much lower concentration than is required to inhibit enzyme activity.

Table I

Partial purification of protein phosphatase from *B. fedtschenkoi* leaves

Purification step	Volume (ml)	Phosphorylase phosphatase activity (mU/ml)	Casein phosphatase activity (mU/ml)
Extract	60.0	0.88	0.22
0–75% $(NH_4)_2SO_4$	8.0	3.71	0.68
Desalted ethanol precipitate	10.0	6.10	1.25
Mono Q concentrated pool	0.7	71.00	6.00

Protein phosphatase activities were determined using ^{32}P-labelled phosphorylase *a* or casein

In order to ascertain whether protein phosphatase 1 or 2A activity in the plant protein phosphatase precipitation was responsible for the dephosphorylation of phosphoenolpyruvate carboxylase, the effects of okadaic acid and inhibitor-2 on the activities were investigated. The results in Table II show that the concentration of okadaic acid required to give complete inhibition of the plant protein phosphatase activity was between 10 and 25 nM. When rabbit muscle protein phosphatase type 2A was used at the same concentration as the plant 2A-type activity in terms of activity against phosphorylase *a* (3 mU/ml), the concentration of okadaic acid required to give complete inhibition was also in the range of 10–25 nM. This is considerably higher than some values quoted for the IC_{50} of okadaic acid against mammalian protein phosphatase 2A using phosphorylase *a* as a substrate (e.g. [9,10]). However, the IC_{50} depends on the protein phosphatase concentration which is significantly higher for assays carried out

Table II

Inhibition by okadaic acid of the effect of mammalian and plant protein phosphatase activity on the malate sensitivity of phosphoenolpyruvate carboxylase

[Okadaic acid] (nM)	K_i value after incubation with plant protein phosphatase		K_i value after incubation with mammalian protein phosphatase	
	0 min	60 min	0 min	60 min
0	3.4	0.7	3.3	0.8
5	3.3	0.7	N.D.	N.D.
10	3.5	0.7	3.0	1.0
25	3.5	3.4	3.0	3.0

Incubations contained 3 mU/ml of type 2A plant or mammalian protein phosphatase activity. N.D., not determined

with phosphoenolpyruvate carboxylase than with phosphorylase *a*. The presence of 1 μM inhibitor-2 did not prevent the dephosphorylation of phosphoenolpyruvate carboxylase by the plant protein phosphatase preparation; the K_i decreased from 3.3 to 0.8 mM in 1 h. These results are thus consistent with the view that the activity in the plant protein phosphatase preparation that can dephosphorylate phosphoenolpyruvate carboxylase is of type 2A.

In a further experiment when the plant protein phosphatase concentration was twenty-fold lower (approximately 0.13 mU/ml of type 2A protein phosphatase activity) in the incubation, a significant increase in the malate sensitivity of phosphoenolpyruvate carboxylase was still observed; the K_i decreased from 2.9 to 1.2 mM in 30 min. Under the standard assay conditions used here, the highest activity of the plant type 2A protein phosphatase that has been measured so far with phosphoenolpyruvate carboxylase as the substrate is about ten-fold less than with phosphorylase *a*. However, the conditions for dephosphorylation of the phosphoenolpyruvate carboxylase have not yet been optimised.

3.3. *General discussion*

MacKintosh and Cohen [8] recently studied the dephosphorylation of phosphorylase *a*, phosphorylase kinase and casein by plant extracts and demonstrated the existence of activities very similar to mammalian protein phosphatases types 1 and 2A. The results presented in the present paper show that two such activities can be detected in leaf tissue of the CAM plant *B. fedtschenkoi* and that the type 2A enzyme is capable of dephosphorylating phosphoenolpyruvate carboxylase. The existence of other phosphoenolpyruvate carboxylase phosphatases in plant tissue cannot be ruled out. However their existence would be difficult to demonstrate owing to the sensitivity of phosphoenolpyruvate carboxylase to proteases present in crude extracts.

The phosphorylation state of *B. fedtschenkoi* phosphoenolpyruvate carboxylase is controlled by an endogenous rhythm rather than directly by light [2,4]. The regulatory properties of the kinase(s) and phosphatase(s) involved in this system are of great interest. The results presented here show that phosphoenolpyruvate carboxylase can be dephosphorylated by plant protein phosphatase type 2A. The activity studied in this work probably represents the catalytic subunit of the phosphatase [7,12]. Investigation of the control of this phosphatase will require isolation of the holoenzyme form.

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