Purification and characterization of two wheat-embryo protein phosphatases

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Two protein phosphatases (enzymes I and II) were extensively purified from wheat embryo by a procedure involving chromatography on DEAE-cellulose, phenyl-Sepharose CL-4B, DEAE-Sephacel and Ultrogel AcA 44. Preparations of enzyme I (M_r 197000) are heterogeneous. Preparations of enzyme II (M_r 35000) contain only one major polypeptide (M_r 17500), which exactly co-purifies with protein phosphatase II on gel filtration and is not present in preparations of enzyme I. However, this major polypeptide has been identified as calmodulin. Calmodulin and protein phosphatase II can be separated by further chromatography on phenyl-Sepharose CL-4B. Protein phosphatases I and II do not require Mg^{2+} or Ca^{2+} for activity. Both enzymes catalyse the dephosphorylation of phosphohistone H1 (phosphorylated by wheat-germ Ca^{2+}-dependent protein kinase) and of phosphocasein (phosphorylated by wheat-germ Ca^{2+}-independent casein kinase), but neither enzyme dephosphorylates a range of non-protein phosphomonoesters tested. Both enzymes are inhibited by Zn^{2+}, Hg^{2+}, vanadate, molybdate, F^-, pyrophosphate and ATP.

INTRODUCTION

Protein phosphorylation–dephosphorylation is an important mechanism involved in stimulus–response coupling in animal cells (Cohen, 1985a,b; Nishizuka, 1986). Protein kinases have been resolved from higher plants, including Ca^{2+}-dependent protein kinases (Hepler & Wayne, 1985; Ranjeva & Boudet, 1987). Plant Ca^{2+}-dependent protein kinases are inactive at 10^{-7} M free Ca^{2+} and fully active at the free Ca^{2+} concentrations of 10^{-6}–10^{-5} M found in the cytosol of excited plant cells (Polya & Micucci, 1984). Accordingly such enzymes are likely to be involved in mediating the effects of extracellular signals that increase cytosolic Ca^{2+} in plant cells (Hepler & Wayne, 1985). Although many elements of a possible cyclic-nucleotide-regulatory system are present in plants (Brown & Newton, 1981; Francko, 1983), cyclic-nucleotide-regulated protein kinase has not yet been purified from plants.

A variety of plant proteins are phosphorylated by endogeneous protein kinases, including Ca^{2+}-dependent protein kinases. However, only a small number of plant enzymes have as yet been shown to be regulated by phosphorylation–dephosphorylation (Ranjeva & Boudet, 1987). Whereas a multiplicity of animal protein phosphatases has been characterized (Ballou et al., 1985; Cohen, 1985a,b; Sparks & Brautigan, 1986), little is known of the nature of plant protein phosphatases. The dephosphorylation of thylakoid phosphoproteins by a membrane-associated protein phosphatase has been described (Yang et al., 1987), as have the properties of pea (Pisum sativum) phosphopyruvate dehydrogenase phosphatase associated with the pyruvate dehydrogenase complex (Miernyk & Randall, 1987). A phosphohistone-H1 phosphatase has been extensively purified from soya-bean (Glycine max) hypocotyls (Lin, 1980). The present paper describes the purification and characterization of two distinct protein phosphatases from wheat (Triticum sp.) embryo.

MATERIALS AND METHODS

Materials

Raw wheat (Triticum aestivum) germ was obtained locally. [γ-^{32}P]ATP was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Dephosphorylated casein, lysine-rich histone (a preparation rich in histone H1, with catalogue specification III-S), nucleotides and other phosphomonoesters and phosphodiesters, polyamines and proteins for gel-filtration-column calibration were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. DEAE-cellulose (DE-52) was obtained from Whatman; DEAE-Sephacel, Sepharose CL-6B and phenyl-Sepharose CL-4B were obtained from Pharmacia; Ultrogel AcA 44 was obtained from LKB.

Purification of protein phosphatases I and II

All procedures were conducted at 0–4 °C. A 500 g portion of raw wheat germ was homogenized for 1 min, by using an Ultra-Turrax blender (Janke und Kunkel, Staufen, Germany) in 2 litres of an extraction medium containing 50 mM-Tris (Cl^-, pH 8.0), 10 mM-2-mercaptoethanol, 0.1 mM-EGTA, 0.5 mM-phenyl-methanesulphonyl fluoride and 0.25 % (v/v) ethanol. The homogenate was filtered through muslin and Miracloth and added to 50 g of DEAE-cellulose (DE-52). The DE-52 was washed successively with 4 litres of buffer A [50 mM-Tris/HCl (pH 8.0)/10 mM-2-mercaptoethanol] containing 0.1 mM-EGTA. Protein phosphatase was eluted with 500 ml of 0.5 M-NaCl/buffer A. The eluate was adjusted to 1 mM-$CaCl_2$ and applied to a 200 ml bed volume of phenyl-Sepharose CL-4B. The phenyl-Sepharose CL-4B was washed with 4 litres of 0.5 M-NaCl/buffer A/1 mM-$CaCl_2$, and the protein phosphatases were eluted in 4 litres of buffer A/1 mM-$CaCl_2$. A subsequent washing of the phenyl-Sepharose CL-4B with 2 litres of buffer A/1 mM-EGTA yielded highly purified pre-
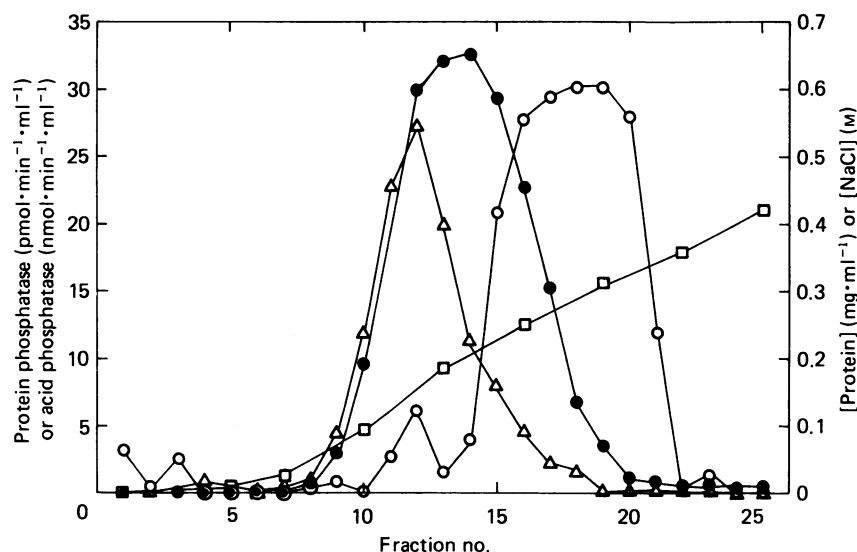
Fig. 1. Resolution of protein phosphatases and acid phosphatase by chromatography on DEAE-Sephacel

For chromatographic details, see the Materials and methods section. Phosphoprotein phosphatase was measured in the standard assay with [^{32}P]phosphocasein as substrate. ○, Protein phosphatase; □, NaCl concentration; ●, protein concentration; △, acid phosphatase measured with p-nitrophenyl phosphate as substrate at pH 5.0.

parations of wheat-embryo Ca^{2+}-dependent protein kinase as described below. The buffer A/1 mm-CaCl$_2$ eluate was added to a 200 ml bed volume of DEAE-Sephacel, which was washed with 500 ml of buffer A and then packed into a column and eluted with a linear gradient of increasing NaCl in buffer A, the protein phosphatase being eluted in 0.25–0.4 m-NaCl in buffer A (Fig. 1). Alternatively the DEAE-Sephacel was washed stepwise with 1 litre of 0.2 m-NaCl/buffer A to remove most of the contaminating protein and acid phosphatase (cf. Fig. 1), the protein phosphatase then being eluted with 0.4 m-NaCl/buffer A. The eluate was concentrated by pressure filtration (Amicon YM-10 membrane) and applied to an Ultrogel AcA 44 column (7 cm^2 × 55 cm) and chromatographed in 0.2 m-NaCl/buffer A to resolve high-M_r protein phosphatase I from low-M_r protein phosphatase II (Fig. 2). The purification schedule is presented in Table 1. The protein phosphatases were routinely stored at 4 °C. Under these conditions protein phosphatases I and II lose 50 % of activity in 11 days and 3 days respectively.

Protein kinase and phosphoprotein preparations

Wheat-germ casein kinase was partially purified as previously described (Davies & Polya, 1983), up to and including affinity chromatography on casein–Sepharose 4B. Ca^{2+}-dependent protein kinase was partially purified from wheat embryo by a procedure involving homogenization, binding to DEAE-cellulose (DE-52) and binding to phenyl-Sepharose CL-4B in the presence of CaCl$_2$, exactly as described above for the purification of protein phosphatases I and II. The protein kinase was eluted from phenyl-Sepharose CL-4B with 2 litres of buffer A/1 mm-EGTA. The eluate was adjusted to 9 % (v/v) ethanediol and 2 mm-CaCl$_2$ and added to a 100 ml bed volume of Cibacron Blue 3G-A–Sepharose CL-6B, which was then washed with 1 litre of buffer B [9 % (v/v) ethanediol/2 mm-CaCl$_2$/buffer A] before elution of the protein kinase in 400 ml of 1 m-NaCl/buffer B. The eluate was concentrated to 4 ml by pressure filtration,
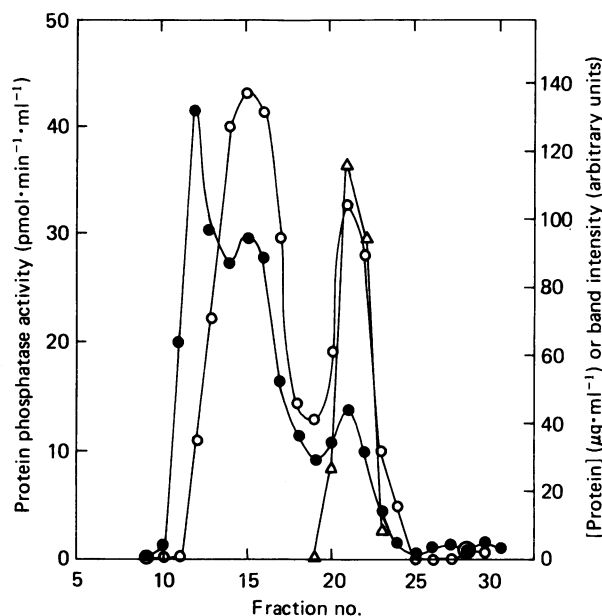

Fig. 2. Resolution of protein phosphatases I and II by chromatography on Ultrogel AcA44

For chromatographic details, see the Materials and methods section. Protein phosphatase was determined with [^{32}P]phosphocasein as substrate. ●, Protein concentration; ○, protein phosphatase; △, mean band intensity from duplicate determinations of the 17.5 kDa polypeptide band (deriving from Coomassie Blue staining of electrophoretograms after sample concentration and SDS/polyacrylamide-gel electrophoresis as described in the Materials and methods section).

and the protein kinase was chromatographed on a Sephacryl S-200 column (5 cm^2 × 80 cm) in buffer A/1 mm-EGTA.

[^{32}P]Phosphocasein was prepared in reaction mixtures (1 ml) containing 50 mm-Tris/HCl, pH 8.0, 10 mm-

Table 1. Purification of protein phosphatase I and II

A 500 g portion of wheat germ was processed as described in the Materials and methods section. Protein phosphatase was assayed in the standard reaction conditions with phosphocasein as substrate.

Purification step	Protein (mg)	Protein phosphatase activity	
		Total (pmol·min⁻¹)	Specific (pmol·min⁻¹·mg of protein⁻¹)
Homogenate	34510	8034	0.23
DE-52	2155	3934	1.83
Phenyl-Sepharose CL-4B	81.5	8316	102
DEAE-Sephacel	9.5	1784	187
Ultrogel AcA 44			
Enzyme I	0.59	232	393
Enzyme II	0.16	132	825

$MgCl_2$, 0.1 mM-[γ-³²P]ATP (~ 50 mCi/mmol), 15 mg of dephosphorylated casein and excess wheat-germ casein kinase. After incubation at 30 °C for 2 h, the reaction mixture was heated to 100 °C for 5 min, chilled on ice and desalted on a Sephadex G-25 column (3 cm² × 29 cm) to resolve [³²P]phosphocasein from unchanged ATP. ³²P-histone was prepared at 30 °C in reaction mixtures containing 50 mM-Tris/HCl, pH 8.0, 10 mM-$MgCl_2$, 0.1 mM-[γ-³²P]ATP (~ 50 mCi/mmol), 0.05 mM-EGTA, 1 mM-$CaCl_2$, 15 mg of lysine-rich histone, 5 mM-dithiothreitol and excess wheat-germ Ca^{2+}-dependent protein kinase. The reactions were terminated by heating at 100 °C for 5 min and the ³²P-histone desalted as described above.

Enzyme assays

All enzyme assays were routinely conducted in duplicate at 30 °C. Protein phosphatase was routinely assayed in a reaction medium (100 μl) containing 50 mM-Mops (sodium salt, pH 6.0), [³²P]phosphoprotein (0.4 mg/ml; ~ 1–2 nmol of phosphate ester/ml) and protein phosphatase preparation. After incubation, two procedures were used to determine protein de-phosphorylation. In procedure 1 (used routinely in the present work), 60 μl aliquots of reaction mixture were spotted on to Whatman 3 MM paper discs that had previously been spotted with 30 μl of 50% (w/v) trichloroacetic acid. Discs were successively washed in 0.5 litre of cold 15% (w/v) trichloroacetic acid (three times), 100% ethanol (twice) and diethyl ether (twice), dried, and counted for radioactivity in 5 ml of 0.5% (w/v) PPO (2,5-diphenyloxazole)/0.03% (w/v) dimethyl-POPOP [1,4-bis(4-methyl-5-phenyloxazol-2-yl)benzene] in toluene to determine residual [³²P]phosphoprotein. In procedure 2 (employed for K_m determinations), reactions were terminated by addition of 0.1 ml 9% (w/v) $HClO_4$. After further addition of 0.2 ml of 5% (w/v) ammonium molybdate in 2 M-H_2SO_4 and 1.2 ml of butan-2-ol/toluene (1:1, v/v), the mixture was agitated and centrifuged at 1400 g for 5 min. A 0.8 ml portion of the non-aqueous upper phase, containing [³²P]-orthophosphate as a phosphomolybdate complex, was added to 5 ml of a scintillation cocktail containing 0.3% (w/v) PPO, 0.02% (w/v) dimethyl-POPOP, 25% (v/v) Triton X-114 and 75% (v/v) xylene, and the

radioactivity counted to determine [³²P]orthophosphate released from [³²P]phosphoprotein during the reaction. Assay values were corrected by subtraction of appropriate controls.

p-Nitrophenyl phosphate hydrolysis was measured in a reaction mixture (1 ml) containing 1 mM-p-nitro-phenyl phosphate and 50 mM-acetate (sodium salt, pH 5.0) or Mops (sodium salt, pH 6.0) or Tris/HCl (pH 8.0). Reactions were terminated by addition of 2 ml of 0.1 M-NaOH, and p-nitrophenol was determined from its absorbance at 400 nm (Andersch & Szcypinski, 1947). Phosphatase activity with other substrates was deter-mined in reaction media (1 ml) containing 1 mM-substrate and 50 mM-Mops (sodium salt, pH 6.0). Reactions were terminated by addition of 1 ml of 12% (v/v) $HClO_4$, and phosphate was determined by the method of Allen (1940). Casein kinase was assayed as described previously (Davies & Polya, 1983). Ca^{2+}-dependent histone kinase was assayed as described previously (Polya & Micucci, 1984), with 10 mM-dithiothreitol included in the assay and lysine-rich histone (1 mg/ml) as substrate.

Other procedures

Electrophoresis in the presence of SDS in 12.5%-(w/v)-polyacrylamide slab gels was performed as described by Laemmli (1970). Electrophoresis in subunit non-dissociating conditions was conducted as described by Janistyn (1986). Samples for electrophoresis were concentrated 10-fold centrifugally by using Amicon Centricon microconcentrators. Gels were stained with 0.25% (w/v) Coomassie Brilliant Blue R in methanol/ acetic acid/water (3:1:6, by vol.), destained in the same solvent, and polypeptide band intensities were deter-mined by means of a Zeineh soft-laser densitometer. High-voltage electrophoresis to separate orthophosphate from phosphoproteins was conducted on Whatman 3 MM paper in acetic acid/pyridine/water (10:1:189), pH 3.5, at 2.5 kV for 2.5 h, a Shandon high-voltage-electrophoresis apparatus being employed. Separation of orthophosphate from phosphoproteins was also effected by descending chromatography on Whatman 3 MM paper in isobutyric acid/aq. conc. NH_3 ($d = 0.88$)/ water (66:1:33, by vol.). Peptides were detected with ninhydrin, and orthophosphate was detected (through formation of a phosphomolybdate complex) by spraying

with 1% (w/v) ammonium molybdate/3% (v/v) $HClO_4/0.1$ M-HCl. Protein was determined by the method of Sedmak & Grossberg (1977), with bovine serum albumin as a standard.

RESULTS AND DISCUSSION

Purification of protein phosphatases I and II

Two protein phosphatases (termed enzymes I and II) were purified 1700- and 3600-fold respectively from wheat embryo by means of a procedure involving chromatography on DEAE-cellulose (DE-52), phenyl-Sepharose CL-4B, DEAE-Sephacel and Ultrogel AcA 44 (Table 1). The overall yield of protein phosphatase after the DEAE-Sephacel step is 22%. However, the subsequent gel-filtration step results in a further 80% loss of protein phosphatase activity and an overall yield of only about 5% (Table 1). The large losses associated with the final gel-filtration step may well derive from the very low amounts of protein involved (Table 1) and the hydrophobic nature of the protein phosphatases. Hydrophobic chromatography on phenyl-Sepharose CL-4B was the most effective single step in the procedure.

Gradient elution from DEAE-Sephacel does not resolve the two protein phosphatases, but separates these enzymes from an acid phosphatase (M_r from gel filtration: 28000) and an associated phosphocasein phosphatase activity (Fig. 1). This gradient-elution step also resolves the major protein phosphatase activity (eluted between 0.2 and 0.35 M-NaCl) from a Ca^{2+}-independent casein kinase (peak elution at 0.13 M-NaCl) and a Ca^{2+}-dependent histone kinase (peak elution at 0.15 M-NaCl). The casein kinase (M_r 34000 from gel

filtration) and the Ca^{2+}-dependent histone kinase (M_r 80000) are similar in size, respectively, to a wheat-embryo casein kinase (Davies & Polya, 1983) and wheat-embryo Ca^{2+}-dependent histone kinase (Polya & Micucci, 1984) described previously.

Subsequent gel filtration resolves the two protein phosphatases from each other (Fig. 2). The M_r values of the protein phosphatases (determined from gel filtration in 0.2 M-NaCl/buffer A on an Ultrogel AcA 44 column calibrated with proteins of known M_r) are 197000 ± 14000 (mean ± S.D. from ten determinations) for protein phosphatase I and 35000 ± 1200 (mean ± S.D. from ten determinations) for enzyme II. The M_r values of the protein phosphatases as determined from gel filtration on a calibrated Sephacryl S-200 column (5 cm² × 80 cm) in buffer A/1 mM-EGTA are 180000 ± 12000 (mean ± S.D. from three determinations) for protein phosphatase I and 32000 for protein phosphatase II. Protein phosphatase II is similar in size to a soya-bean phosphoprotein phosphatase (M_r 28000; Lin, 1980).

The peaks of protein phosphatase obtained from gel filtration correspond to peaks of protein (Fig. 2). However, protein phosphatase I preparations, unlike protein phosphatase II preparations, are very heterogeneous, as revealed by gel electrophoresis under subunit-dissociating (Fig. 3) and non-dissociating (Fig. 4) conditions. The band intensity on SDS/polyacrylamide-gel electrophoresis of a major 31 kDa polypeptide present in the peak fractions of protein phosphatase I on gel filtration does not exactly correlate with protein phosphatase I activity, as determined in a number of experiments with different preparations of the enzyme (e.g. cf. Figs. 2 and 3).
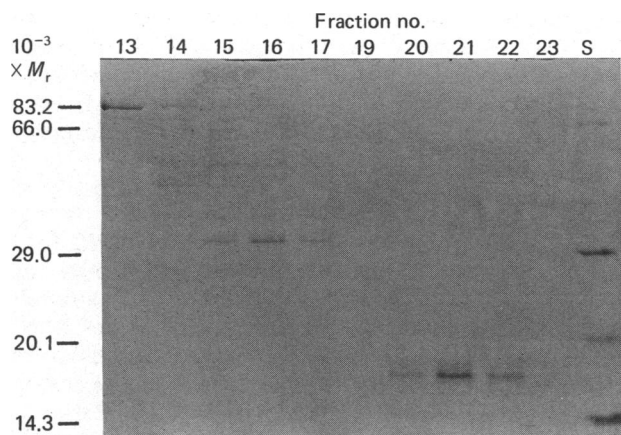

Fig. 3. Electrophoresis of enzyme I and II preparations under subunit-dissociating conditions

The lane numbers correspond to fractions 13–17 and 19–23 of Fig. 2, 1 ml samples of which were concentrated to 200 μl and 25 μl samples were subjected to SDS/polyacrylamide-gel electrophoresis as described in the Materials and methods section. The amount of protein loaded (μg) was as follows (fraction number in parentheses): 12.1 μg (fraction 13), 10.9 (14), 11.9 (15), 11.0 (16), 6.5 (17), 3.6 (19), 4.3 (20), 5.5 (21), 3.9 (22) and 1.8 (23). Lane S contains standards of known M_r. The molecular size of the wheat-germ '83 kDa' polypeptide in Lane 13 was estimated from five separate determinations (83.2 ± 4.0 kDa).
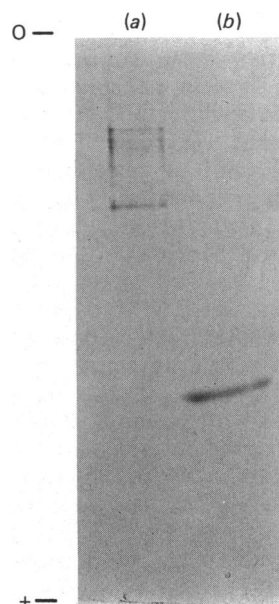

Fig. 4. Electrophoresis of enzyme I and II preparations in subunit-non-dissociating conditions

Final preparations of enzyme I and II from the purification procedure were subjected to electrophoresis in subunit-non-dissociating conditions as described in the Materials and methods section. (a) Enzyme I preparation (35.6 μg); (b) enzyme II preparation (16.5 μg); O, cathodic origin; +, anodic end of gel.

Conversely, protein phosphatase II-containing fractions from gel filtration exhibit only one major polypeptide band (M_r 17500±800; mean±s.d. from five determinations), together with a very minor companion band of slightly higher M_r on gel electrophoresis under subunit-dissociating conditions (Fig. 3). Protein phosphatase II preparations exhibit only one major band on electrophoresis under non-dissociating conditions (Fig. 4). The major 17.5 kDa polypeptide exactly co-purifies with protein phosphatase II activity on gel filtration (Figs. 2 and 3). However, this co-purification is fortuitous, and this major polypeptide has been identified as the Ca^{2+}-binding protein calmodulin. Wheat-germ calmodulin exhibits large differences in mobility when subjected to SDS/polyacrylamide-gel electrophoresis in the presence or absence of Ca^{2+} (Polya et al., 1983). The major polypeptide in protein phosphatase II preparations exactly co-purifies with wheat-germ calmodulin [isolated as described previously (Polya et al., 1983)] when subjected to SDS/polyacrylamide-gel electrophoresis in the presence of 0.1 mM-$CaCl_2$ (mobility corresponding to M_r 14000) or in the presence of 1.0 mM-EGTA (mobility corresponding to M_r 18100). Wheat-germ calmodulin binds to phenyl-Sepharose CL-4B in the presence of Ca^{2+} and is eluted in the absence of Ca^{2+} (e.g. with EGTA present) (Polya et al., 1983). However, in the protein phosphatase isolation procedure described in the Materials and methods section, evidently some calmodulin may be bound to certain hydrophobic proteins in the presence of Ca^{2+} and may be eluted from phenyl-Sepharose CL-4B together with such proteins in buffer A/1 mM-$CaCl_2$. We estimate that 0.5 mg of calmodulin/kg fresh wt. is eluted from phenyl-Sepharose CL-4B in buffer A/1 mM-$CaCl_2$ (together with the protein

phosphatases), as compared with 22 mg of calmodulin/kg fresh wt. eluted in a subsequent buffer A/1 mM-EGTA wash. Calmodulin can be removed from protein phosphatase II by further chromatography on phenyl-Sepharose CL-4B, protein phosphatase II being eluted in buffer A/1 mM-$CaCl_2$ and calmodulin being eluted by buffer A/1 mM-EGTA. Protein phosphatase II preparations are free of the polypeptides found in protein phosphatase I preparations, and vice versa, as determined from electrophoresis under subunit-dissociating (Fig. 3) and non-dissociating (Fig. 4) conditions, and we conclude that protein phosphatases I and II are distinct enzymes.

Both protein phosphatases exhibit maximal activity with phosphocasein as substrate over the pH range 6–7 (Fig. 5). Inclusion of 10 mM-$MgCl_2$ or 10 mM-dithiothreitol in the reactions had no significant effect on the activity of either enzyme (less than 16% change). The protein phosphatases were therefore routinely assayed at pH 6.0 with no added dithiothreitol or bivalent cation as described in the Materials and methods section. Protein phosphatase rate was constant over 2 h with either phosphocasein or phosphohistone as substrate. The product of the reaction with [^{32}P]phosphocasein is orthophosphate. The ^{32}P-labelled reaction product is resolved from the protein substrate by high-voltage electrophoresis and by paper chromatography as described in the Materials and methods section. In either separation system the ^{32}P-labelled product co-purifies with orthophosphate and was not ninhydrin-positive.

Substrate specificity of protein phosphatases I and II

Both protein phosphatases catalyse the dephosphorylation of [^{32}P]phosphocasein phosphorylated by wheat-germ casein kinase on serine residues (Davies & Polya, 1983) and of a histone H1 preparation phosphorylated by wheat-embryo Ca^{2+}-dependent protein kinase. The latter protein kinase phosphorylates serine residues (Polya & Micucci, 1984). The phosphocasein phosphatase and phosphohistone phosphatase activities associated with protein phosphatase I exactly co-purify on gel filtration, and the same is observed with protein phosphatase II.

The K_m values for the phosphohistone preparation used were determined by using protein phosphatase assay procedure 2 (see the Materials and methods section) and fitting the data to the Michaelis–Menten equation by means of a non-linear least-squares curve-fitting program. With protein phosphatase I the K_m is 2.1±0.7 μM-phospho residue (mean±s.e.m.), corresponding to 0.35±0.11 mg of protein·ml^{-1}. With protein phosphatase II the K_m is 2.5±0.6 μM-phospho residue, corresponding to 0.49±0.12 mg of protein·ml^{-1}. With [^{32}P]phosphocasein as substrate, saturation was not observed at phosphocasein concentrations up to 0.8 mg/ml.

The specificity of the protein phosphatases for non-protein phosphomonoester substrates was examined. It should be noted that an acid phosphatase that dephosphorylates phosphocasein at relatively low rates (compared with the rate of p-nitrophenyl phosphate hydrolysis) is resolved from the phosphatases on elution from DEAE-Sephacel (Fig. 1). In contrast, protein phosphatases I and II do not catalyse the dephosphorylation of p-nitrophenyl phosphate at pH 6.0 or 8.0. No significant dephosphorylation of the following substrates by protein phosphatases I and II was detected: O-phospho-L-serine,O-phospho-L-threonine,O-phospho-
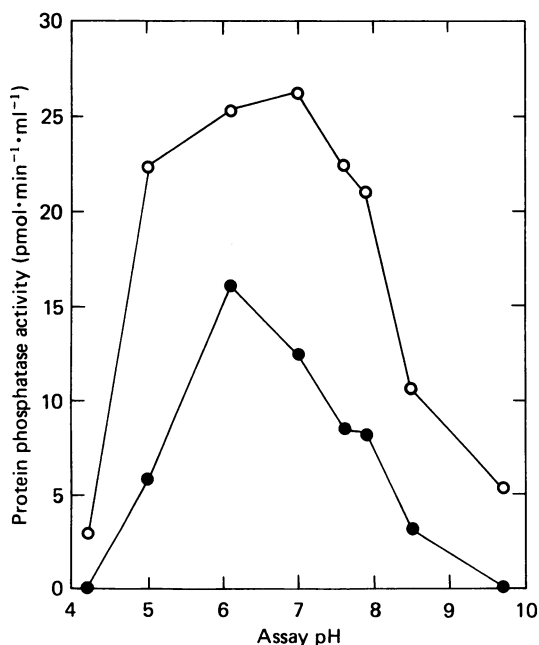

Fig. 5. pH-dependence of enzymes I and II

Protein phosphatase was measured with [^{32}P]phosphocasein as substrate and 50 mM buffer [acetate (pH 4.2 and 5.0), Mops (pH 6.1), Hepes (pH 7.0 and 7.6), Tris (pH 7.9 and 8.5) and glycine (pH 9.7)]. ○, Enzyme I; ●, enzyme II.

L-tyrosine, ADP, 2′-AMP, galactose 6-phosphate, phosphoglycollate, phenolphthalein phosphate, phytic acid, ribose 5′-phosphate and 5′-AMP. With a 1 mM concentration of the above substrates under the standard assay conditions at pH 6.0, less than 2 % of substrate was degraded over 4 h by either enzyme; under the standard assay conditions with same amount of protein phosphatase I or II, [^{32}P]phosphocasein was nearly completely dephosphorylated over the same period.

Inhibitors of the protein phosphatases

Neither protein phosphatase I or II requires a bivalent metal ion for activity. With phosphocasein as substrate, inclusion of 1 mM concentrations of EDTA, EGTA, $MgCl_2$ or $CaCl_2$ has no significant effect (less than 10 % activation or inhibition) on either protein phosphatase. The soya-bean protein phosphatase is similarly Mg^{2+}- and Ca^{2+}-independent (Lin, 1980), whereas pea phosphopyruvate dehydrogenase phosphatase is Mg^{2+}- dependent (Miernyk & Randall, 1987). In the standard reaction conditions with casein as substrate with 1 mM-$CaCl_2$, -$NiSO_4$, -$MnCl_2$ or -$BaCl_2$ included, phospho-protein phosphatase I is 70, 53, 52 and 53 % of control respectively; with the same inclusions, protein phosphatase II activity is 107, 87, 60 and 96 % of control respectively. $ZnCl_2$ and $HgCl_2$ at 10^{-4}–10^{-3} M inhibit both protein phosphatases (Table 2). $ZnCl_2$ also inhibits the soya-bean protein phosphatase (Lin, 1980). Both protein phosphatases are inhibited at elevated ionic strength, $(NH_4)_2SO_4$ being more inhibitory on a molar basis than NaCl (Table 2).

NaF has previously been described as an inhibitor of both animal (Sparks & Brautigan, 1986) and plant (Lin, 1980; Yang et al., 1987; Miernyk & Randall, 1987) protein phosphatases. NaF inhibits both casein and histone dephosphorylation catalysed by protein phosphatases I and II (Table 2). Vanadate and molybdate have also been shown to inhibit animal (Sparks & Brautigan, 1986) and plant (Lin, 1980) protein phos-

phatases and also inhibit both protein phosphatases I and II (Table 2).

While O-phospho-L-serine, O-phospho-L-threonine and O-phospho-L-tyrosine are not substrates for the protein phosphatases, at 1 mM these phosphoamino acids inhibit casein dephosphorylation by protein phosphatase I by 16, 8 and 36 % respectively and inhibit protein phosphatase II by 35, 36 and 75 % respectively. Similarly, the phosphomonoesters 5′-AMP, ribose 5′- phosphate, p-nitrophenyl phosphate and phenol-phthalein phosphate (none being substrates for either protein phosphatase) at 1 mM inhibit casein dephos-phorylation by protein phosphatase I by 21, 21, 40 and 96 % respectively and inhibit protein phosphatase II by 75, 74, 60 and 98 % respectively. Phytic acid at 1 mM inhibits protein phosphatases I and II 33 % and 72 % respectively, but 1 mM-galactose 6-phosphate has little inhibitory effect (< 13 % inhibition of either enzyme). 3′,5′-Cyclic AMP and 3′,5′-cyclic GMP at 1 mM have no effect on phosphocasein dephosphorylation catalysed by either enzyme.

ATP and pyrophosphate are potent inhibitors of both protein phosphatases, substantially inhibiting dephos-phorylation of both phosphocasein and phosphohistone at 10^{-5}–10^{-4} M (Table 2). In contrast, concentrations of ADP, orthophosphate and phosphoglycollate of about 10^{-3} M are required for substantial inhibition of protein phosphatases I and II (Table 2). The substantial inhibition of wheat-germ protein phosphatases I and II by micromolar concentrations of ATP is surprising, in view of the ATP levels in plant tissue of ∼ 40 nmol/g fresh wt. (Polya & Atkinson, 1969). This corresponds to a cytosolic ATP concentration of 0.2 mM if one assumes that the ATP is confined to the cytoplasm, the cyto-plasmic volume is 10 % of the total volume and that only half of the ATP is located in the cytosol as opposed to compartmentation in organelles (e.g. see Stitt et al., 1982). Since much ATP in vivo may exist as an Mg–ATP complex, the effect of millimolar Mg^{2+} on this inhibition

Table 2. Inhibition of protein phosphatases I and II

Protein phosphatases I and II were assayed with either phosphocasein (A) or phosphohistone (B) as substrates in the absence (in sextuplet) or presence (assays in duplicate) of increasing concentrations of the test compounds to determine IC_{50} (concentration causing half-maximal inhibition) values. All assay results were corrected by application of appropriate controls.

		IC_{50} (mM)			
	Substrate ...	A		B	
Compound	Phosphatase ...	I	II	I	II
ATP		0.02	0.006	0.09	0.01
Sodium pyrophosphate		0.007	0.002	0.02	0.08
Phenolphthalein phosphate		0.04	0.009	0.20	0.08
Phosphoglycollate		0.07	0.08	0.3	0.1
ADP		3.0	1.0	0.4	0.09
Sodium phosphate		4.0	0.5	3.0	0.4
NaF		0.8	0.6	1.0	0.5
$ZnCl_2$		0.2	0.2	–	–
Sodium vanadate		0.3	0.3	0.7	0.8
Ammonium molybdate		0.1	0.1	–	–
NaCl		600	500	–	–
$(NH_4)_2SO_4$		60	100	–	–
$HgCl_2$		–	–	0.6	0.9

was examined. Inclusion of 2.5 mM-$MgCl_2$ does not prevent inhibition by 0.5 mM-ATP of casein dephosphorylation by either enzyme, nor does it prevent inhibition of either enzyme by 0.5 mM-ATP, phenolphthalein phosphate, orthophosphate or pyrophosphate. GTP, CTP, ITP, UTP and ATP at 1 mM inhibit casein dephosphorylation catalysed by both protein phosphatases I and II by 89–100%. The inhibition of both enzymes by ATP, pyrophosphate, phenolphthalein phosphate, phosphoglycollate and NaF is greater at a phosphohistone concentration of 0.4 mg/ml than at 0.04 mg/ml (results not shown), suggesting that these compounds do not inhibit the protein phosphatases in a competitive fashion. The substantial inhibition of both protein phosphatases by ATP concentrations much lower than the inferred cytosolic concentrations suggests the possible activation of these enzymes in vivo by an effector that can overcome the ATP inhibition.

Polyamines act as growth regulators in plants (Smith, 1985), and polyamines such as spermine markedly activate particular animal protein phosphatases (Cohen, 1985a). However spermine inhibits phosphocasein dephosphorylation by protein phosphatases I and II [IC_{50} (concn. causing half-maximal inhibition) values 0.4 mM and 0.7 mM respectively]; about 10 mM-1,6-diaminohexane is required to inhibit either enzyme by 50%. Spermine-inhibited plant protein phosphatases of the kind described here could account for the observations of stimulation by 0.3–1.0 mM-spermine of net protein phosphorylation in some plant systems (Veluthambi & Poovaiah, 1984; Datta et al., 1986).

General discussion

Phosphoprotein phosphatases I and II are distinct enzymes that nevertheless have a similar substrate specificity and exhibit a similar pattern of sensitivity to a variety of inhibitors (Table 2). The specific activities of the phosphatase preparations (~ 1 nmol·min^{-1}·mg^{-1}) are much lower than for a partially purified soya-bean phosphoprotein phosphatase (10 nmol·min^{-1}·mg^{-1}) (Lin, 1980). However, it is possible that these wheat-germ enzymes are much more active with their specific endogenous substrates (as yet to be resolved) and may be subject to activation by endogenous effectors. Particular animal protein phosphatases are subject to marked MgATP-dependent activation (Ballou et al., 1985; Cohen, 1985a,b) or to activation by Ca^{2+} or polyamines (Cohen, 1985a,b). The inhibition of both enzymes I and II by ATP concentrations much lower than those found in vivo strongly suggests the existence of a mechanism to overcome this inhibition in vivo. Both enzymes dephosphorylate a histone-H1-rich preparation phos-

phorylated by a wheat-germ Ca^{2+}-dependent protein kinase. Thus protein phosphatases I and II can reverse consequences of activation of this protein kinase by signal-induced increases in cytosolic free Ca^{2+} concentration.

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