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Plant protein phosphatases

Subcellular distribution, detection of protein phosphatase 2C and identification of protein phosphatase 2A as the major quinate dehydrogenase phosphatase

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Protein phosphatases 1 and 2A (PP1 and PP2A) were identified in a variety of plant cells and found to be particulate or soluble depending on the species. In extracts prepared from oilseed-rape seeds these enzymes were associated with microsomes and more rapidly sedimenting fractions, whereas in wheat leaf extracts they were largely microsomal, the remainder being present in the soluble fraction. In pea leaf and carrot cell extracts PP1 and PP2A were almost entirely soluble. No PP1 or PP2A activity was associated with the membranes or stroma of chloroplasts in oilseed-rape seeds, pea leaves and wheat leaves. An Mg^{2+}-dependent okadaic acid-insensitive protein phosphatase that resembles protein phosphatase 2C (PP2C) was detected in carrot cells, pea leaves and wheat leaves, but not in oilseed-rape seeds. In wheat leaf extracts PP2C was mostly present in the soluble fraction, a different location from PP1 or PP2A. The rapid inactivation of the cytosolic enzyme quinate dehydrogenase (QDH) in a fraction prepared from light-grown carrot cells was completely blocked by either okadaic acid or microcystin (two potent and specific inhibitors of PP1 and PP2A), whereas inhibitor 2 (a specific inhibitor of PP1) inhibited inactivation by only about 10%. Addition of the purified PP2A catalytic subunit from mammalian skeletal muscle increased the rate of QDH inactivation, whereas addition of mammalian PP1 did not. It is concluded that PP2A is the major enzyme responsible for dephosphorylating (inactivating) QDH in carrot cells. These observations indicate that okadaic acid and microcystin may be useful for identifying other plant processes that are controlled by phosphorylation/dephosphorylation mechanisms. Okadaic acid did not prevent the rapid inactivation of phosphoribulokinase or activation of glucose-6-phosphate dehydrogenase in a fraction prepared from light-grown pea leaves, and addition of the purified catalytic subunits of PP1 and PP2A did not accelerate either process. These observations, in conjunction with the absence of PP1 and PP2A activity in chloroplasts, suggest that these phosphatases are not involved in the regulation of chloroplast metabolism.

INTRODUCTION

Signals, such as hormones, light and gravity, control diverse physiological processes in plants. However, the molecular details of plant signal-transduction pathways, and how far they resemble the well-studied intracellular signalling mechanisms in animal cells, are not yet clearly understood. There is increasing evidence that the reversible phosphorylation of proteins is important in the regulation of plant metabolism [1], but compared with mammalian cells relatively few proteins whose activities are controlled by phosphorylation have so far been identified. Two cytosolic enzymes that are known to be controlled in this manner are phosphoenolpyruvate carboxylase [2] and quinate dehydrogenase (QDH) [3], phosphorylation of the latter being mediated by a Ca^{2+}/calmodulin-dependent protein kinase [4].

Recently, we reported that two of the major serine/threonine-specific protein phosphatases (PP) present in animal cells are present at similar levels in extracts prepared from the seeds of *Brassica napus* (oilseed rape) and that their properties are virtually indistinguishable from the corresponding enzymes in mammalian tissues [5]. Similarities included substrate-specificity (with mammalian phosphoprotein substrates), sensitivity to the heat-stable proteins inhibitor 1 and inhibitor 2 (from rabbit skeletal muscle), which inhibit PP1 specifically, inhibition by the tumour-promoter okadaic acid [5] and inhibition by the cyanobacterial toxin microcystin LR [6]. Okadaic acid and microcystin LR are potent and specific inhibitors of PP1 and PP2A [7,8]. Under standard assay conditions okadaic acid inactivates PP2A at 1 nM and can be used as a specific inhibitor of this enzyme, because PP1 is unaffected at this concentration. The IC_{50} for inhibition of PP1 by okadaic acid is approx. 10 nM, complete inhibition occurring at 1 μM [5,9].

PP1 and PP2A are capable of dephosphorylating a variety of regulatory proteins in animal cells [10], raising the question of their physiological roles in higher plants. In order to begin to answer these questions, we have extended our studies to other plant extracts and investigated the subcellular distribution of PP1 and PP2A, as well as their ability to regulate some cytosolic and chloroplast enzymes whose activities change dramatically in response to light. These studies have demonstrated that PP2A is the major enzyme responsible for inactivating the cytosolic enzyme QDH in carrot cells, and that PP1 and PP2A are not involved in the regulation of chloroplast metabolism. In addition, an Mg^{2+}-dependent okadaic acid-insensitive protein phosphatase (PP2C), which is not detectable in oilseed-rape seed extracts, has now been identified in carrot cells and in both pea leaf and wheat leaf extracts.

MATERIALS AND METHODS

Materials

Oilseed-rape seed (*Brassica napus* var. Jet Neuf) was provided by Dr. A. R. Slabas (Unilever Research, Sharnbrook, Beds., U.K.). Seeds were collected 38–41 days after flowering, just at the onset of lipid accumulation [11], placed directly into liquid N_2

Abbreviations used: PP, protein phosphatase; QDH, quinate dehydrogenase.
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and stored at −70 °C until use. Carrot cell suspension cultures were obtained from Dr. J. M. Widholm [12] (University of Illinois at Urbana–Champaign, IL, U.S.A.), cultured under continuous light [3], harvested within 2 min by filtration under suction, frozen in liquid N_2 and stored at −70 °C until use. Pea seedlings (*Pisum sativum* L. cv. Onward) and wheat (*Triticum aestivum* var. Slepner) were grown under normal daylight/dark conditions in Levington Multipurpose compost. The catalytic subunits of PP1 and PP2A [13] and inhibitors 1 and 2 [14] were purified to homogeneity from rabbit skeletal muscle, okadaic acid was generously donated by Dr. Y. Tsukitani (Fujisawa Chemical Co., Tokyo, Japan), and microcystin LR was provided by Professor G. Codd (Department of Biological Sciences, University of Dundee, Dundee, U.K.). Dehydroquinate was made chemically from quinic acid [15], and dehydroquinase was purified from an *Escherichia coli* overproducing strain [16]. BSA, histone type IIIS and ribulose 5-phosphate were purchased from Sigma Chemical Co. (Poole, Dorset, U.K.), NADH and NADPH from Boehringer Corp. (Lewes, Sussex, U.K.) and Superose 12 and Sephadex PD10 columns from Pharmacia (Milton Keynes, Bucks., U.K.).

Isopycnic sucrose-density-gradient centrifugation

Oilseed-rape seeds (5–10 g) were homogenized in a Polytron (Ystral, Dottingen, Germany) for 135 s (3 × 45 s) in 2 vol. of ice-cold extraction medium comprising 50 mM-Tricine/KOH, pH 7.6, 0.4 M-sorbitol, 17 mM-NaCl, 2 mM-sodium D-isoascorbate, 5 mM-$MgCl_2$ and 0.1 % BSA. The top three leaf pairs from 14-day-old *Pisum sativum* seedlings or leaves from 10–15-day-old *Triticum aestivum* were cut, and homogenized at full speed in a Waring blender for only 2 s in 2 vol. of ice-cold extraction medium to minimize lysis of chloroplasts. The homogenate was filtered through four layers of cheesecloth, and 5–7 ml of extract applied to the top of 30 ml linear continuous 25–60 % (w/w) sucrose density gradients in 20 mM-Tricine/KOH (pH 7.6)/0.1 % 2-mercaptoethanol/1 mM-benzamidine. Gradients were centrifuged at 4 °C in a Beckman SW27 swing-out rotor for 3 h at 84000 ***g***, and removed by pumping through a capillary pipette at 1 ml/min from the bottom of the tube. Approx. 1 ml fractions were collected. Sucrose concentrations were determined by refractometry.

Preparation of ammonium sulphate fractions from light-grown carrot cells and pea seedlings

All operations were performed at 0–2 °C and the entire procedure was completed within 25 min. Carrot cells (5 g) were taken from storage at −70 °C and ground for 2 min with a pestle and mortar with 0.5 g of acid-washed sand, 0.5 g of polyvinylpolypyrrolidone and 5 ml of 0.1 M-Tris/HCl (pH 7.5 at 0 °C)/2 mM-EDTA/100 mM-NaCl/0.1 % 2-mercaptoethanol/1 mM-benzamidine/0.1 mM-phenylmethanesulphonyl fluoride (buffer A). The resulting paste was squeezed through four layers of cheesecloth. The solid residue was re-extracted with another 5 ml of buffer, and the pooled soluble fractions were centrifuged for 5 min at 20000 ***g***. The supernatant was decanted and 1.25 vol. of 90 %-saturated ammonium sulphate added, maintaining the pH at 7.5 by addition of a few drops of conc. NH_3 solution. The suspension was centrifuged for 10 min at 20000 ***g***, the supernatant discarded, and the pellet redissolved in 1 ml of extraction buffer and desalted by passage through a PD10 column equilibrated in extraction buffer minus NaCl. The protein-containing fractions (2 ml) were pooled and portions frozen in liquid N_2 and stored at −70 °C.

The leaves of *Pisum sativum* were homogenized in buffer A with a Waring blender (5 s at the high-speed setting), and after being squeezed through cheesecloth the suspension was fractionated with ammonium sulphate and desalted as described above.

Preparation of ^{32}P-labelled substrates and protein phosphatase assays

^{32}P-labelled phosphorylase was prepared by phosphorylation with phosphorylase kinase [13], and ^{32}P-labelled phosphorylase kinase [17] and casein [18] with protein kinase A as described in the references cited. Histone (type IIIS) was phosphorylated by protein kinase A in an identical manner with ^{32}P-labelled phosphorylase kinase and freed from [γ-^{32}P]ATP by gel filtration on a PD10 column. PP1 and PP2A were routinely assayed at 30 °C [13] using phosphorylase (10 μM), although casein (6 μM in terms of ^{32}P) and phosphorylase kinase (1 μM) were also employed where indicated. PP1 was the phosphorylase phosphatase activity not inhibited by 1 nM-okadaic acid and/or inhibited by inhibitor 2, whereas PP2A was the activity inhibited by 1 nM-okadaic acid and/or resistant to inhibitor 2 [9]. PP2C was the Mg^{2+}-dependent casein phosphatase activity measured in the presence of 5 μM-okadaic acid [9] or 1 μM-microcystin [6]. Release of [^{32}P]P_i from ^{32}P-labelled histone (3 μM in terms of ^{32}P) was assayed after extraction of the phosphomolybdate complex into 2-methylpropan-1-ol/toluene [19]. One unit of activity was the amount that catalysed the release of 1 μmol of [^{32}P]phosphate in 1 min from each substrate.

Assay of plant enzymes

All assays were carried out at 30 °C. QDH activity was monitored by the oxidation of NADH at 340 nm in the presence of dehydroquinate [3]. The cuvette contained 5 mM-dehydroquinate, 0.18 mM-NADH, 20 mM-NaF and 0.5 M-Tris/HCl, pH 8.5 (measured at 20 °C), in a total volume of 1 ml. The concentration of dehydroquinate was measured spectrophotometrically following conversion into 3-dehydroshikimate using 3-dehydroquinase [16]. Phosphoribulokinase was assayed in 100 mM-Tris/HCl (pH 7.8)/0.5 mM-ribulose 5-phosphate/0.2 mM-ATP/20 mM-$MgCl_2$, coupling the ADP formed in the reaction to the oxidation of NADH via pyruvate kinase and lactate dehydrogenase [20]. Ribulose-bisphosphate carboxylase [21] and CDP-choline:diacylglycerol acyltransferase [22] were assayed as described, except that 1,2-dipalmitin and Tween 20 were excluded from assays of the latter enzyme. Inclusion of 1,2-dipalmitin and Tween 20 resulted in lower activities being measured, presumably because endogenous diacylglycerols in the microsomal fractions were used preferentially. One unit of activity was that amount that catalysed the formation of 1 μmol of product in 1 min.

Measurement of protein and chlorophyll

Protein was determined by the procedure of Bradford [23], with BSA ($A_{280\,nm}^{1\%}$ = 6.5) as standard. Chlorophyll was determined by the method of Winterman & de Mots [24].

RESULTS

Subcellular location of PP1 and PP2A

We reported previously that, when oilseed-rape seed homogenates were prepared in the absence of Triton X-100 and centrifuged at 20000 ***g***, most of the PP1 and PP2A was recovered in a dark-green particulate fraction [5]. PP1 and PP2A in this fraction appeared to be associated with membranes, because complete solubilization occurred when 2 % Triton X-100 was included in the homogenization buffer. In order to identify the membranes with which PP1 and PP2A were associated in the absence of Triton X-100, the extracts were subjected to sucrose-density-gradient centrifugation and fractions from the gradient

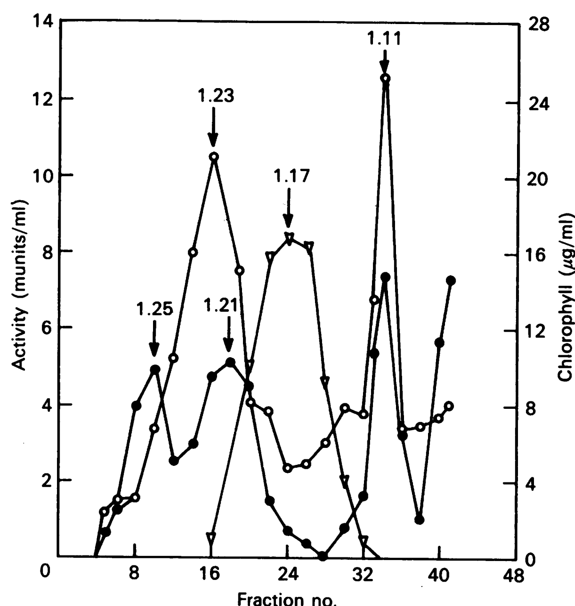

Fig. 1. Sucrose-density-gradient centrifugation of *Brassica napus* extracts prepared in the absence of Triton X-100

Extracts were filtered through cheesecloth and subjected to density-gradient centrifugation as described in the Materials and methods section. Symbols: ○, PP1; ●, PP2A; ▽, chlorophyll. The numbered arrows show densities (g/ml). CDP-choline:diacylglycerol acyltransferase activity was co-eluted exactly with the peaks of PP1 and PP2A activity centred at a density of 1.11 g/ml. Similar results were obtained in experiments performed with two different extracts.

assayed for PP1 and PP2A activity in the absence of bivalent cations with ^{32}P-labelled phosphorylase as substrate. These studies showed that PP1 was associated with fractions sedimenting at densities of 1.23 and 1.11 g/ml respectively (Fig. 1). PP2A was also associated with the fraction sedimenting at 1.11 g/ml, as well as two other regions sedimenting at 1.25 and 1.21 g/ml (Fig. 1). The lighter opaque fraction ($\rho = 1.11$ g/ml) contained the membranes of the endoplasmic reticulum, as judged by co-elution of the marker enzyme CDP-choline: diacylglycerol acyltransferase activity [25]. PP1 and PP2A in the lighter fraction could be solubilized with 2% Triton X-100, but not by extraction with 0.5 M-NaCl (results not shown), demonstrating that they were associated with membranes. The identities of the heavier fractions ($\rho = 1.21$, 1.23 and 1.25 g/ml) are unclear, but, in contrast with the lighter fraction, PP1 and PP2A could be solubilized with either 2% Triton X-100 or 0.5 M-NaCl. Neither fraction coincided with the elution position of broken plastids (identified by the location of chlorophyll), which sedimented as a broad peak centred at $\rho = 1.17$ g/ml (Fig. 1). Little PP1 and PP2A were present in the cytosol at the top of the gradient (fraction 38). No PP1 or PP2A activity was associated with either the oil-body layer that floated on the top of the gradient [25] or with the starch granules that pelleted at the bottom of the gradient (results not shown).

Oilseed-rape seeds contain few chloroplasts and these are ruptured under conditions required to lyse whole seeds. In order to determine whether PP1 and PP2A were associated with intact chloroplasts it was therefore necessary to use different plant tissues. Accordingly, pea leaves were homogenized in a Waring blender for < 2 s, and after filtering through cheesecloth were subjected to sucrose-density-gradient centrifugation as before. Under these conditions about 50% of the chloroplasts were intact ($\rho = 1.23$ g/ml) and 50% were broken ($\rho = 1.17$ g/ml) (Fig. 2*a*). In contrast with oilseed-rape seed extracts, however,

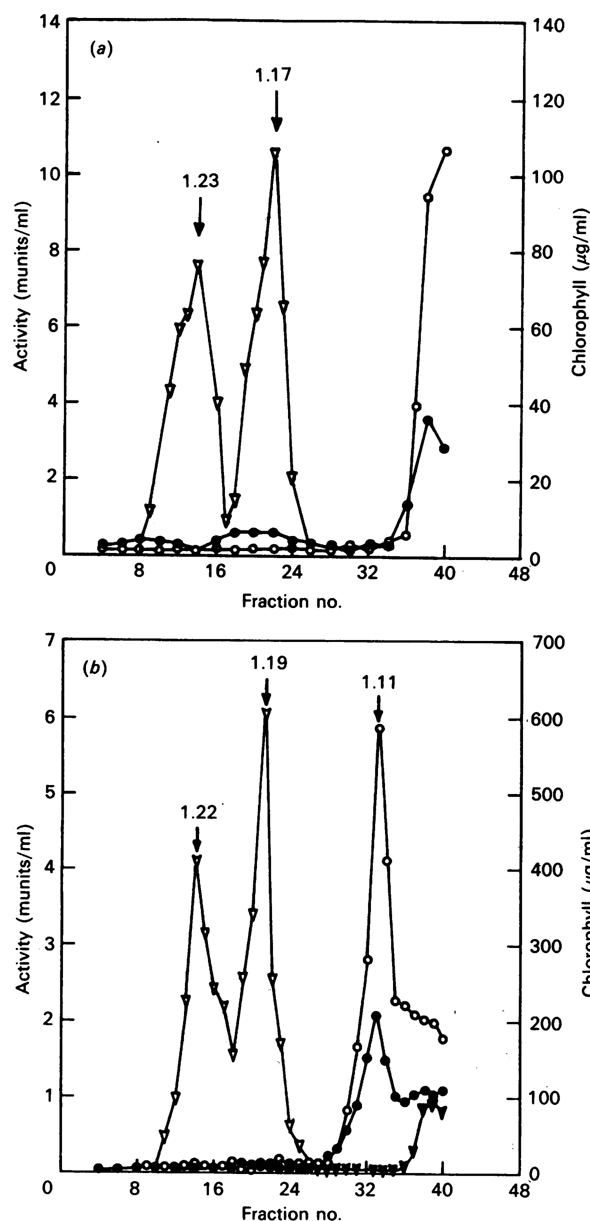

Fig. 2. Sucrose-density-gradient centrifugation of pea leaf extracts (*a*) and wheat leaf extracts (*b*)

Extracts were filtered through cheesecloth and subjected to density-gradient centrifugation as described in the Materials and methods section. Symbols: ○, PP1; ●, PP2A; ▼, PP2C; ▽, chlorophyll. The arrows show the positions of intact chloroplasts (sedimenting at 1.22–1.23 g/ml), broken chloroplasts (1.17–1.19 g/ml) and microsomes (1.11 g/ml). A peak of ribulose-bisphosphate carboxylase activity was co-eluted with intact chloroplasts, the remaining activity (released from broken chloroplasts) being found at the top of the gradient (results not shown). Similar profiles were seen in two further experiments for each species.

virtually all the PP1 and PP2A activity was soluble (Table 1) and sedimented at the top of the gradient (Fig. 2). Very little phosphatase activity was associated with either broken or intact chloroplasts, even when the latter were lysed either by dilution (Fig. 2) or with 0.5% Triton X-100 (results not shown). Essentially the same profiles were obtained when assays were performed with casein, histone and phosphorylase kinase (results not shown) instead of phosphorylase.

Similarly, when extracts of wheat leaves were subjected to sucrose-density-gradient centrifugation, very little protein phos-

Table 1. Specific activities of protein phosphatases in homogenates of higher plants

Each tissue was ground finely in liquid N_2, resuspended in 2 vol. of buffer A (4 vol. for wheat leaves) and filtered through four layers of cheesecloth to give the homogenate. PP1 and PP2A were assayed for 10 min at high dilutions to minimize the effects of inhibitory substances in the extracts. Final dilutions from the extracts were 150-fold (rape seed), 120-fold (wheat leaf), 60-fold (pea leaf) and 240-fold (carrot cells). PP2C was assayed for 20 min at the highest possible dilutions, which were 30-fold (rape seed), 9-fold (wheat leaf), 9-fold (pea leaf) and 45-fold (carrot cells). Each value (mean ± S.D.) represents data from three separate samples taken from the same batch of seeds, plants or cells.

Plant	Activity (munits/mg of protein)			Protein (mg/ml)	Chlorophyll (μg/ml)
	PP1	PP2A	PP2C		
Rape seed	2.22±0.17	1.29±0.12	0.004±0.003	4.2±0.2	23.5±3.5
Wheat leaf	3.23±0.42	1.18±0.15	0.11±0.016	1.2±0.04	94.5±8.3
Pea leaf	2.36±0.12	0.80±0.03	0.032±0.008	2.9±0.15	112.5±12
Carrot cells	7.91±0.44	1.80±0.19	0.21±0.02	3.5±0.15	–

phatase activity sedimented with either intact or broken chloroplasts when assayed with ^{32}P-labelled phosphorylase in the absence of bivalent cations (Fig. 2*b*). A substantial proportion of the PP1 and PP2A activity in wheat leaf extracts sedimented at a density of 1.13 g/ml, characteristic for microsomes.

Identification of protein phosphatase 2C in plants

We reported in ref. [5] that extracts of oilseed-rape seeds did not contain detectable amounts of the Mg^{2+}-dependent okadaic acid-insensitive protein phosphatase 2C (PP2C), and these results were confirmed in the present study. However, carrot cell extracts did contain significant amounts of a PP2C-like activity (Table 1). In the presence of 5 μM-okadaic acid or 1 μM-microcystin LR the dephosphorylation of casein was almost completely dependent on Mg^{2+}, 50% activation occurring at 1.3 mM (Fig. 3), similar to mammalian PP2C. Like the mammalian enzyme, the PP2C of carrot cells dephosphorylated the α-subunit of phosphorylase kinase preferentially (Fig. 3). Pea and wheat leaf extracts contained lower, but detectable, levels of PP2C (Table 1). PP2C from carrot cells, pea and wheat leaves had very low phosphorylase phosphatase activity (results not shown), like mammalian PP2C.

The subcellular location of PP2C was studied in wheat leaf extracts by sucrose-density-gradient centrifugation (Fig. 2*b*). These experiments demonstrated that most of the Mg^{2+}-dependent okadaic acid-insensitive (PP2C) activity assayed with ^{32}P-labelled casein (Fig. 2*b*) was in the soluble fraction at the top of the gradient.

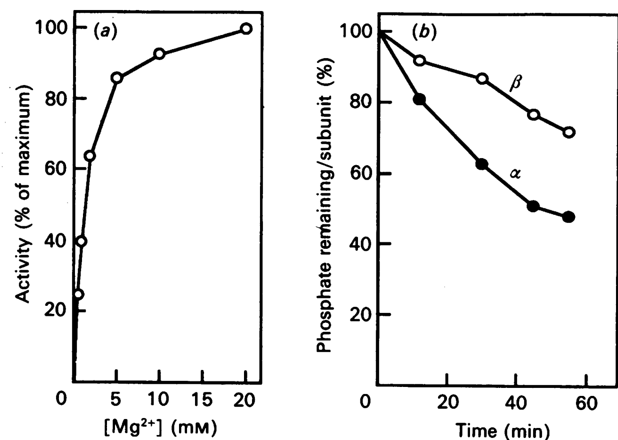

Fig. 3. Dephosphorylation of casein (*a*) and phosphorylase kinase (*b*) by protein phosphatase 2C from carrot cells

The ammonium sulphate fraction was desalted into buffer A (minus EDTA) and assayed in the presence of 5 μM-okadaic acid and Mg^{2+}. Assays were performed at 60-fold (*a*) or 9-fold (*b*) dilutions of the fraction. In (*a*), 100% activity corresponds to 4 munits/ml in the undiluted fraction. In (*b*), the assays were performed in the presence of 20 mM-magnesium acetate. The release of phosphate from the α-subunit (●) and β-subunit (○) of phosphorylase kinase was quantified as in ref. [17]. Similar results were obtained in three separate experiments.

Inactivation of QDH by PP1 and PP2A

QDH is present in an active phosphorylated form in carrot cells grown in continuous light [3]. When extracts from these cells were fractionated from 0–45%-saturated ammonium sulphate in the presence of NaF to inhibit protein serine/threonine phosphatases and then desalted (to remove ammonium sulphate and NaF), the activity of QDH decreased by 90% within 30 min upon incubation at 30 °C. The decrease in activity could be blocked by re-addition of 50 mM-NaF and reversed by incubation with MgATP [3].

In the present work, it was found that QDH retained > 95% of its activity when a 0–50%-saturated-ammonium sulphate fractionation was performed in the absence of NaF, provided that the temperature was kept at 0 °C. Subsequent incubation at 30 °C led to the expected rapid inactivation, which could be completely prevented by addition of 2 μM-okadaic acid or 1 μM-microcystin LR (Fig. 4) or 50 mM-NaF (results not shown). The effects of okadaic acid and microcystin confirmed that inactivation resulted from dephosphorylation, and indicated that PP1 and/or PP2A were involved. These enzymes are present at high concentrations in carrot cells (Table 1). Okadaic acid had no effect on QDH when included in the assays at 2 μM. Addition of 10 mM-$MgCl_2$ or 1 mM-$MnCl_2$ did not affect the rate of inactivation in the presence or absence of 2 μM-okadaic acid (results not shown). This demonstrated that the PP2C-like activity in carrot cell extracts was incapable of inactivating QDH. The stimulation of QDH inactivation by $MgCl_2$ reported in ref. [3] may be explained by the inclusion of NaF during ammonium sulphate fractionation, which is well known to convert PP1 and PP2A from bivalent-cation-independent into bivalent-cation-dependent enzymes (e.g. see ref. [26]).

In order to determine the relative contributions of PP1 and PP2A to the inactivation of QDH, the desalted ammonium sulphate fraction was supplemented with inhibitor 2. Inhibitor 2 (0.5 μM) only inhibited the rate of inactivation of QDH by about 10% (Fig. 5), suggesting that PP2A was the dominant QDH phosphatase in the fraction. Control experiments established

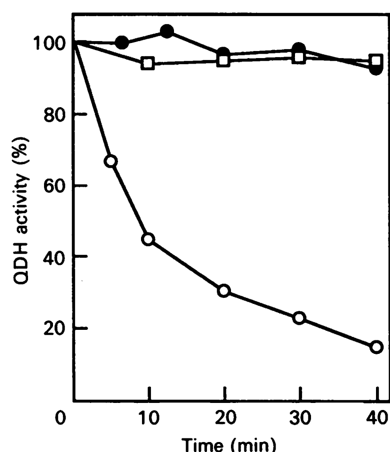

Fig. 4. Effect of okadaic acid and microcystin LR on the inactivation of QDH from light-grown carrot cells

The ammonium sulphate fraction from light-grown carrot cells was desalted and diluted to 1.6 mg/ml in buffer A containing 2 μM-okadaic acid (●), 1 μM-microcystin LR (□) or no additions (○). At the times indicated, 200 μl portions of the incubation were assayed for QDH activity as described in the Materials and methods section. Similar results were obtained in three experiments with okadaic acid and two with microcystin LR. 100% activity corresponds to ΔA_{340}/min of 0.0073 in the assay.

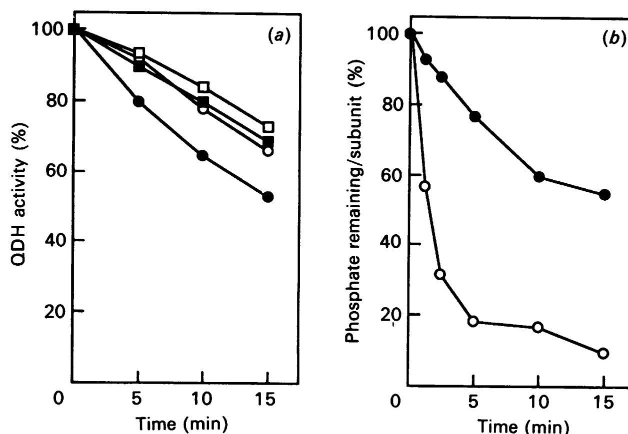

Fig. 5. Effect of inhibitor 2 on the inactivation of QDH and dephosphorylation of phosphorylase kinase in light-grown carrot cell extracts

(*a*) The ammonium sulphate fraction from carrot cells was desalted and diluted to 1.4 mg/ml in buffer A supplemented with 0.5 μM-inhibitor 2 (□), or 120 munits of mammalian PP1 catalytic subunit/ml (■), or 120 munits of mammalian PP2A catalytic subunit/ml (●), or no additions (○). QDH activity (100% activity corresponds to a ΔA_{340}/min of 0.0047) was then measured after incubation at 20 °C for various times. The added PP1 and PP2A activities were severely depressed by substances present in the carrot cell fraction and precise activities under the incubation conditions are given in the text. Similar results were obtained in experiments with three different extracts. (*b*) Dephosphorylation of the β-subunit of phosphorylase kinase by the light-grown carrot cell fraction in the presence (●) and in the absence (○) of 0.5 μM-inhibitor 2. The experiment was carried out under the same conditions as in (*a*). The rabbit skeletal-muscle phosphorylase kinase substrate contained 0.77 mol of phosphate/mol of α-subunit and 0.83 mol of phosphate/mol of β-subunit.

that, under the same conditions used to measure inactivation of QDH, inhibitor 2 inhibited by about 90% the initial rate of dephosphorylation of the β-subunit of phosphorylase kinase catalysed by the PP1 activity in the fraction [10,17] (Fig. 5). In contrast, dephosphorylation of the α-subunit was unaffected (results not shown).

PP1 accounted for 77% and PP2A for 23% of the phosphorylase phosphatase activity in the ammonium sulphate fraction when assays were performed at the same dilution used to study inactivation of QDH. Under these conditions the phosphorylase phosphatase activity of PP1 was 4.8 munits/ml, and that of PP2A was 1.4 munits/ml. The amount of PP2A activity in the fraction was then increased by 4.6 munits/ml by addition of the catalytic subunit of PP2A from mammalian muscle, which increased the rate of QDH inactivation (Fig. 5). In contrast, increasing the PP1 activity by 6.4 munits/ml had no effect on the rate of QDH inactivation (Fig. 5). These results, in conjunction with the effects of okadaic acid, microcystin LR and inhibitor 2, establish that PP2A is the major QDH phosphatase in carrot cells.

It should be noted that, as in mammalian tissue extracts [26], PP1 and PP2A activities were both strongly suppressed in the concentrated fraction compared with dilute solutions. The activities of PP1 and PP2A in this carrot cell fraction both increased approx. 5-fold when assayed at a 200-fold higher dilution, whereas the activities of the PP1 and PP2A catalytic subunits from muscle decreased by 95% when added to the ammonium sulphate fraction. The carrot enzymes were not free catalytic subunits, as judged by gel filtration on a column (25 cm × 1 cm) of Superose 12 equilibrated in 50 mM-Tris/HCl (pH 7.5)/1.0 mM-EDTA/1.0 mM-EGTA/0.1% 2-mercaptoethanol/1 mM-benzamidine/0.02% Triton X-100/100 mM-KCl. Both enzymes were eluted as single peaks earlier than BSA (66 kDa) and close to its dimer. The molecular masses of the mammalian catalytic subunits are 36–37 kDa and they are eluted from Superose 12 after BSA (results not shown).

Lack of effect of PP1 and PP2A on chloroplast enzymes

The activities of a number of chloroplast enzymes are known to alter rapidly in response to light, enzymes of the reductive pentose phosphate shunt (e.g. phosphoribulokinase) being activated and enzymes of the oxidative pathway (e.g. glucose-6-phosphate dehydrogenase) being inactivated [27]. An ammonium sulphate fraction was isolated from pea seedlings that had been exposed to sunlight of approx. 1000 μE for 1 h. Incubation of this fraction at 30 °C induced a rapid inactivation of phosphoribulokinase and activation of glucose-6-phosphate dehydrogenase. Although inhibition of phosphoribulokinase was largely prevented by 50 mM-NaF, okadaic acid (2 μM) had no effect. Similarly, okadaic acid did not prevent the activation of glucose-6-phosphate dehydrogenase. Activation of glucose-6-phosphate dehydrogenase was also unaffected by 50 mM-NaF. Inactivation of phosphoribulokinase and activation of glucose-6-phosphate dehydrogenase upon incubation at 30 °C were also inhibited by 20 mM-$MgCl_2$, which is not an inhibitor of PP1, PP2A or other known protein phosphatases.

The ammonium sulphate fraction was also prepared from pea seedlings that had been kept in the dark for 4 h. Incubation at 30 °C had no effect on the activities of phosphoribulokinase or glucose-6-phosphate dehydrogenase in the presence or in the absence of 2 μM-okadaic acid, 50 mM-NaF or 20 mM-$MgCl_2$ (results not shown).

DISCUSSION

The results described in this paper demonstrate that PP1 and PP2A are present in a wide variety of plant extracts, where they may be particulate or soluble, depending on the species. Although largely particulate in extracts prepared from oilseed rapeseeds (Fig. 1) and microsomal in wheat extracts (Fig. 2*b*), they were

almost entirely soluble in pea leaf extracts (Fig. 2*a*) as well as in carrot cell extracts (Table 1). However, in no plant extracts tested were significant amounts of PP1 and PP2A associated with either the membranes or stroma of chloroplasts. Nor was any other bivalent-cation-dependent or -independent protein phosphatase found to be specifically associated with chloroplasts with a variety of substrates, including histones. Markwell and co-workers reported that an Mg^{2+}-stimulated histone phosphatase activity was associated with the thylakoid membranes of wheat chloroplasts [28]. However, this activity appeared to be extremely low, and these investigators did not report whether it represented only a trace of the total phosphatase activity present in the cell extracts.

Consistent with its subcellular location, PP2A was found to be the major protein phosphatase capable of inactivating the cytosolic enzyme QDH in carrot cell extracts (Figs. 3 and 4). We have also found that okadaic acid prevents the re-activation of another cytosolic enzyme, namely sucrose-phosphate synthetase in spinach leaf extracts. This enzyme is converted from a phosphate-inhibited (less active, phosphorylated) form into a phosphate-insensitive (more active, dephosphorylated) form in the light [29]. Similarly, the cytosolic enzyme phosphoenolpyruvate carboxylase in the Crassulacean acid metabolism plant *Bryophylum fedtschenkoi* was recently shown to be dephosphorylated by PP2A, but not by PP1 [30], converting it from a malate-insensitive (active) into a malate-sensitive (less active) form [2].

Although the results suggest a role for PP2A in the regulation of cytosolic metabolism, more work is needed to understand the role of PP1 *in vivo*. It seems likely that, as in animal cells, PP1 and PP2A play important roles in the control of other plant processes, such as growth. For example mutant yeast, *Aspergillus* and *Drosophila* lacking PP1 are defective in chromosome separation and fail to complete mitosis (reviewed in ref. [31]), and PP2A is involved in preventing the activation of cdc2, the protein kinase that plays a central role in driving cell-cycle events in eukaryotic cells [32].

Phosphoribulokinase and glucose-6-phosphate dehydrogenase, which are activated and inactivated respectively in response to light, could not be interconverted by PP1 or PP2A, and okadaic acid did not prevent the reversion of these enzymes to the 'dark' state. These observations are consistent with the absence of PP1 and PP2A from chloroplasts. Although the inactivation of phosphoribulokinase in extracts from light-grown pea seedlings was largely prevented by 50 mM-NaF, this compound is known to inhibit enzymes other than protein phosphatases, and it is noteworthy that it did not prevent the re-activation of glucose-6-phosphate dehydrogenase. Regulation of chloroplast enzymes by light appears to be exerted by a different post-translation modification, namely thiol–disulphide interchange [33]. Nevertheless, several phosphoproteins have been identified in chloroplasts (e.g. ref. [34]), and the switch between Photosystems I and II is believed to be triggered by the phosphorylation of certain thylakoid membrane proteins [35]. Presumably, chloroplast phosphoproteins are regulated by protein phosphatases with completely different specificities from PP1 and PP2A. This situation would be similar to that in *E. coli* which contains over 100 phosphoproteins [36], and yet is devoid of protein phosphatase activity towards all the substrates used to assay eukaryotic protein phosphatases [37].

Finally, the present work has demonstrated that okadaic acid and microcystin LR are important compounds for identifying plant processes that are controlled by phosphorylation, which further emphasizes their potential as new probes for the study of cellular regulation [38].

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