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Biochemical characterization of two forms of 3-hydroxy-3-methylglutaryl-CoA reductase kinase from cauliflower (Brassica oleracia)

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We recently reported the existence of a protein kinase cascade in higher plants, of which the central component is a 3-hydroxy-3-methylglutaryl (HMG-)CoA reductase kinase functionally related to mammalian AMP-activated protein kinase [MacKintosh, R. W., Davies, S. P., Clarke P. R., Weekes, J., Gillespie, S. G., Gibb, B. J. & Hardie, D. G. (1992) Eur. J. Biochem. 209, 923–931]. We have now purified this protein kinase 9000-fold from cauliflower inflorescences. During the course of this work we noticed a second minor form (form B) which separated from the major form (A) on ion exchange and gel filtration. Both forms phosphorylate the catalytic fragment of mammalian HMG-CoA reductase. Both forms are markedly inactivated by incubation with the reactive ATP analogue p-fluorosulphonylbenzoyl adenosine (FSO,PhCOAdo), and also by mammalian protein phosphatase 2C, indicating that form B, like form A, is activated by phosphorylation. Form A has an apparent native molecular mass of 200 kDa by gel filtration and, after labelling with [14C]FSO,PhCOAdo, of 150 kDa by electrophoresis in non-denaturing gels. The catalytic subunit was identified as a polypeptide of 58 kDa after labelling with [14C]FSO,PhCOAdo and [γ-32P]ATP. Form B has an apparent native molecular mass of 45 kDa by gel filtration, and was identified as a polypeptide of 45 kDa after labelling with [14C]FSO,PhCOAdo and [γ-32P]ATP. Using a series of variants of the synthetic peptide substrate, the substrate specificities of the two forms are similar but not identical. Form B does not appear to be a proteolytic fragment of form A, and we therefore propose that it represents a closely related member of the same protein kinase sub-family.

It is now clear that reversible protein phosphorylation is the major mechanism for the regulation of protein function in animal cells, its functions being particularly to mediate the response to extracellular signals such as hormones and cytokines, and to control events which occur discontinuously in the cell cycle such as DNA replication and mitosis. A third function which is just emerging may be to mediate the response of cells to environmental stresses such as heat shock or hypoxia [1]. At least in the case of cell-cycle control, the protein kinases involved appear to be highly conserved between plants and animals [3, 4]. Plant cells also respond to a variety of external stimuli such as hormones, light and environmental stress. The systems which mediate these responses inside the plant cell remain largely undefined, although by analogy with animal systems it appears that protein kinases and phosphatases will play an important role. A small number of enzymes have been demonstrated to be regulated by phosphorylation in higher plants, e.g. sucrose phosphate synthase [5, 6], quinate dehydrogenase [7] and phosphoenolpyruvate carboxylase [8, 9]. A number of higher plant protein kinases [10–13] and protein phosphatases [14] have also been defined by cDNA cloning.

Recently we obtained the first direct biochemical evidence for a protein kinase cascade in higher plants [15]. The protein kinase which is the central component of this cascade appears by functional criteria to be a homologue of the AMP-activated protein kinase, an activity which in mammalian cells is a component of the response to cellular stress [1] such as heat shock and hypoxia (unpublished results). Both the animal and plant kinases inactivate mammalian acetyl-CoA carboxylase and 3-hydroxy-3-methylglutaryl(HMG)-CoA reductase, apparently by phosphorylation at the same sites. The system also appears to be highly conserved in that, like the mammalian kinase, the plant kinase is inactivated by mammalian protein phosphatases and reactivated by mammalian kinase. The one difference found to date is that the plant protein kinase is not activated by AMP. Since the plant kinase inactivates HMG-CoA reductase from potato tubers, and the phosphorylation site defined on mammalian HMG-CoA reductase is conserved in a number of plant HMG-CoA reductases, we have provisionally named the plant kinase HMG-CoA reductase kinase [15].

In this study we report the further purification and characterization of the HMG-CoA reductase kinase protein from cauliflower inflorescences. During the course of this work we identified a second HMG-CoA reductase kinase activity which appears to represent a distinct gene product.
MATERIALS AND METHODS

Materials

Cauliflower (Brassica oleracea var. Botrytis) was obtained from a local supermarket. All column materials were obtained from Pharmacia, except for phosphocellulose P11, which was from Whatman Biosystems Ltd, and the C₁₈ reverse-phase column which was from Vydac, USA. Radiochemicals were from Amersham International, except for [³⁵S]Fluorosulphonylbenzoyl adenosine (FSO₂PhCoAδ) which was from New England Nuclear. The catalytic subunit of PP2C was purified from rabbit skeletal muscle [16]. Peptides were synthesised by Cambridge Research Biochemicals (Northwich, UK) using polyamide chemistry on a solid-phase pepsyn KB resin. All other reagents were of the highest commercial grade available.

Enzyme assay

The synthetic peptide HMRSAAMSGHLVKRR, whose sequence is derived from the major phosphorylation site (Ser79) on acetyl-CoA carboxylase, was routinely used as the substrate to assay kinase activity. Kinase activities are expressed as nmol phosphate incorporated into peptide/min at 30°C in the standard assay [17].

Purification of two kinase activities from cauliflower

The purification procedure used previously [15] to partially purify a single kinase activity from cauliflower, has been extensively modified. Extracts were prepared in a Waring blender by homogenizing 1.5 kg cauliflower inflorescence plus 25 g polyvinylpyrrolidone with 1.5 l homogenization buffer (50 mM Tris/HCl, pH 8.2, 0.25 M mannitol, 50 mM NaF, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 1 mM benzamidine, 0.1 mM phenylmethane sulphonyl fluoride). This and all subsequent procedures were carried out at 4°C. Following the addition of Triton X-100 to give a final concentration of 0.5% (by vol.), the homogenate was centrifuged at 18000×g for 30 min. The supernatant was removed and the pellets were re-extracted in an equal volume of homogenization buffer and centrifuged as above. The combined supernatants were passed through two layers of cheese cloth and one layer of mirror cloth, before ammonium sulphate was added to give a 35% saturated solution. The suspension was stirred for 20 min and the precipitate collected by centrifugation at 24000×g for 20 min. The precipitate was redissolved in approximately 300 ml of 50 mM Tris/HCl, pH 8.0, containing 50 mM NaF, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 1 mM benzamidine, 0.1 mM phenylmethane sulphonyl fluoride. This and all subsequent procedures were carried out at 4°C. Following the addition of Triton X-100 to give a final concentration of 0.5% (by vol.), the homogenate was centrifuged at 18000×g for 30 min. The supernatant was removed and the pellets were re-extracted in an equal volume of homogenization buffer and centrifuged as above. The combined supernatants were passed through two layers of cheese cloth and one layer of mirror cloth, before ammonium sulphate was added to give a 35% saturated solution. The suspension was stirred for 20 min and the precipitate collected by centrifugation at 24000×g for 20 min. The precipitate was redissolved in approximately 300 ml of 50 mM Tris/HCl, pH 8.0, containing 50 mM NaF, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 1 mM benzamidine, 0.1 mM phenylmethane sulphonyl fluoride, 0.02% (by vol.) Brij and 10% (by vol.) glycerol, and applied to a Mono Q HR 5/5 column (1 ml/min) equilibrated in buffer D. The kinase was eluted with a gradient (Fig. 2) from 0 to 100 mM NaCl in buffer D. Total amounts of each enzyme were calculated from the areas under the peaks on the traces at 320 nm. Peak A was concentrated to 250 µl using a Centricon-30 concentrator (Amicon Corp.), dialysed against 2×500 ml buffer D [50 mM sodium Hepes, pH 7.4, containing 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 1 mM benzamidine, 0.1 mM phenylmethane sulphonyl fluoride, 0.02% (by vol.) Brij and 10% (by vol.) glycerol] and applied to a Mono Q HR 5/5 column (1 ml/min) equilibrated in buffer D. The kinase was eluted with a gradient (Fig. 2) from 0 to 100 mM MgCl₂ in buffer D. Active fractions were pooled, concentrated to 0.5 mg/ml protein, frozen in liquid N₂ and stored at -70°C with no loss of activity.

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Purification of two kinase activities from cauliflower

Partial purification of mammalian AMP-activated protein kinase

Mammalian AMP-activated protein kinase was purified from rat liver as described previously [18].

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Radioactive labelling with [³⁵S]FSO₂PhCoAδ and [γ⁻³²P]ATP

Peaks A, B or mammalian AMP-activated protein kinase (4 U peak A; 8 U peak B; 2 U AMP-activated protein kinase) were incubated with 25 mM sodium Hepes, pH 7.0 containing 169 mM [³⁵S]FSO₂PhCoAδ (53.2 Ci/mmol), for 60 min at 30°C. Reactions which were to be analysed by
Table 1. Purification of HMG-CoA reductase kinases A and B from cauliflower florets. Data refer to a purification from four cauliflower plants (1.6 kg inflorescence).

<table>
<thead>
<tr>
<th>Sample source</th>
<th>Protein Activity</th>
<th>Specific Activity</th>
<th>Yield (%)</th>
<th>Purification</th>
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<tbody>
<tr>
<td></td>
<td>mg</td>
<td>U</td>
<td>U/mg</td>
<td>-fold</td>
</tr>
<tr>
<td>Crude extract</td>
<td>5997</td>
<td>8813</td>
<td>1.47</td>
<td>100</td>
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<tr>
<td>Ammonium sulphate precipitate</td>
<td>1578</td>
<td>8150</td>
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<td>92</td>
</tr>
<tr>
<td>DEAE-Sepharose</td>
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<td>6813</td>
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<td>Blue-Sepharose</td>
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<td>5101</td>
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<td>Mono Q</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>3.0</td>
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<td>3717</td>
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<td>B</td>
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<td>353.3</td>
<td>33.9</td>
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<td></td>
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<td>600</td>
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<td>3325</td>
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<td>221</td>
<td>3281</td>
<td>2.5</td>
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<tr>
<td>Mono Q (MgCl₂ gradient)</td>
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<td></td>
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<td></td>
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<tr>
<td>A</td>
<td>0.07</td>
<td>927</td>
<td>13102</td>
<td>10.5</td>
</tr>
</tbody>
</table>

* Purification factors are underestimates because they are given relative to the specific activity in the crude extract, which will be a mixture of kinases A and B.

SDS/PAGE were stopped by adding an equal volume of SDS sample buffer and boiling for 2 min. Samples were run on a 10% polyacrylamide gel as described by Laemmli [19] then transferred to nitrocellulose. Reactions which were analysed by non-denaturing gel electrophoresis were made up to 20% (mass/vol.) glycerol then immediately loaded and separated in a 4% polyacrylamide gel in 30 mM Tris/borate, pH 8.0, 0.67 mM EDTA, 0.1% Triton X-100. For autophosphorylation, 4 U peaks A or B were incubated for 30 min at 30°C in 50 mM Na Hepes, 10% (mass/vol.) glycerol, 1 mM dithiothreitol, 1 mM benzamidine in the presence of [γ-32P]ATP (300 μM; 2 × 10⁶ dpm/μmol) and 25 mM MgCl₂. Reactions were stopped by the addition of SDS sample buffer and boiling for 3 min, and separated by electrophoresis in 10% polyacrylamide gels in the presence of SDS.

All labelled polypeptides were detected, either on nitrocellulose or dried gels, using a Molecular Dynamics Phorimager.

Purification of synthetic peptides

Synthetic peptides were made up to 2 ml with de-ionized water containing 0.1% (by vol.) trifluoroacetic acid. An aliquot (1 ml) of each peptide was applied to a Vydac C₁₈ reverse-phase HPLC column equilibrated in 0.1% trifluoroacetic acid and run at 1 ml/min. The peptides were eluted using a linear gradient from 0–15% in 0.1% trifluoroacetic acid over 5 min, then 15–35% acetonitrile in 0.1% trifluoroacetic acid over 15 min. Fractions which contained the major A₃₅ peptide from each run were pooled, dried down to approximately 50 μl to remove trifluoroacetic acid and acetonitrile, and made up to 200 ml with deionized water. The structures of all peptides were verified by fast-atom-bombardment mass spectrometry on a VG Analytical Model 70-250SE, and concentrations were determined by amino acid analysis using a Millipore-Waters PICO-TAG system.

RESULTS

Purification of two plant peptide kinases

The procedure reported previously for partial purification of HMG-CoA reductase kinase from cauliflower [15] was based on our method for purification of mammalian AMP-activated protein kinase [18]. This procedure has now been extensively modified in order to achieve further purification of the enzyme (Table 1). Poly(ethylene glycol) precipitation was omitted and replaced with a modified ammonium sulphate fractionation, which enhanced the yield while still achieving a 3.5-fold purification. The buffer and fractionation conditions were optimized at each stage and two additional purification steps (phosphocellulose chromatography and Mono-Q chromatography with a Mg²⁺ gradient) were added.

When the ammonium sulphate fraction was applied to a DEAE-Sepharose column, and eluted with a gradient of NaCl, two discrete peaks of kinase activity were detected (data not shown). The major peak (A), elutes at 0.20–0.25 M NaCl while a minor peak (B) elutes at 0.15–0.20 M NaCl. Peak B was not reported by MacKintosh et al. [15] but, using the improved purification procedure in this study, we consistently find that 10–20% of total activity is present as peak B. To cut down on the number of columns that had to be run, peaks A and B were combined at this stage, and applied to a Blue-Sepharose column. Although two activities could be discerned if the dye column was eluted with a salt gradient, they were not completely resolved, and the column was therefore eluted with a single 0.5 M NaCl step. The two activities were clearly separated on a Mono Q column (Fig. 1A) with peak A eluting as a broad peak between 100–250 mM NaCl and peak B eluting as a sharp peak at 50–100 mM NaCl. At this stage the two activities were pooled separately for further purification. Phosphocellulose chromatography (data not shown) removed several major contaminants which were difficult to resolve from peak A using a variety of other separation procedures tested. Peak A was finally applied to a Mono Q column and eluted with a gradient of MgCl₂ (Fig. 2), instead of NaCl as used at an earlier stage in the purification. This resulted in a preparation that had been purified approximately 9000-fold with a specific activity of 13.1 μmol · min⁻¹ · mg⁻¹.

Following chromatography on phosphocellulose, peak B was applied to a Sephacryl-S200 gel-filtration column, which removed contaminating peak A activity (Fig. 3B). Further purification of peak B was not attempted due to the very small amounts of activity remaining. The final preparation of peak B had a specific activity of 3.3 μmol · min⁻¹ · mg⁻¹.

Inactivation of kinases A and B by FSO₃-PbCOAdo and protein phosphatase 2C

The reactive ATP analogue FSO₃-PbCOAdo has been shown to irreversibly inactivate protein kinases by reacting
at a conserved lysine residue in the ATP-binding site [20]. It inactivates rat liver AMP-activated kinase [21, 22], and \(^{[14C]}\)FSO\(_2\)PhCOAdo has been used to identify the catalytic subunit of that enzyme [18]. Fig. 4 shows that both peaks A and B are inactivated by incubation with 200 \(\mu\)M FSO\(_2\)PhCOAdo, with inactivation of peak A being particularly rapid. Inactivation exhibits linear plots when the logarithm of activity remaining is plotted against time, with half times of 3.5 min for peak A and 17 min for peak B. This is consistent with a single site of reaction for both peaks, in contrast to results with mammalian AMP-activated protein kinase, where the plots are biphasic due to reaction at both the AMP (allosteric) and ATP (catalytic) sites [18, 21, 22]. In the presence of Mg\(^{2+}\), ATP (2.5 mM) markedly reduces the rate of inactivation for both peaks. However AMP (2.5 mM) was ineffective in providing protection against FSO\(_2\)PhCOAdo (data not shown).

We have shown previously that peak A can be inactivated by endogenous cauliflower protein phosphatases with the characteristics of protein phosphatases 2A and 2C, and by incubation with purified mammalian protein phosphatase 2A [15]. Fig. 5 shows that both peaks A and B are rapidly inactivated by incubation with purified mammalian protein phosphatase 2C. Using equivalent units of each protein kinase of similar specific activity, and identical amounts of protein phosphatase, peak B was inactivated much more rapidly, with a half-time of 1 min versus 13 min for peak A.

Rechromatography of peaks A and B

To determine whether peak B might have been derived from peak A during the purification procedure, a small-scale
crude extract was prepared and immediately applied to a Mono Q column as described in Materials and Methods. The activity eluted in two peaks as in Fig. 1A, with peak B comprising approximately 15% of total activity. Exactly the same results were obtained if the extract was incubated for 1 h at 30°C prior to loading on the Mono Q column (data not shown). In a second experiment, following the normal chromatography on Mono Q (Fig. 1A), peaks A and B were dialysed and aliquots were reapplied to the column. Fig. 1B and C show that the two peaks eluted in exactly the same positions on re-chromatography (note that peak B is always contaminated with a small amount of peak A after a single Mono Q separation). Peaks A and B were subsequently dephosphorylated using protein phosphatase 2C and reapplied to the column. Although the activity was greatly reduced in both cases as expected, the peaks eluted at the same ionic strengths as before (Fig. 1B and C).

Determination of native molecular mass of peaks A and B

The apparent molecular mass of peaks A and B were determined by gel filtration using a Sephacryl S-200 FPLC column as described in Materials and Methods. This gave an apparent molecular mass of 200 ± 50 kDa for peak A and 45 ± 5 kDa for peak B (Fig. 3, note that before gel filtration peak B is always contaminated with small amounts of peak A). As these results for peak A were not in agreement with those obtained previously [15] we carried out further studies with this form. Peak A from the phosphocellulose step was also analysed by gel filtration on Superose 12 (30 cm × 1 cm) either in buffer D or buffer D plus 0.5 M NaCl. Although the higher ionic strength buffer changed the elution of other proteins, as judged by differences in the A_{280} profile, the protein kinase eluted in both cases at the same position with an apparent molecular mass of 200 ± 40 kDa (data not shown).

Peak A was also analysed by PAGE under non-denaturing conditions at pH 8.0, after labelling with [14C]FSO_{2}PhCOAdo. We also analysed a sample of purified rat liver AMP-activated protein kinase under the same conditions. Fig. 6 shows that the two preparations comigrated as single radioactive species, corresponding to an apparent molecular mass of 160 ± 30 kDa.

Subunit structure of peaks A and B

Fig. 7 shows that several polypeptides were detectable by Coomassie Blue staining after SDS/PAGE electrophoresis of the final preparation of peak A, with a prominent doublet migrating with apparent molecular masses of 56/58 kDa. Prior labelling of the preparation with [14C]FSO_{2}PhCOAdo, followed by detection by phosphorimaging, showed that the 56/58-kDa bands were the only polypeptides that reacted with FSO_{2}PhCOAdo (Fig. 7). ATP (2.5 mM) greatly reduced the labelling of both polypeptides (data not shown). When peak A was incubated with [y-32P]ATP a single labelled polypeptide was seen at 58 kDa (Fig. 7).

When the final preparation of peak B was analysed on a SDS/polyacrylamide gel, there were no prominent polypeptides at 56/58 kDa (Fig. 7). There were higher molecular mass polypeptides, and several polypeptides migrating at 30–45 kDa, where we would expect peak B based on its native molecular mass of 45 ± 5 kDa on gel filtration. When peak B was labelled with [14C]FSO_{2}PhCOAdo, three or more polypeptides migrating between 30–45 kDa were labelled (Fig. 7): all were greatly reduced in intensity when 2.5 mM ATP was included in the incubation (data not shown). When peak B was incubated with [y-32P]ATP a single polypeptide corresponding to the largest of the FSO_{2}PhCOAdo-labelled bands (43 kDa) was detected. No polypeptides of 56/58 kDa were labelled in the peak B preparation either with [14C]FSO_{2}PhCOAdo or [y-32P]ATP, although we analysed twice the quantity of enzyme based on kinase units.

Fig. 7 also compares the migration of [14C]FSO_{2}PhCOAdo-labelled polypeptides in the preparations of peaks A and B with mammalian AMP-activated kinase. The results clearly show that the labelled doublet in peak A (56/58 kDa) is slightly smaller than the 63-kDa catalytic subunit (Carling et al., 1989) of mammalian AMP-activated protein kinase.
Methods, and analysed by SDS-PAGE. Polypeptides were detected using \[^{14}C\]FSO, PhCOAdo (FSBA), as described in Materials and Methods, and analysed by SDS/PAGE. Polypeptides were detected by Coomassie Blue staining (as indicated), or phosphorimaging (Autophosphorylation and \[^{14}C\]FSBA labelling).

**Fig. 7. Identification of the catalytic subunits of** (A) peak A, (B) peak B, and (An) rat liver AMP-activated protein kinase.

The results show that the specificities of peaks A and B are similar, but not identical. As with many other protein kinases, the specificities of peaks A and B for peptide substrates are those obtained with the parent SAMS-containing peptide. The specificities of peaks A and B for peptide substrates differ from the parent SAMS-containing peptide (peptide 1) are highlighted by underlining and bold type. To allow easy comparison, the data are also given as values for the enzyme (Table 2). The data were estimated on three separate occasions, and these differences were reproducible.

Both the A and B forms also phosphorylate the purified 53-kDa catalytic fragment of HMG-CoA reductase from rat liver (data not shown).

**DISCUSSION**

We have previously shown that a calcium-independent protein kinase, which appears by functional criteria to be a homologue of the mammalian AMP-activated protein kinase, is present in higher plants [15]. In the present study we have improved and extended the purification protocol for this enzyme and identified the catalytic subunit. In addition, we have identified a second peptide kinase activity in cauliflower which we believe to be a distinct but related gene product.

With our new procedure we have purified peak A approximately 9000-fold, increased its specific activity by an order of magnitude, and removed several contaminating polypeptides that interfered with attempts to identify the catalytic

**Table 2. Kinetics data obtained with kinase A and kinase B using variants of the SAMS-containing peptide as substrate.** Residues which differ from the parent SAMS-containing peptide (peptide 1) are highlighted by underlining and bold type. To allow easy comparison, values for V/Km are expressed relative to those obtained with the parent SAMS-containing peptide. n.d., activity not detectable.

<table>
<thead>
<tr>
<th>Peptide no. sequence</th>
<th>Kinase A</th>
<th>Kinase B</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>V (nmol/min)</td>
<td>Km (μM)</td>
</tr>
<tr>
<td>HMRSAMSGLHLVKRR</td>
<td>1467 ± 32</td>
<td>49.8 ± 3.6</td>
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The specificities of peaks A and B was investigated using a series of variants of the synthetic peptide (HMRSAMSGLHLVKRR, SAMS-containing peptide) used to routinely assay the enzyme (Table 2). The V values cannot be directly compared because of differences in the purity of the preparations, but the data are also given as V/Km values relative to those obtained with the parent SAMS-containing peptide. The results show that the specificities of peaks A and B are similar, but not identical. As with many other protein kinases, basic residues on the N-terminal side of the phosphorylation site appear to be important for recognition. For both peaks A and B, replacement of the arginine at P-4 (i.e. 4 residues N-terminal to the phosphorylated serine) with another basic residue (lysine or histidine) slightly reduced V/Km, while replacement with a non-basic residue (glycine) dramatically reduced this parameter. We also tested the effect of the presence of hydrophobic residues in the peptide. Replacement of the methionine at P-5 or the leucine at P+4 with glycine also greatly reduced V/Km, but replacement of the methionine at P-1 or the valine at P+5 had little effect. Replacement of two or three of these hydrophobic residues with glycine had larger effects than replacing one. Finally, the peptide with threonine in place of serine was still a substrate for both peaks A and B, but neither phosphorylated the peptide containing tyrosine in place of serine.

Although these results show that similar specificity determinants are required by both peaks A and B, there were quantitative differences in the kinetics data. In particular the peptide with histidine in place of arginine at P-4 gave a K_m 3-4-fold higher with peak B than peak A, while the peptide with glycine in place of leucine at P+4 was a much poorer substrate for peak B. For both peaks A and B the kinetics data were estimated on three separate occasions, and these differences were reproducible.

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Both the A and B forms also phosphorylate the purified 53-kDa catalytic fragment of HMG-CoA reductase from rat liver (data not shown).
activity to homogeneity. However the final specific activity by MgATP, suggesting that the reagent reacted at the ATP-binding site on the enzyme. Consistent with reaction at a single site, a plot of the logarithm of activity remaining against time was linear, suggesting a pseudo-first-order reaction where the rate is proportional to the concentration of unreacted kinase remaining. By contrast, incubation of mammalian AMP-activated protein kinase gives biphasic plots, with FSO\textsubscript{2}PhCOAdo rapidly reducing the AMP-stimulated activity, then more slowly reducing the basal catalytic activity. The initial rapid phase is protected by both AMP and ATP, whereas the second slower phase is only protected by ATP, suggesting that the reagent reacts rapidly at the allosteric (AMP) site then more slowly at the catalytic (ATP) site [18, 21, 22]. By contrast, peaks A and B give monophasic plots with FSO\textsubscript{2}PhCOAdo (Fig. 6) and are protected by ATP but not AMP. These results are consistent with previous findings that peak A is not activated by AMP, and suggest that neither form of the plant kinase has an AMP-binding site. The high specific activity of peak A might suggest that there is not a missing allosteric activator, and that the plant kinase is regulated solely by phosphorylation.

When purified peak A was incubated with FSO\textsubscript{2}PhCOAdo, a doublet of 56/58 kDa were the only labelled peptides, whereas only the 58-kDa polypeptide was labelled using \( \gamma\textsuperscript{32}P\)ATP. When mammalian AMP-activated protein kinase is inactivated with protein phosphatases, the 63-kDa catalytic subunit shifts to a molecular mass of 61 kDa, whereas autophosphorylation only labels the 63-kDa polypeptide (Carling, D. and Hardie, D. G., unpublished results). By analogy, we would like to suggest that the 56-kDa and 58-kDa polypeptides of plant peaks A are the dephospho-form and phospho-form of the catalytic subunit, respectively. Since the 58-kDa phosphorylated form would be much more active, this would explain why it, and not the 56-kDa form, becomes autophosphorylated on incubation with \( \gamma\textsuperscript{32}P\)ATP. Attempts to confirm this hypothesis by incubating peak A with protein phosphatases were hampered by the presence of contaminants of the same size range in the protein phosphatase preparations used. However this hypothesis is consistent with the finding that a single radioactive species was obtained when FSO\textsubscript{2}PhCOAdo-labelled peak A was analysed by electrophoresis in non-denaturing gels (Fig. 6).

Analysis of peak A by gel filtration on two different columns suggested a native molecular mass of 200 ± 40 kDa. Mammalian AMP-activated protein kinase migrated closely with this on at least one of these columns (data not shown). This disagrees with the previous report where both plant and animal kinases were reported to comigrate on gel filtration with apparent molecular masses of 65 ± 5 kDa [15]. The reasons for this discrepancy are not clear, but the question of the native molecular mass of peak A was addressed very extensively for this study. A native molecular mass greater than 150 kDa for both the plant and animal kinases was obtained by gel filtration, and was confirmed by the migration of the [\(^{14}\text{C}\)FSO\textsubscript{2}PhCOAdo-labelled proteins on polyacrylamide gel electrophoresis in non-denaturing conditions (Fig. 6). While the present data do not allow us to unequivocally determine the native subunit structure, the peak A protein kinase, and rat liver AMP-activated protein kinase, must be oligomers. Whether they are multimers of the 58/63-kDa catalytic subunits, or whether other subunits are involved, remains to be determined.

When the ammonium sulphate fraction from cauliflower inflorescences was applied to a DEAE-Sepharose column and eluted with a NaCl gradient, two peaks of peptide kinase activity were detected. The major peak (A) corresponds to the HMG-CoA reductase kinase which we have previously described [15]. Peak B consistently represents only 10–20% of the total activity recovered after DEAE-Sepharose, and it is possible that it was missed in our previous study because the DEAE-Sepharose was eluted with a step rather than a gradient, and the overall yield in that procedure was lower. Our present data do not allow us to unequivocally identify the catalytic subunit of peak B. However the finding that the activity migrates at 45 ± 5 kDa on gel filtration, and that the only polypeptides labelled with [\(^{14}\text{C}\)FSO\textsubscript{2}PhCOAdo are in the range 35–43 kDa, suggest that, unlike peak A, it is a monomer. Since protein kinases often autophosphorylate, the 43-kDa polypeptide which labels with both [\(^{14}\text{C}\)FSO\textsubscript{2}PhCOAdo and [\(^{32}\text{P}\)]ATP is the prime candidate for the catalytic subunit.

An obvious possibility was that peak B arose from peak A during the purification, either by proteolysis or some other modification. Although we cannot yet rule out conclusively, we consider it to be very unlikely for the following reasons. The ratio of activities in peak A to peak B was consistent from preparation to preparation at between 5:1 and 6:1. This was true even if a freshly prepared homogenate was applied directly to Mono Q, and the ratio was not affected even if the homogenate was previously incubated for 60 min at 30°C (data not shown). When peak A and peak B were separated by Mono Q chromatography and were then reapplied to the Mono Q column, they eluted in the same positions as before. Peaks A and B were not the phospho-form and dephospho-form of the same protein, because dephosphorylation reduced their activities but did not shift their elution positions on Mono Q (Fig. 1 B and C), and because they gave polypeptides of different molecular mass when labelled with [\(^{14}\text{C}\)FSO\textsubscript{2}PhCOAdo. The specificities of peaks A and B (Table 2) were similar, but sufficiently different to render it unlikely that peak B is a catalytic fragment derived by proteolytic removal of a regulatory region from peak A. The homogenization and purification buffers contained a cocktail of proteinase inhibitors.

A definitive answer to the question of the relationship between peaks A and B will have to await sequencing and molecular cloning of these entities. However we would like to suggest that they represent distinct gene products, but are members of the same protein kinase sub-family. Clearly their specificities for peptide/protein substrates are similar, and both are regulated by phosphorylation, but the presence of additional sequence (and possibly even subunits) in peak A suggests that their regulation may be different.

The specificities of peaks A and B for variants of the SAMS-containing peptide are very similar to that of mammalian AMP-activated protein kinase, and the significance of this specificity is discussed elsewhere [29].

The physiological functions of the two forms of HMG-CoA reductase kinase remain unclear at present. In rat hepato-
ocytes we have shown that cellular stress (particularly heat shock) dramatically activates the AMP-activated protein kinase, the signal for this being elevation of AMP and depletion of ATP. Activation of the kinase in turn causes phosphorylation of acetyl-CoA carboxylase and HMG-CoA reductase, leading to total cessation of fatty acid and cholesterol synthesis. We have proposed that the kinase exerts a protective function in animal cells, switching off biosynthetic pathways whenever the cell is compromised for energy, and preserving ATP for other purposes such as maintenance of ion gradients. Since both the A and B forms of HMG-CoA reductase kinase phosphorylate mammalian HMG-CoA reductase (this study) and at least the A form inactivates HMG-CoA reductase from potato tubers [15], it seems possible that they may fulfil a similar function in plants. However since neither the A nor the B forms are activated by AMP, the signal which switches them on must be different.

We have not yet shown that peak B phosphorylates HMG-CoA reductase from plants, but it does have a very similar specificity to peak B for peptides, including a requirement for hydrophobic residues at P-5 and P+4, and a basic residue at P-4 (Table 2). Since these features are conserved in all twelve of the plant HMG-CoA reductase sequences currently available [29] it seems almost certain that peak B will phosphorylate HMG-CoA reductase from plants. We therefore feel justified in referring to peaks A and B as HMG-CoA reductase kinase A and B. However since it is likely that both protein kinases have additional targets in plants, this nomenclature should be regarded as provisional.

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REFERENCES
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