Three spinach leaf nitrate reductase–3-hydroxy-3-methylglutaryl-CoA reductase kinases that are regulated by reversible phosphorylation and/or Ca²⁺ ions

Pauline DOUGLAS*¶, Emmanuelle PIGAGLIO†, Albert FERRER‡, Nigel G. HALFORD§ and Carol MacKINTOSH*

*Medical Research Council Protein Phosphorylation Unit, Department of Biochemistry, University of Dundee, Dundee, DD1 4HN, Scotland, U.K., †INRA, Laboratoire Cellulaire, Versailles F-78000, France, ‡Facultat de Farmacia, Universitat de Barcelona, Avda. Diagonal 643, 08028-Barcelona, Spain, and §Cell Biology Department, IACR-Long Ashton Research Station, University of Bristol, Long Ashton, Bristol, BS18 9AF, U.K.

In spinach (*Spinacea oleracea* L.) leaf extracts, three protein kinases (PK_I, PK_{II} and PK_{III}) were identified each of which phosphorylated spinach nitrate reductase on serine-543, and inactivated the enzyme in the presence of nitrate reductase inhibitor, 14-3-3. PK_{III} was also very active in phosphorylating and inactivating *Arabidopsis* (Landsberg *erecta*) 3-hydroxy-3-methylglutaryl-coenzyme A reductase 1 (HMGR1). PK_I and PK_{II} phosphorylated HMGR1 more slowly than PK_{III}, compared with their relative rates of phosphorylation of nitrate reductase. All three kinases gave phosphopeptide CNBr-cleavage maps of HMGR1 identical with those that are seen after phosphorylation of serine-577 by the sucrose non-fermenting (SNF1)-like PK, 3-hydroxy-3-methylglutaryl-Co A reductase kinase A (HRK-A), from cauliflower [Dale, Arró, Becerra, Morrice, Boronat, Hardie and Ferrer (1995) Eur. J. Biochem. **233**, 506–513]. PK_I was Ca²⁺-dependent when prepared in the absence of protein phosphatase (PP) inhibitors, and largely Ca²⁺-dependent when prepared in the presence of PP inhibitors (NaF and EGTA). The Ca²⁺-independent portion of PK_I was inactivated by either PP2A or PP2C, while the Ca²⁺-dependent portion of PK_I became increasingly activated during storage, which we presume was mimicking the effect of an unidentified PP. These findings indicate that PK_I is regulated by two functionally distinct phosphorylations. PK_I had a molecular mass of 45 kDa on gel filtration and was active towards substrate peptides that terminated at the +2 residue from the phosphorylation site, whereas PK_{III} was inactive towards these peptides. PK_{II} was Ca²⁺-stimulated under all conditions tested. PK_{III} was Ca²⁺-independent, inactivated by PP2A or PP2C, had a requirement for a hydrophobic residue in the +4 position of peptide substrates, had a molecular mass by gel filtration of ∼ 140 kDa, and an antibody against the rye SNF1-related PK (RKIN1) recognised a 58 kDa subunit in fractions containing PK_{III}. These properties of PK_{III} are identical with those reported previously for the SNF1-like enzyme, HRK-A. Our results indicate a considerable complexity of kinase cascades mediating the regulation of assimilatory and biosynthetic pathways in response to environmental stimuli in plants.

INTRODUCTION

In leaves, the cytosolic pathways of nitrate assimilation and sucrose and organic acid synthesis are sometimes termed 'light-coupled' to indicate their intimate links with photosynthetic provision of reducing equivalents and fixed carbon. However, photosynthesis is not simply a provider of precursors but has also been identified as a 'signal' source that promotes the activation/dephosphorylation of nitrate reductase (NR) (nitrate assimilation) and sucrose-phosphate synthase (SPS) (sucrose synthesis), and the activation/phosphorylation of phosphoenolpyruvate carboxylase (PEPC) (organic acid synthesis) in the cytosol. The overall effect would tend to activate sucrose and amino acid synthesis to co-ordinate with activation of photosynthesis. When photosynthetic rates are reduced, in the dark or when CO_2 is depleted, NR, SPS and PEPC are converted into low-activity forms [1–4]. Superimposed on the light/dark and CO_2 effects, many other parameters (circadian rhythms, hormones, water status, nitrogen status, anoxia and build-up of photosynthetic products over the course of the day) modulate the activities and phosphorylation states of these enzymes in leaves [1,4]. In roots and other organs, NR, SPS and PEPC are also regulated by reversible phosphorylation in response to a variety of effectors [1,4].

These findings raise many questions. What are the controlling signals? How are the effects of photosynthesis on the protein kinases (PKs) and protein phosphatases (PPs) mediated? Are any other cytosolic pathways, such as isoprenoid synthesis, also co-ordinately regulated with photosynthesis? How do we account for both parallel control of NR, SPS and PEPC in response to photosynthesis, and opposite control of these enzymes by some other stimuli? For example, high nitrate promotes the activation of NR and PEPC (and hence amino acid biosynthesis), but not SPS [1].

One approach to answering these questions is to identify the regulatory phosphorylation sites on the target enzymes and to characterize the relevant PKs and PPs. Such investigations of NR have revealed that the regulation of this enzyme cannot be explained by a simple phosphorylation/dephosphorylation mechanism. Instead, NR is inactivated by an unusual two-step

process in which NR is phosphorylated on serine-543 [5] which has no direct effect on enzyme activity, but instead allows it to bind to a nitrate reductase inhibitor protein (NIP) [6,7]. It is the interaction of NIP with phosphorylated NR that is responsible for inhibition. Purified NIP has recently been found to be composed of a mixture of 14-3-3 protein isoforms [8,9] that bind directly to the '14-3-3 binding consensus' [R(S/T) XS*XP, where S* is phosphoserine] encompassing the phosphorylated serine-543 [9]. The low-activity complex of phosphorylated NR and NIP-14-3-3s can be reactivated in vitro by dissociation of the two proteins either during purification or by dephosphorylation of NR [7].

These findings focus attention on the nature of the protein kinase(s) that phosphorylate serine-543 on NR. Plants have been shown to contain several homologues of well-characterized mammalian PKs [10] including mitogen-activated protein (MAP) kinase [11], glycogen synthase kinase III [12], rye sucrose non-fermenting (SNF1) PK (RKIN1), which is one of several plant homologues of the SNF1/AMP-activated PKs that are activated by stresses in yeasts and mammals [13,14] and calmodulin-dependent (CaM) kinase II [15]. There are also PKs that are unique to plants, such as the calmodulin domain protein kinase (CDPK) family which comprises a calmodulin domain fused to a PK domain [16]. Interest in cAMP, cGMP and Ca^{2+}/phospholipid signalling in plants has stimulated searches for plant versions of PKA, PKG and PKC but no direct plant homologues of these kinases have been found to date [10]. So far, only two plant PKs have been well characterized by both activity and sequence, namely, 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR) kinase A (HRK-A) [17,18] which has been identified as belonging to RKIN1/SNF1/AMP-activated PK [13,14] sub-family, and the CDPK group, whose candidate targets include the plasma membrane H^+-ATPase and the nodulin-26 protein of legumes [10,16].

In this paper we report the identification, in spinach and Arabidopsis leaf extracts, of three NR kinases that are regulated by reversible protein phosphorylation and/or Ca^{2+} ions, and which are able also to phosphorylate and inactivate plant HMGR. The properties of the NR/HMGR kinases are compared with those of previously identified plant PKs.

MATERIALS AND METHODS

Plants

Spinach seeds (Spinacia oleracea L. cv. Polka F1, a gift from Werner M. Kaiser, University of Würzburg, or var. Medania, from Nuttings and Son Ltd., U.K.) were planted in a mixture of Levington's compost and horticultural vermiculite. Plants were maintained in a growth cabinet (Vindon Scientific Ltd., Oldham, Lancs., U.K.) for ~ 5 weeks on a cycle of 11 h light (~ 350 μmol·m^{-2}·s^{-1}; tungsten and fluorescent lamps) at 22 °C, followed by 13 h dark at 17 °C, or in a greenhouse during winter with supplementary lighting. Plants were irrigated twice daily with a general feed (Chempak Formula 3, which contains ammonium and nitrate). Air humidity was ~ 75%. Leaves were harvested after 2 h in the normal light period. Routinely, leaves were frozen immediately and stored at −80 °C. Arabidopsis (Landsberg erecta) were grown to the leaf rosette phase (but without feed), and were harvested and frozen as for spinach.

Other materials

PP2A [19] was purified from bovine cardiac muscle by Bob MacKintosh (MRC Unit, Dundee, U.K.), and mammalian PP2C

was expressed in Escherichia coli [20] and purified by Nicholas Helps (MRC Unit, Dundee).

The SP synthetic peptide, KGRJRRISSVEJ, is a shortened version of SP2 (HKGRJRRISSVEJ) comprising residues 150–161 flanking the serine-158 phosphorylation site on SPS [21], except that the peptide contains 2-aminohexanoic acid (norleucine), J, in place of methionine in the natural sequence. SP2 has been used previously to identify NR and SPS kinases [21]. The sequence of the synthetic peptide SAMS, HMRSAMSGLHLVKRR, is derived from the major phosphorylation site (Ser-79) on mammalian acetyl-CoA carboxylase [22]. Both SAMS and the AMARA synthetic peptides (Table 2) were used previously as substrates for the mammalian AMP-activated PK [23], and the plant HRK-A [24]. The synthetic peptides starting with KK (Table 2) were used previously as substrates for mammalian MAP kinase-activated protein (MAPKAP) kinases-1, -2 and -3 and CaM kinase II [25,26]. Peptides were synthesized on an Applied Biosystems 431A peptide synthesizer by Barry Caudwell (MRC Unit, Dundee, U.K.).

Partial purification of peptide kinases from spinach leaf extracts

Frozen spinach leaves (~ 100 g) were powdered in a Waring Blender. Then 1.5 vol. of buffer A (50 mM Hepes/NaOH, pH 7.5, 10 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 mM PMSF) was added and the mixture was homogenized for an additional 30 s. Homogenates were centrifuged at 10 000 *g* for 30 min and the supernatant, designated the extract, was filtered through two layers of glass wool. The extract was made 18% (w/v) in poly(ethylene glycol) (PEG)-8000 by addition of a solution of 50% (w/v) PEG-8000 (Sigma) in buffer A and the mixture was incubated with gentle stirring at 4 °C for 30 min, followed by centrifugation at 4500 *g* for 15 min. The pellet was resuspended by gentle Dounce homogenization in a minimal volume of buffer A and loaded on to a 10 ml column of Q-Sepharose equilibrated with buffer A. After washing with buffer A, NR kinase was eluted in buffer A containing 0.3 M NaCl (~ 100 ml) and dialysed against 2 × 2 litres of buffer A. Protein was measured by the Bradford method [27] and extracts containing 20 mg of protein (~ 1/20 of the total pool) were filtered through a 0.22 μm syringe filter, and applied at 1 ml/min to a Mono Q HR5/5 (Pharmacia) anion-exchange column equilibrated with buffer A. The column was washed with buffer A and the absorbance of the eluate was monitored until the A_{280} had returned to baseline. The column was eluted with buffer B (buffer A containing 1 M NaCl) from 0–50 mM NaCl in 1 ml followed by 50 mM–400 mM NaCl over 21 ml. Fractions (0.5 ml) were collected and mixed immediately with 0.5 ml of glycerol and were either assayed immediately or stored at −20 °C until required.

Where it is stated that extractions and chromatography were carried out in the presence of PP inhibitors, the procedure was identical with that described above except that buffer C [50 mM Tris/HCl, pH 7.5 at 4 °C, 50 mM NaF, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 0.1 mM PMSF, 0.02% (v/v) Brij-35 and 10% (v/v) glycerol] was used in place of buffer A. Buffer D (buffer C containing 1 M NaCl) was used in place of buffer B.

Scaled-down procedures were used to chromatograph extracts from Arabidopsis leaves (10 g).

Peptide kinase assays

Peptide kinases were assayed by measuring the incorporation of ^{32}P from [γ-^{32}P]ATP into substrate peptides. Incubations con-

tained 15 μl of PK fraction, 40 μM SP-peptide, AMARA series or KK series peptides, or 200 μM SAMS peptide; 300 μM CaCl$_2$ or 150 μM EGTA in buffer A in a total volume of 45 μl. Reactions were initiated by adding 5 μl of 40 mM MgCl$_2$ and 1 mM [γ-^{32}P]ATP (200–500 c.p.m./pmol). After 15 min at 30 °C, 20 μl aliquots were applied to phosphocellulose papers (2 × 2 cm^2), which were then immersed in 75 mM orthophosphoric acid, washed four times (1 min per wash) in orthophosphoric acid to remove unreacted [γ-^{32}P]ATP and once in acetone, and the radio-activity on each paper was measured by scintillation spectroscopy in the presence of 1 ml of scintillation fluid. Phosphorylation was normally limited to < 0.2 mol of phosphate/mol of substrate to ensure that initial-rate conditions were maintained. In control incubations the substrate was replaced by buffer.

Inactivation of NR

NR [7] and NIP [9] were purified to homogeneity from spinach leaves. NR ($\sim$ 500 nmol/min per ml), NIP-14-3-3s (100 nM) and 15 μl of the NR kinase fraction were assayed by preincubation at 30 °C for 20 min in a total volume of 50 μl of buffer A containing 1 mM ATP. NR activity assays were initiated by the addition of 50 μl of buffer A containing 2 mM KNO$_3$ and 500 μM NADH. After 5 min, the reactions were stopped with 10 μl of 0.5 M zinc acetate. Sulphanilamide [50 μl of 1 % (w/v) in 3 M HCl] and 50 μl of 0.02 % (w/v) N-(1-naphthyl)ethylenediamine dihydro-chloride were added, the mixtures clarified by centrifuging at 15 000 g for 2 min and nitrite was determined by measuring the absorbance at A_{540} [7].

Gel-filtration chromatography

Spinach leaf extracts were prepared and chromatographed on Q-Sepharose, as above. Active fractions ($\sim$ 3 ml) were pooled, concentrated to 0.75 ml in a Centricon-30 concentrator (Amicon), filtered through a 2.2-μm filter, and applied at 1 ml/min to a FPLC Superdex 200 HR16/60 (Pharmacia) gel-filtration column (1.6 cm × 60 cm) equilibrated in buffer A containing 0.2 M NaCl. The column was eluted at 1 ml/min and after the void volume of 43 ml, 1 ml fractions were collected and assayed for SP-peptide kinase activity in the presence of Ca^{2+} or EGTA. The column was calibrated using standards from Bio-Rad; thyroglobulin (670 kDa), γ-globulin (158 kDa), BSA (66 kDa), ovalbumin (44 kDa), myoglobulin (17 kDa) and vitamin B$_{12}$ (13.5 kDa).

Phosphorylation of *Arabidopsis* HMGR1

Arabidopsis HMGR1 expressed in *E. coli* [18,28] and purified was a gift from Albert Ferrer (Universitat de Barcelona, Spain). HMGR1 ($\sim$ 2 μg) was phosphorylated at 30 °C in incubations containing 15 μl of a Mono Q fraction, 1 μM microcystin-LR, 300 μM Ca^{2+} (or 150 μM EGTA) in buffer A, in a total volume of 25 μl. Reactions were initiated by adding 5 μl of 1 mM [γ-^{32}P]ATP (200–500 c.p.m./pmol). Phosphorylation was ter-minated by boiling in SDS sample buffer and the mixture was separated by SDS/PAGE (10 % gels). Gels were stained with Coomassie Blue and then autoradiographed.

CNBr cleavage of ^{32}P-labelled HMGR1 and separation of phosphopeptides

^{32}P-labelled HMGR1 was excised from gels, the pieces were washed in Milli-Q water (5 × 1 ml) for 1 h, brought to near dryness by rotary evaporation and each was ground in 400 μl of 70 % (v/v) formic acid. CNBr ($\sim$ 10 mg) was added and the

mixture was incubated for 16 h at 4 °C, in the dark. The supernatants containing $\sim$ 70 % of the radioactivity were dried to $\sim$ 100 μl, diluted to 1 ml in water, dried and reconstituted in 400 μl of 0.1 % (v/v) trifluoroacetic acid. Samples were chromatographed on a Vydac C18 column equilibrated with 0.1 % (v/v) trifluoroacetic acid, pH 1.9, and developed with a linear water/acetonitrile gradient, increasing by 0.5 % (v/v) acetonitrile per min. Fractions (1 ml) were collected and ^{32}P radioactivity was recorded with an on-line monitor. All maps were identical and a representative phosphopeptide from one sample was subjected to phosphoamino acid sequencing after rechromatography on an RP300 column (Applied Biosystems) run at 0.2 ml/min with a gradient of water/acetonitrile (increasing by 0.5 % acetonitrile/min) in 0.1 % (v/v) trifluoro-acetic acid. The absorbance at A_{214} was recorded on-line and fractions of $\sim$ 100 μl were collected manually and radioactivity was monitored by Čerenkov radiation to identify phospho-peptides. Phosphopeptides ($\sim$ 10 pmol) were sequenced on a 476A protein sequencer (Applied Biosystems). In order to identify phosphorylation site(s), phosphopeptides were covalently attached to a Sequelon arylamine PVDF membrane, subjected to Edman degradation (470A sequencer; Applied Biosystems) and the ^{32}P radioactivity released after each cycle of Edman degra-dation was extracted and counted as described in [5,29].

HMGR1 inactivation assays

HMGR1 (2 μg per assay) was preincubated at 30 °C with 2 mM ATP, 25 μl PK$_I$, PK$_{II}$ or PK$_{III}$ (see the Results section) in the presence of 300 μM Ca^{2+} or in the presence of 150 μM EGTA, in buffer A in a total volume of 50 μl. At timed intervals HMGR1 assays were started by adding 10 μl of the preincubation medium to a cuvette containing 1 ml of 0.3 mM HMGCoA, 0.2 mM NADPH and 4 mM dithiothreitol in 100 mM potassium phos-phate (pH 7.0). Oxidation of NADPH was monitored by fol-lowing the decrease in A_{340}. Controls were performed in the absence of HMGR1 or HMGCoA. One unit of HMGR1 reduces 1 nmol of HMGCoA (2 nmol of NADPH oxidized) per min [18,28]

RESULTS

Resolution of three SP-peptide kinases by anion-exchange chromatography of spinach leaf extracts

SP-peptide is a shorter version (see the Materials and methods section) of the SP2 peptide that was used previously to identify NR and SPS kinases [21]. Both SP2 [21] and SP-peptide (results not shown) block inactivation/phosphorylation of NR in leaf extract. We found three major peaks of SP-peptide kinase activity after chromatography of a spinach leaf extract on a Mono Q anion-exchange column, termed hereafter PK$_I$, PK$_{II}$ and PK$_{III}$ (Figure 1a). The SP-peptide kinase activity of PK$_I$ was Ca^{2+}-dependent, whereas PK$_{II}$ was Ca^{2+}-stimulated (not completely Ca^{2+}-dependent), and PK$_{III}$was Ca^{2+}-independent (Figure 1a). The Ca^{2+}-dependency of PK$_I$ was found to be in the micromolar range in separate experiments where free Ca^{2+} ions were added to solutions containing EGTA (results not shown).

NIP-14-3-3-dependent inactivation of NR by PK$_I$, PK$_{II}$ and PK$_{III}$

PK$_I$, PK$_{II}$, and PK$_{III}$ were each able to phosphorylate and inactivate NR in the presence of Mg-ATP and NIP-14-3-3s (Table 1), and PK$_I$, PK$_{II}$ and PK$_{III}$ each had similar ratios of activities towards SP-peptide and NR (Table 1). Similar to the SP-peptide kinase activities, the NR-inactivating activity of PK$_I$
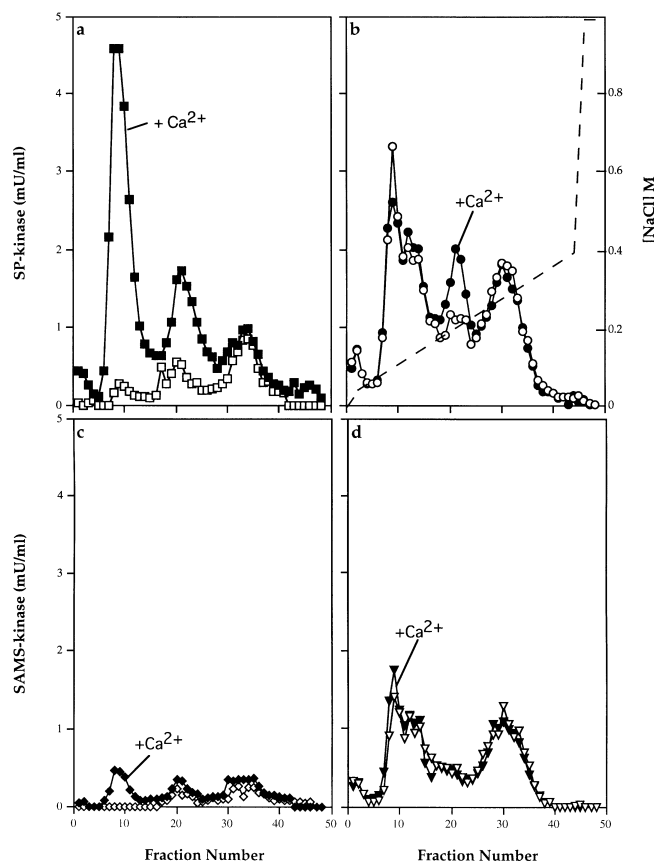
Figure 1   Resolution of three SP/SAMS peptide kinase activities (PK$_I$, PK$_{II}$ and PK$_{III}$) by anion-exchange chromatography of spinach leaf extracts

Extracts were prepared in the absence (**a** and **c**) or presence (**b** and **d**) of EGTA and NaF and subjected to Q-Sepharose and Mono Q anion-exchange chromatography as described in the Materials and methods section. The Mono Q fractions (0.5 ml) were assayed for SP-peptide kinase activity (**a** and **b**) and SAMS-peptide kinase activity (**c** and **d**) in the presence of 300 μM Ca^{2+} ions (closed symbols) or 150 μM EGTA (open symbols). No SP-peptide or SAMS PK activity was detected in the column flow-through. Similar overall patterns of activities were obtained in three other experiments although the relative activities of each peak varied considerably between experiments (see the Results section).

Table 1   Comparison of the NIP-14-3-3-dependent inactivation of NR and inactivation of HMGR1 by PK$_I$, PK$_{II}$ and PK$_{III}$

Assays were carried out in the presence of Ca^{2+} ions or of EGTA as indicated. In Preparation 1 (prepared in the absence of EGTA and NaF, as described in the legend to Figure 1a), fractions containing PK$_I$, PK$_{II}$ or PK$_{III}$ activity were assayed for both SP-peptide kinase activity and NR kinase activity, measured as the ATP- and NIP-14-3-3-dependent inactivation of NR. Each NR kinase incubation contained 15 μl of PK fraction and 1 m-unit of NR in a preincubation volume of 50 μl. In Preparation 2 (which was similar to Preparation 1 but was prepared separately), fractions of PK$_I$, PK$_{II}$ and PK$_{III}$ were assayed for both SP-peptide kinase activity and HMGR1 kinase activity, measured as the ATP-dependent inactivation of HMGR1. Each HMGR1 kinase incubation contained 100 μl of PK fraction and 1.75 units (12.5 μg) of HMGR1 in a preincubation volume of 1 ml.

	PK$_I$		PK$_{II}$		PK$_{III}$	
	Ca^{2+}	EGTA	Ca^{2+}	EGTA	Ca^{2+}	EGTA
Preparation 1						
SP-peptide kinase (m-units/ml)	21.2	0.1	7.6	2.6	25.7	23.2
NR inactivation (%)	70.0	0	17.0	0	85.0	85.0
Preparation 2						
SP-peptide kinase (m-units/ml)	12.3	0.5	4.1	2.1	5.0	5.6
HMGR inactivation (%)	24.0	10.0	60.0	42.0	72.0	75.0

was Ca^{2+}-dependent, whereas PK$_{II}$ was Ca^{2+}-stimulated and PK$_{III}$ was Ca^{2+}-independent (Table 1). The SP-peptide completely blocked inactivation of NR by all three peaks (results not shown). Together, these results suggested that the SP-peptide kinases PK$_I$, PK$_{II}$ and PK$_{III}$ (Figure 1a and Table 1) are identical with the NR-inactivating kinases, PK$_I$, PK$_{II}$ and PK$_{III}$ that are shown in Table 1.

Comparison of the properties of PK$_I$, PK$_{II}$ and PK$_{III}$ prepared in the presence or absence of PP inhibitors

The finding that a major portion of the NR-inactivating kinase activities (i.e. PK$_I$ and PK$_{II}$) was Ca^{2+}-dependent was unexpected, because we had found previously that phosphorylation and inactivation of NR in a crude extract of spinach leaves was unaffected by addition of the Ca^{2+} chelator, EGTA [30]. The NR inactivation experiments were, therefore, repeated at various stages of purification, and variable degrees of Ca^{2+}-dependency of NR inactivation were observed.

One possible explanation for these inconsistencies was that the crude extract contained PK$_I$ and/or PK$_{II}$ in Ca^{2+}-independent

forms that were converted into Ca^{2+}-dependent form(s) during purification by some process, such as protein dephosphorylation. Another possibility was that the major NR kinase in the crude extract was the Ca^{2+}-independent enzyme (PK$_{III}$) but that much of this activity had been lost on purification. In support of both of these possibilities, when the extraction and anion-exchange chromatography were repeated in buffers containing PP inhibitors (NaF and EGTA) several changes in the profile of SP-peptide kinase activities were observed (Figure 1b). PK$_I$ chromato-graphed as a split peak that was Ca^{2+}-independent (Figure 1b), in contrast with the single Ca^{2+}-dependent PK$_I$ in the absence of PP inhibitors (Figure 1a). PK$_{II}$ was largely unchanged, being Ca^{2+}-activated in both preparations (Figures 1a and 1b). PK$_{III}$ was Ca^{2+}-independent in both preparations (Figures 1a and 1b) but PK$_{III}$ activity was generally greater when prepared in the presence of (Figure 1b) than in the absence (Figure 1a) of PP inhibitors.

In some preparations, in the presence of PP inhibitors, PK$_I$ was not completely Ca^{2+}-independent and this peak was some-times split to a greater extent than is shown in Figure 1(b), making it difficult to distinguish PK$_{II}$ from the split sub-peaks of PK$_I$. The relative activities of PK$_I$, PK$_{II}$ and PK$_{III}$ varied considerably from one preparation to another. These variations in the properties of the PKs presumably depended on differences in the enzymes when the leaves were harvested and frozen.

When *Arabidopsis* extracts were chromatographed in the absence and presence of PP inhibitors, the profiles and properties of the three SP-peptide kinases were similar to those seen with spinach (results not shown).

Effects of storage and dephosphorylation on the activity of PK$_I$ and PK$_{III}$

When the properties of PK$_I$ prepared in the absence and in the presence of PP inhibitors were compared (Figures 1a and 1b respectively), protein dephosphorylation during purification was indicated (which can be seen in Figure 1a but not Figure 1b) and this was having at least two effects on PK$_I$, (a) increasing the Ca^{2+}-dependent activity and (b) decreasing the Ca^{2+}-independent activity. This hypothesis was tested by examining the effects of
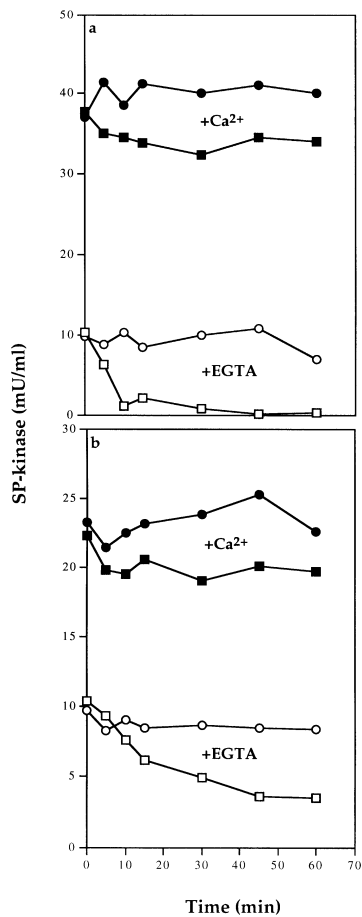
Figure 2 Effects of PP2A and PP2C on PK$_I$ activity

(**a**) PK$_I$ (15 μl of a Mono Q fraction) was preincubated at 30 °C with 20 m-units/ml PP2A in the absence (squares) or presence (circles) of 1 μM microcystin-LR and assayed for SP-peptide kinase activity in the presence of Ca^{2+} (filled symbols) or EGTA (open symbols) at the times indicated. (**b**) PK$_I$ (15 μl of a Mono Q fraction) was preincubated at 30 °C with 10 m-units/ml PP2C in the presence of 10 mM MgCl$_2$, and the presence (circles) or absence (squares) of 50 mM EDTA, and assayed for SP-peptide kinase activity in the presence of Ca^{2+} (filled symbols) or EGTA (open symbols) at the times indicated. The dilution in the SP-peptide kinase assay meant that the concentration of EDTA carried over into the final assay was negligible.

PPs on PK$_I$ activity from the preparation shown in Figure 1(b). We saw no change in the Ca^{2+}-dependent portion of PK$_I$ by incubating the extract with PP1, PP2A, or PP2C (results not shown). However, during the course of these experiments, we found that the Ca^{2+}-dependent SP-peptide kinase activity of PK$_I$ was extensively activated during storage of the fractions. For example, the SP-peptide kinase activity of freshly assayed PK$_I$ fraction in the presence of Ca^{2+} was 2.5 m-units/ml and in the presence of EGTA was 3.2 m-units/ml. After storage for 21 days at -20 °C the activity was 11.1 m-units/ml in the presence of Ca^{2+} but in the presence of EGTA the activity had not changed (2.6 m-units/ml) (and results not shown). In contrast, the Ca^{2+}-independent activity of PK$_I$ was completely inactivated by either PP2A (Figure 2a) or PP2C (Figure 2b). The effect of PP2A was blocked by the PP inhibitor, microcystin-LR (Figure 2a), and the effect of PP2C was blocked by EDTA (PP2C is a Mg2+-dependent enzyme) (Figure 2b).

PK$_{III}$ kinase was completely inactivated by incubation with either PP2A (Figure 3a) or PP2C (Figure 3b). The effect of PP2A was blocked by the inhibitor, microcystin-LR (Figure 3a).
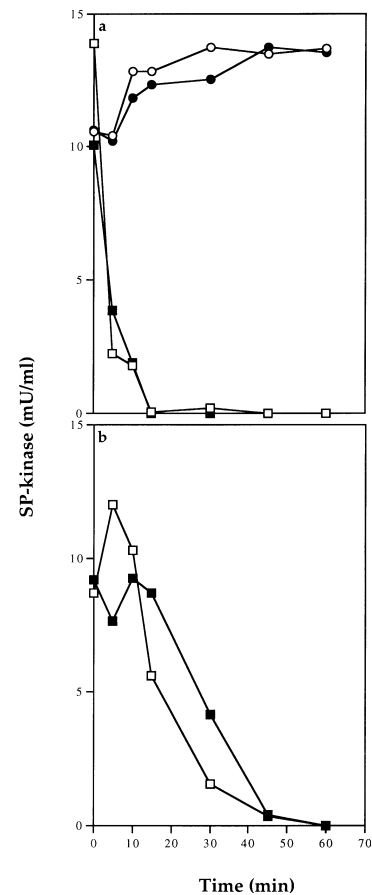

Figure 3 Effects of PP2A and PP2C on PK$_{III}$ activity

(**a**) PK$_{III}$ (15 μl of a Mono Q fraction) was preincubated at 30 °C with 20 m-units(mU)/ml PP2A in the absence (squares) or presence (circles) of 1 μM microcystin-LR, and assayed for SP-peptide kinase activity in the presence of Ca^{2+} (closed symbols) or EGTA (open symbols) at the times indicated. (**b**) PK$_{III}$ (15 μl of a Mono Q fraction) was preincubated at 30 °C with 10 m-units/ml PP2C in the presence of 10 mM MgCl$_2$ and assayed for SP-peptide kinase activity in the presence of Ca^{2+} (closed symbols) or EGTA (open symbols) at the times indicated.

Phosphorylation of the SAMS peptide by PK$_I$, PK$_{II}$ and PK$_{III}$

The inactivation of PK$_{III}$ by PP2A and PP2C (Figures 3a and 3b) is similar to that of the PK, HRK-A, found in higher plants which is also dephosphorylated and inactivated by PP2A and PP2C [31]. HRK-A phosphorylates a synthetic peptide (HMRSAMSGLHLVKRR), the SAMS peptide, and also inactivates HMGR by phosphorylating this protein on serine-577 (in the *Arabidopsis* HMGR1 isoform) [18]. We therefore tested SAMS peptide and HMGR1 as substrates for SP-peptide/NR kinases PK$_I$, PK$_{II}$ and PK$_{III}$ from spinach leaf extracts.

Figures 1(c) and 1(d) show that the profiles of SAMS-peptide kinase activity after anion-exchange chromatography of spinach leaf extracts closely mirrored the SP-peptide kinase activity profiles. However, the SAMS peptide kinase activities were generally lower than the SP-peptide kinase activities.

Phosphorylation of HMGR1 by PK$_I$, PK$_{II}$ and PK$_{III}$

Bacterially expressed *Arabidopsis* HMGR1 was phosphorylated using [γ-^{32}P]ATP and the anion-exchange column fractions from
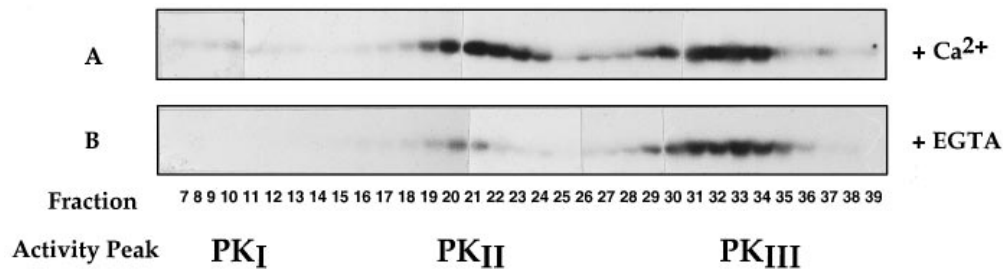
Figure 4   Autoradiographs of HMGR1 after phosphorylation with PK$_I$, PK$_{II}$ and PK$_{III}$

HMGR1 was phosphorylated in the presence of $[\gamma\text{-}^{32}P]$ATP, run on SDS/PAGE (10% gel), stained with Coomassie Brilliant Blue and autoradiographed. Reactions were carried out (**A**) in the presence of 300 μM Ca^{2+} or (**B**) in the presence of 150 μM EGTA.

the preparation shown in Figure 1(a). HMGR1 was found to be phosphorylated (Figure 4) and inactivated (Table 1) by PK$_I$, PK$_{II}$ and PK$_{III}$. The Ca^{2+}-dependencies for phosphorylation of HMGR1 (Figure 4) or SP-peptide (Figure 1a) were identical. However, PK$_{II}$ and PK$_{III}$ inactivated HMGR1 faster than did PK$_I$, relative to their SP-peptide kinase activities (Table 1). The inactivation of HMGR1 was reversed by PP2A (results not shown).

HMGR1 was phosphorylated using $[\gamma\text{-}^{32}P]$ATP by the peak fractions of PK$_I$, PK$_{II}$, PK$_{III}$ or cauliflower HRK-A, and cleaved with CNBr. The CNBr phosphopeptide maps for each sample were identical, with two major phosphopeptides eluting at 6.8% and 7.4% acetonitrile respectively, on C18 HPLC chromatography. The two phosphopeptides derived from one sample were sequenced and taken as representative. The sequence of each phosphopeptide was KYNRSSRDISGATTT, which is identical with residues 573–587 of HMGR1. Solid-phase sequencing showed that each peptide was phosphorylated on the fifth cycle of Edman degradation, which corresponds to serine-577 in the sequence of HMGR1, the same residue phosphorylated by HRK-A identified by Dale et al. [18].

Detection of PK$_{III}$ using an antibody raised against the SNF1-like RKIN1 from rye

It has been shown previously that antibodies raised against the RKIN1 gene product from rye recognize the 58 kDa subunit of HRK-A purified from cauliflower [32]. Similarly, Figure 5 demonstrates that an antibody raised against a bacterially expressed fusion of RKIN1 with maltose-binding protein recognized a 58 kDa protein in Western blots of Mono Q fractions containing PK$_{III}$. Interestingly, the 58 kDa protein detected by the antibody did not exactly co-migrate with PK$_{III}$ activity. The strongest antibody signal was seen in fractions immediately after the peak fraction of PK$_{III}$ activity (fraction 35; Figure 5), which may indicate that the inactive (dephosphorylated) form of PK$_{III}$ is eluted from the column later that the active form. The anti-maltose binding protein–RKIN1 antibody did not recognize any proteins in fractions containing PK$_I$ or PK$_{II}$ (results not shown).

Gel filtration

Superdex 200 gel filtration of spinach leaf extracts revealed four peaks of SP-peptide kinase activity, which were eluted at 59, 64, 69 and 73 ml respectively and corresponded to molecular masses of 140 kDa, 93 kDa, 63 kDa and 46 kDa (results not shown). In order to identify which of these molecular masses corresponded
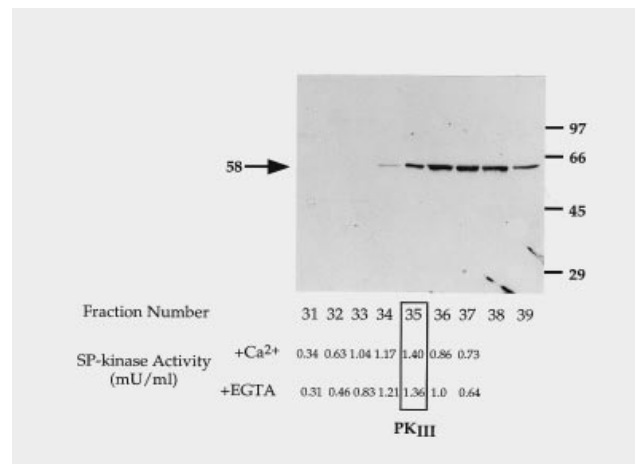

Figure 5   Western blots of fractions with antisera raised against RKIN1 after Mono Q chromatography

Samples (20 μl) of fractions from a Mono Q profile similar to that shown in Figure 1(**a**) were subjected to SDS/PAGE (10% gels), transferred to nitrocellulose membranes and blotted with antisera raised against a full-length, expressed RKIN1 fusion protein [32] at a dilution of 1:250. Blots were visualized by enhanced chemiluminescence (Amersham International). The positions of molecular-mass markers, phosphorylase (97 kDa), BSA (66 kDa), ovalbumin (45 kDa) and carbonic anhydrase (29 kDa) are shown on the right. The SP-peptide kinase activity contained in fractions from the PK$_{III}$ peak is shown below the fraction number. The box indicates the fraction containing the peak of PK$_{III}$ activity.

to PK$_I$, PK$_{II}$ and PK$_{III}$, each peak from the Superdex 200 was individually chromatographed on Mono Q anion-exchange chromatography. This analysis showed that the 46 kDa protein represented PK$_I$ kinase activity and the 140 kDa protein contained PK$_{III}$ activity. The proteins with molecular masses of 63 kDa and 93 kDa were either PK$_{II}$ or a sub-peak of PK$_I$ but could not be assigned definitively.

Comparison of the substrate specificity of PK$_I$ and PK$_{III}$

The substrate specificities of PK$_I$ and PK$_{III}$ were distinct. PK$_I$ was active towards a series of peptides that began with KK- and ended at the +2 residue from the phosphorylation site, whereas PK$_{III}$ was inactive towards these substrates (Table 2). This KK-series of peptides has been used previously to identify the substrate specificity of mammalian MAPKAP kinases-1, -2 and -3, and CaM kinase II [25,26]. However, PK$_I$ specificity does not

Table 2 Substrate specificity of PK$_I$ and PK$_{III}$ using synthetic peptide substrates

The following three peptides show the sequences flanking the residues that have been shown to be phosphorylated after ^{32}P-labelling of the intact proteins.

NR protein	GPTLKRTA<u>S</u>TPFMNTTSK [5]
HMGR1 protein	HMKYNR<u>S</u>SRDISGA [18]
SPS protein	KGRMRRIS<u>S</u>VGMMDNWAN [21]

Peptide kinase activities in fractions prepared in the absence of protein phosphatase inhibitors (as described in the legend to Figure 1a) are expressed as a percentage relative to the activity towards the SP-peptide (100%) in the presence of Ca^{2+}. The activities of PK$_I$ against all substrates tested were Ca^{2+}-dependent. The underlined residues are the phosphorylated residues. The residues indicated by bold characters are those which differ from the parent peptide. For peptides marked with an asterisk the phosphorylated residue was identified by solid-phase sequencing in the present study (results not shown).

| | | Peptide kinase activity (%) | |
Peptide	Sequence	PK$_I$	PK$_{III}$
1 *SP	KGRJRRIS<u>S</u>VEJ	100	100
2 SAMS	HMRSAMS<u>S</u>GLHLVKRR	6	11
3 AMARA series	AMARAA<u>S</u>AAA**L**ARRR	2	32
4	AMARAA<u>S</u>AAA**I**ARRR	6	61
5	AMARAA<u>S</u>AAA**F**ARRR	14	44
6	AMARAA<u>S</u>AAA**M**ARRR	6	38
7	AMARAA<u>S</u>AAA**V**ARRR	5	12
8	AMARAA<u>S</u>AAA**G**ARRR	5	< 1
9 *KK series	KKLNRTL<u>**T**</u>VA	73	< 1
10	KKLNRTL<u>S</u>**E**A	86	10
11	KKLNRTL<u>S</u>**A**A	83	15
12	KKLN**K**TL<u>S</u>VA	78	5
13	KKLNRTL<u>S</u>**P**A	42	1
14	KK**K**NRTL<u>S</u>VA	26	1
15	KK**E**NRTL<u>S</u>VA	12	< 1
16	KKLNRTL<u>S</u>**K**A	< 1	< 1

match that of any of the mammalian kinases. For example, MAPKAP kinases-1 and -2 did not phosphorylate threonine [25], whereas PK$_I$ did. PK$_I$ could tolerate a glutamic acid or proline residue in the +1 position, whereas CaM kinase II could not phosphorylate these substrates [25].

Compared with PK$_I$, PK$_{III}$ had high activity towards the AMARA peptide, commonly used as a substrate for the SNF1 PK subfamily [23,24], which includes HRK-A [17]. Similar to the SNF1 PKs, a hydrophobic residue located four residues to the C-terminal of the phosphorylation site (position +4) was critical for phosphorylation of the AMARA peptides by PK$_{III}$. Changing the +4 residue to a glycine virtually abolished activity, and PK$_{III}$ had no activity towards peptides that ended at the +2 residue (Table 2). We were surprised, therefore, that PK$_{III}$, HRK-A and the mammalian AMP-activated PK (Steve Davies, personal communication) were each able to phosphorylate residue 9, which corresponds to the second serine in the SP-peptide even though this site has no +4 residue. It is possible that the long side-chain of the 2-aminohexanoic acid (norleucine) residue in the +3 position of SP-peptide is flexible enough to compensate for lack of the +4 residue.

NR was phosphorylated and inactivated by HRK-A purified from cauliflower (results not shown). Similarly to their substrate specificities, the NR inactivating activity of PK$_I$ was blocked by both a KK peptide (No. 11 in Table 2) and the AMARA peptide (No. 3 in Table 2), whereas NR inactivation by HRK-A was blocked by the AMARA peptide, but not by the KK peptides (results not shown).

DISCUSSION

Regulation of three NR kinases by Ca^{2+} ions and reversible phosphorylation

The results in this paper suggest that the control of the phosphorylation events that lead to the inactivation of NR (nitrate assimilation), HMGR (isoprenoid synthesis) and SPS (sucrose synthesis) are likely to be more complex than has hitherto been realized.

Recently three PKs (termed I, II and III) were identified by McMichael et al. [21] and Bachmann et al. [33,34] after anion-exchange chromatography of spinach leaf extracts. PKI and PKII phosphorylated and inactivated NR (in the presence of NIP), whereas PKI and PKIII phosphorylated and inactivated SPS. Following a similar procedure and using a synthetic peptide as substrate, we also found three PKs (termed PK$_I$, PK$_{II}$ and PK$_{III}$) which we presume are the same enzymes as those detected by McMichael et al. [21] and Bachmann et al. [33,34]. However, in contrast with these authors we found that all three enzymes were able to phosphorylate and inactivate NR (in the presence of NIP) (Table 1).

PKs I and II were reported previously [21,33,34] to be strictly Ca^{2+}-dependent, whereas peak III was Ca^{2+}-independent. We also found that PK$_I$ was Ca^{2+}-dependent, whereas PK$_{II}$ was Ca^{2+}-stimulated (not absolutely Ca^{2+}-dependent) and PK$_{III}$ was Ca^{2+}-independent, provided that the experiments were carried out in the absence of PP inhibitors. However, we found that when extracts were prepared and chromatographed in the presence of general PP inhibitors (NaF and EGTA) a different profile was revealed (compare Figures 1a and 1b) demonstrating a intricate regulation of these enzymes.

Glucose 6-phosphate was implicated previously as a selective regulatory inhibitor of NR/SPS kinases [35]. However, we found no inhibition of NR kinases by glucose 6-phosphate at concentrations up to 10 mM (results not shown).

A speculative model for the regulation of PK$_I$

The most noticeable effects of purifying PK$_I$ in the presence of PP inhibitors was that this activity separated into two or more sub-peaks and was completely Ca^{2+}-independent. These effects, together with the finding that PPs inactivated the Ca^{2+}-independent activity of PK$_I$, and that the Ca^{2+}-dependent activity of PK$_I$ increased on storage are consistent with the model shown in Figure 6. In this model we propose that PK$_I$ is a single enzyme (although we emphasize that this point is not proved) which can be regulated by covalent modification at two distinct sites. Phosphorylation at site 1 is essential for activity in the absence of Ca^{2+} ions. Site 1 can be dephosphorylated and inactivated by PP2A or PP2C (Figure 6). Phosphorylation (or some other modification that is stabilized by NaF and EGTA) at site 2 renders the enzyme insensitive to activation by Ca^{2+} ions (Figure 6). We failed to find a PP that acted on site 2, thereby increasing Ca^{2+} sensitivity of PK$_I$. However, PK$_I$ did become progressively more sensitive to activation by Ca^{2+} ions during storage, presumably due to removal of site 2 perhaps by proteolysis. These findings explain why NR kinase activity in a rapidly prepared leaf extract was found to be Ca^{2+}-independent previously [30], although variability in the Ca^{2+}-sensitivity of PK$_I$, even in the presence of PP inhibitors, was noted which presumably reflects its activity state in the leaves at the time of harvesting.

The role of reversible protein phosphorylation in determining the Ca^{2+}-dependency of PK$_I$ is particularly intriguing; perhaps there are physiological circumstances where it would be im-
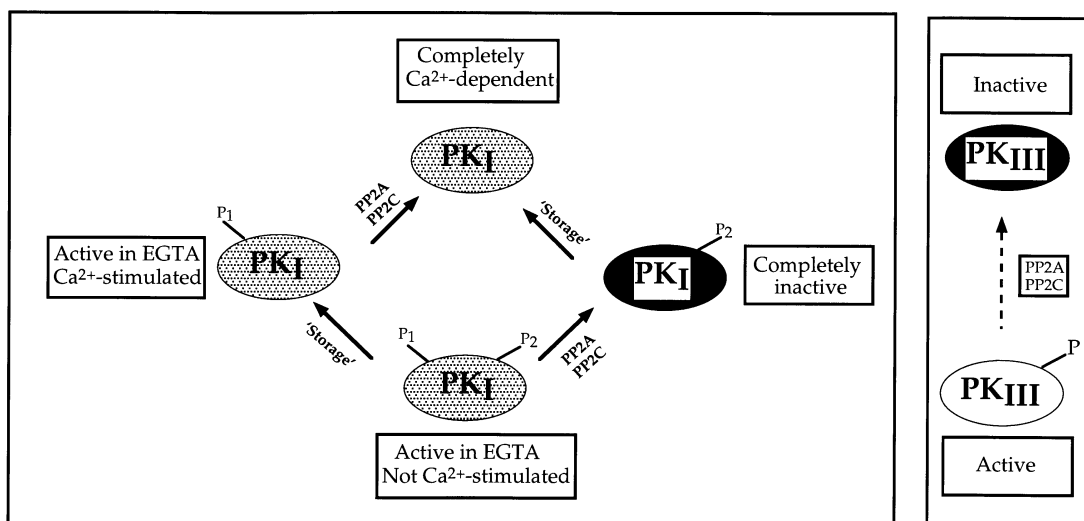
Figure 6   A model of the regulatory properties of PK$_I$ and PK$_{III}$

The scheme indicates enzyme of high activity (white), intermediate activity (hatched) and no activity (black). The model for PK$_I$ assumes that Ca^{2+}-dependent and -independent activities of PK$_I$ are catalysed by the same enzyme, although whether there are one or two co-migrating enzymes is not certain. PK$_I$ is proposed to be regulated by covalent modification at two distinct sites. Phosphorylation at site 1 (P1) is essential for activity in the absence of Ca^{2+} ions. Site 1 can be dephosphorylated and inactivated by PP2A or PP2C. Phosphorylation (or some other modification that is stabilized by NaF and EGTA) at site 2 (P2) renders the enzyme insensitive to activation by Ca^{2+} ions. We were unable to identify a PP that acted on site 2, thereby increasing Ca^{2+} sensitivity of PK$_I$. However, PK$_I$ did become progressively more sensitive to activation by Ca^{2+} ions during storage, presumably due to removal of site 2, perhaps by proteolysis. PK$_{III}$ is inactivated by dephosphorylation by PP2A or PP2C.

portant for this enzyme to be insensitive to activation by Ca^{2+} ions, even if Ca^{2+} is present in the cell cytoplasm.

Identity of PK$_{II}$

The activity of PK$_{II}$ was stimulated by Ca^{2+} ions, but was not totally Ca^{2+}-dependent. It is likely that the PK$_{II}$ here is the same as the peak II kinase that was shown recently [34] to be immunoprecipitated by a polyclonal antibody raised against the calmodulin-like domain of a soybean CDPK, a class of PKs that are unique to plants and protozoa, and which carry a calmodulin-domain and a PK domain on the same polypeptide [10,16]. However, we did find it difficult to distinguish PK$_{II}$ from sub-peaks of PK$_I$.

Similarity in properties of PK$_{III}$ and HRK-A

The activity of PK$_{III}$ was abolished by PP2A or PP2C (Figures 3a and 3b) demonstrating that this enzyme is activated by phosphorylation. These distinctive regulatory properties, together with the phosphorylation and inactivation of HMGR1 by PK$_{III}$ (Figure 4 and Table 1), the apparent molecular mass of 140 kDa and the requirement for a hydrophobic residue in the +4 position from the phosphorylation site of substrates (Table 2), suggest strongly that the PK$_{III}$ kinase described here is likely to be a homologue of the plant PK, HRK-A. Furthermore, NR was inactivated by HRK-A purified from cauliflower, and the inactivation was specifically blocked by competition with the AMARA peptide, which is a substrate for both PK$_{III}$ and HRK-A (results not shown). HRK-A has not yet been sequenced but is thought to be a homologue of the rye RKIN-1, the mammalian AMP-activated PK and SNF1 from *Saccharomyces cerevisiae* [10,13,17,23,24]. Unfortunately, no immunoprecipitating antibodies are available for these enzymes. However, we did find that a protein of 58 kDa was recognized by an antibody raised against

RKIN1 [32]. This molecular mass is similar to the 65 kDa PK detected by McMichael et al. [21] using a recombinant fragment of SPS as a substrate for in-gel assays of fractions of their kinase III, and is identical with the 58 kDa subunit of HRK-A [32]. It is very likely, therefore, that PK$_{III}$ belongs to the RKIN1/SNF1/AMP-activated PK family [13,14].

SNF1 is required for the response to glucose starvation in yeast, and activation of the AMP-activated PK in response to environmental and nutritional stresses in mammalian cells leads to inactivation of several biosynthetic enzymes [13]. Similarly, the PK$_{III}$ activity identified by the present work inactivates several biosynthetic and assimilatory enzymes in plants, namely NR, SPS and HMGR1. This poses the question of which stimuli activate PK$_{III}$ *in vivo*. Potentially, the activity of PK$_{III}$ could be affected by changes in either the kinase kinase which phosphorylates and activates this enzyme and/or the relevant inactivating PP. In this connection, it is interesting to note that a plant PP2C has recently been identified as the *Arabidopsis* ABI1 gene product [36,37]. ABI1 mutant plants are insensitive to abscisic acid (a hormone associated with responses to water stress) and have a wilted phenotype. It would be very interesting to know whether the ABI1 PP2C can dephosphorylate and inactivate kinases PK$_I$ and/or PK$_{III}$ *in vivo*.

Phosphorylation of HMGR1

We found that all three SP-peptide kinases phosphorylated and inactivated bacterially expressed *Arabidopsis* HMGR1 but with different relative activities compared with their activities towards SP-peptide and NR (Table 1). In agreement with a previous study, we found that HRK-A from cauliflower phosphorylated HMGR1 on serine-577. The spinach kinases PK$_I$, PK$_{II}$ and PK$_{III}$ gave CNBr HMGR1 phosphopeptide maps identical with those of HRK-A, suggesting that these enzymes also phosphorylated serine-577.

In plants, HMGR catalyses the synthesis of mevalonate, which is used in the synthesis of a huge array of isoprenoids including photosynthetic pigments and growth regulators, induced isoprenoids for plant defences and specialized compounds such as fragrant oils and rubber polymers. Manipulation of the activity of HMGR in transgenic plants has clearly demonstrated that HMGR activity is rate-limiting for the total flux of carbon into certain isoprenoids, while later enzymes are rate-limiting for some branches of the pathway [38]. Finding that three NR/SPS kinases can also act on HMGR raises many questions about global co-ordination of nitrate assimilation, sucrose and isoprenoid metabolism. There have been no studies on how these pathways interact but from a regulatory viewpoint, NR, SPS and HMGR do have several factors in common. The activities of all three enzymes are influenced by many environmental and metabolic signals [1–4,38,39]. For example, in leaves, the mRNAs for NR, SPS and HMGR are all induced by light [38,39]. All three enzymes are cytosolic but their activities presumably need to be co-ordinated with the other steps in their pathways that are located in different compartments. Furthermore, nitrate assimilation into amino acids and sucrose and isoprenoid synthesis all require carbon and energy from photosynthesis.

In order to understand how the NR/SPS/HMGR PKs, PK_I, PK_{II} and PK_{III}, link environmental stimuli with metabolic changes it is clearly crucial to know which of these kinases is regulating which substrate under which physiological condition.

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