Isolation and Preliminary Characterization of a Casein Kinase from Cauliflower Nuclei

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ABSTRACT

A casein-type protein kinase has been isolated from cauliflower (Brassica oleracea Gars.) nuclei and purified to a specific activity of 23,000 units/mg of protein (1 unit is defined as the transfer of 1 picoequivalent of 32P from γ-[32P]ATP to substrate per minute at 28°C). The enzyme has a molecular weight of approximately 39,000 as judged by sucrose density gradient sedimentation. The casein kinase requires ATP as the phosphate donor and will phosphorylate casein and phosphatase, but not histones. The enzyme activity is not affected by cAMP or cGMP. The casein kinase appears to be analogous to casein kinases described in other plant and animal systems.

Protein phosphorylation is known to be involved in the regulation of a number of enzyme activities (17, 20). Evidence correlating increased RNA synthesis with enhanced nuclear protein phosphorylation suggests that chromosomal protein phosphorylation may play a role in the regulation of transcription (for reviews see refs. 9 and 20). Consequently, there is substantial interest in the properties of the protein kinases which phosphorylate the two major classes of nuclear protein, histones and nonhistone proteins. The histone kinases are specific for histone and proteamines, located in the cytoplasm and, in animal systems, often dependent on cyclic nucleotides for maximal activity (17). The nonhistone protein kinases will phosphorylate nonhistone nuclear proteins, but not histones, and are not affected by cyclic nucleotides (17). The nonhistone protein kinases are operationally defined as "casein" or "phospho" kinases by virtue of their ability to phosphorylate these two acidic substrates. Protein kinase activities have been detected in various subcellular fractions in a number of higher plants, and ribosome-associated protein kinase activities have been described in Lemna, peas, and wheat (15, 16, and refs. in 20). However, few nuclear protein kinases have been extensively purified and characterized in higher plants. A cytoplasmic cAMP-independent histone kinase and a chromatin-associated casein kinase have been characterized in soybean hypocotyl (12, 15).

Cauliflower (Brassica oleracea Gars.) inflorescences contain a casein kinase activity that co-purifies with RNA polymerase during solubilization from nuclei. Purification and preliminary characterization of casein kinase from cauliflower nuclei are described here. Cauliflower casein kinase phosphorylates casein and phosphatase but not histones, and it is not affected by cyclic nucleotides. This casein kinase is similar to the chromatin-associated casein kinase described previously in soybean (15).

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MATERIALS AND METHODS

Buffers. Buffer A contained 2 mM MES (pH 6.0), 20 mM MgCl2, 20 mM KCl, 0.25 M sucrose, 10 mM 2-mercaptoethanol, and 40% (v/v) glycerol. Buffer B contained 50 mM Tris-HCl (pH 8.0), 0.1 mM EDTA, 10 mM DTT, 0.5 mM phenylmethylsulfonylfluoride, 1% (v/v) dimethylsulfoxide, 0.1% (v/v) Triton X-100, and 30% (v/v) glycerol. Buffer O contained 50 mM Tris-HCl (pH 8.0), 10 mM DTT, 0.5 mM phenylmethylsulfonylfluoride, and 1% (v/v) dimethylsulfoxide.

Casein Kinase Assays. γ-[32P]ATP was prepared enzymically (18). Unless otherwise stated, casein kinase assays contained in a final volume of 0.2 ml, 100 mM Tris-HCl (pH 7.5), 10 mM MgCl2, 10 mM DTT, 25 μM ATP (75–150 cpm/pmol) and 500 μM casein (Sigma). Casein kinase assays were initiated with 5 μl of casein kinase preparation, incubated for 30 min at 28°C, and terminated with 3 ml of cold 10% (w/v) trichloroacetic acid containing 10 mM Na-pyrophosphate. Precipitates were collected on GF/A glass fiber discs (Whatman) with 15 ml of cold 10% (w/v) trichloroacetic acid followed by 3 ml of 95% ethanol, and dried. Radioactivity was determined in a toluene-based scintillator. Assays were performed in triplicate and values corrected for nonspecific 32P trapping during precipitation (typically 80–150 cpm/assay). A unit of casein kinase is defined as the amount of enzyme that catalyzes the transfer of 1 pmol of 32P from γ-[32P]ATP to casein/min at 28°C.

Casein Kinase Solubilization and Gel Filtration. Solubilization and gel exclusion chromatography follow the techniques described by Guilfoyle (6) for the isolation of RNA polymerase from cauliflower. A nucleus-enriched fraction was prepared from cauliflower inflorescence with buffer A as described by Chen et al. (2). Protein kinase was solubilized from the nuclear fraction by shearing the preparation in buffer B containing 0.5 mM ammonium sulfate with a polytron (Brinkmann Instruments) followed by sonication with a Branson microtip sonicator. The solubilized casein kinase was recovered in the supernatant after centrifugation for 60 min at 50,000 rpm (Spinco type 60 rotor). The supernatant was diluted to 100 mM ammonium sulfate with buffer B. After recentrifugation (60 min, 50,000 rpm) the casein kinase in the supernatant was precipitated by the gradual addition of solid ammonium sulfate (0.35 g/ml) and stirring for 30 min. The precipitate was recovered by centrifugation (60 min, 55,000 rpm) and suspended in 40 ml of buffer B containing 500 mM ammonium sulfate.

The soluble casein kinase was loaded onto a column (90 x 2.5 cm) of agarose A-1.5m (Bio-Rad) equilibrated with buffer B containing 500 mM ammonium sulfate and eluted with the same buffer at a flow rate of 0.25 ml/min. Five-ml fractions were collected, aliquots assayed for casein kinase activity, and the peak fractions were pooled and precipitated with ammonium sulfate as described above.

Ion Exchange Chromatography. DEAE-cellulose (Whatman) and phosphocellulose (Whatman) were prepared as described previously (6). The ammonium sulfate precipitate from the agarose
fractionation was suspended in buffer B containing 25 mM ammonium sulfate and loaded onto a column (30 x 1.5 cm) of DEAE-cellulose equilibrated in the same buffer. The column was washed with 1 column volume of buffer C containing 25 mM ammonium sulfate. Essentially none of the casein kinase activity is retained on the column under these conditions. Elution of the DEAE-cellulose column with 25 to 400 mM ammonium sulfate gradient did not elute any additional casein kinase activity.

After passage over DEAE-cellulose, the casein kinase was loaded directly onto a column (30 x 1.5 cm) of phosphocellulose equilibrated in buffer C containing 25 mM ammonium sulfate. After the column was washed with 2 column volumes of buffer C containing 25 mM ammonium sulfate, casein kinase was eluted with a 25 to 400 mM ammonium sulfate gradient. The volume of the gradient was 2 column volumes and 1.5-ml fractions were collected. Casein kinase eluted from 50 to 150 mM ammonium sulfate. Peak fractions were concentrated by ammonium sulfate precipitation as described above.

Sucrose Gradient Sedimentation. The ammonium sulfate precipitate after phosphocellulose was dissolved in buffer C containing 250 mM ammonium sulfate and loaded onto 5 to 20% (w/v) isokinetic sucrose gradients in buffer C containing 250 mM ammonium sulfate. The gradients were centrifuged for 16 hr at 48,000 rpm in a Beckman SW 50.1 rotor. Fractions were fractionated and aliquots of each fraction assayed for casein kinase activity. By the sedimentation of BSA (included as an internal standard in one gradient), the mol wt was calculated according to Martin and Ames (14).

Ammonium Sulfate and Protein Determinations. Ammonium sulfate concentrations were determined with a Bausch & Lomb refractometer. Protein was determined according to Lowry et al. (13) after the samples were precipitated with cold 10% (w/v) trichloroacetic acid.

RESULTS

Casein Kinase Purification. Cauliflower inflorescence is a rich source of casein kinase from higher plants. The nucleus-enriched fraction from which the casein kinase was solubilized contains approximately equal amounts of RNA polymerase I and II activity (6). Both casein kinase and RNA polymerase activities are solubilized quantitatively by the procedure described. Casein kinase was initially resolved from RNA polymerase by agarose A-1.5m gel filtration at high ionic strength (Fig. 1a). The high ionic strength used during gel filtration is necessary to prevent aggregation of both casein kinase and RNA polymerase with contaminating nucleic acids and proteins. Elution of the agarose column at low ionic strengths results in both enzyme activities eluting as an aggregate fraction in the void volume. Casein kinase may be further purified by DEAE-cellulose ion exchange chromatography. Although cauliflower casein kinase does not bind to DEAE-cellulose at 25 mM ammonium sulfate, passage over this anion exchange resin does give significant purification by removal of contaminating proteins. Further purification of the casein kinase activity is achieved by phosphocellulose ion exchange chromatography (Fig. 1b). Sucrose density gradient sedimentation gives a further 3-fold purification (Fig. 1c). Casein kinase activity sediments as a single symmetrical peak of 3.1 ± 0.9S corresponding to a mol wt of 39,000 ± 6,000 daltons (average and standard deviation of eight determinations).

The purification scheme for casein kinase is summarized in Table I. The over-all purification based on the enzyme applied to the agarose column was approximately 80-fold with a recovery of 24%. Although the final specific activity is higher than generally reported for comparable casein kinases from animal systems, the preparation was not homogeneous. Electrophoresis of the preparation on polyacrylamide in the presence of SDS revealed the presence of three major polydisperse peptides of approximately 32,000, 43,000, and 73,000 daltons. The casein kinase is stable indefinitely if frozen at −20°C in the presence of 30% glycerol and 0.5 mM phenylmethylsulfonylfluoride.

Casein Kinase Characterization. Casein kinase activity shows a broad pH curve reaching a plateau at about pH 7.5 (Fig. 2). Broad pH curves have been observed in a number of other systems and are thought to be the result of increased casein solubility at higher pH (19). Phosphorylation by casein kinase requires a
standard mixtures were tested (data not shown). Additional substrate indicates that the enzyme preparation is not contaminated with casein kinase substrates. SDS-polyacrylamide gel electrophoresis followed by autoradiography failed to show any autophosphorylation of casein kinase after incubation in the presence of labeled ATP (data not shown).

Fig. 2. Casein kinase activity as a function of pH. Standard assay mixtures were modified with 100 mM Tris-maleate buffers at the indicated pH. Means of three replications are shown and vertical lines denote standard deviation. Values at each pH have been corrected for nonenzymic 32Pi incorporation.

Table II. Casein kinase phosphate donor specificity and effect of cyclic nucleotides.

<table>
<thead>
<tr>
<th>Addition</th>
<th>Conc.</th>
<th>pmol 32Pi incorp.</th>
<th>% control</th>
</tr>
</thead>
<tbody>
<tr>
<td>A Control</td>
<td>---</td>
<td>264 ± 18</td>
<td>100</td>
</tr>
<tr>
<td>ATP</td>
<td>150 µM</td>
<td>40 ± 8</td>
<td>15</td>
</tr>
<tr>
<td>GTP</td>
<td>150</td>
<td>280 ± 41</td>
<td>106</td>
</tr>
<tr>
<td>CTP</td>
<td>150</td>
<td>233 ± 8</td>
<td>88</td>
</tr>
<tr>
<td>UTP</td>
<td>150</td>
<td>231 ± 41</td>
<td>88</td>
</tr>
<tr>
<td>B cAMP</td>
<td>10</td>
<td>262 ± 42</td>
<td>99</td>
</tr>
<tr>
<td>cAMP</td>
<td>1</td>
<td>271 ± 15</td>
<td>103</td>
</tr>
<tr>
<td>cGMP</td>
<td>10</td>
<td>275 ± 42</td>
<td>104</td>
</tr>
<tr>
<td>cGMP</td>
<td>1</td>
<td>251 ± 7</td>
<td>95</td>
</tr>
</tbody>
</table>

divalent cation. When tested over the range of 0 to 50 mM Mg2+, casein kinase showed optimal activity with 5 mM Mg2+. Mn2+ at 1 mM gave about 60% of the maximal activity observed with Mg2+, but Ca2+ would not support phosphorylation at any concentration tested (data not shown).

Phosphate donor specificities were investigated by attempting to compete out incorporation from labeled ATP with excess unlabeled CTP, GTP, or UTP (Table II). Addition of 6-fold excess unlabeled ATP competes out label incorporation proportionally, but CTP, GTP, or UTP will not compete incorporation from labeled ATP. The apparent Kₘ for ATP with the optimal Mg2+ concentration (5 mM) is about 2 × 10⁻⁸ M (data not shown), and activity is inhibited by ATP concentrations greater than 200 µM. While the Kₘ for ATP is higher than that reported for either of the soybean kinases (12, 15), it is substantially lower than is typically observed in animal systems (4, 8). Neither cAMP nor cGMP has any effect on casein kinase activity.

Phosphate acceptor specificities are shown in Table III. Casein kinase will phosphorylate casein and phospho vitin, but not histones, to high levels. The low levels of incorporation in the absence of any additional substrate indicate that the enzyme preparation is not contaminated with casein kinase substrates. SDS-polyacrylamide gel electrophoresis followed by autoradiography failed to show any autophosphorylation of casein kinase after incubation in the presence of labeled ATP (data not shown).

Table III. Casein kinase phosphate acceptor specificity.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>pmol 32Pi incorp.</th>
<th>% Casein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein</td>
<td>385 ± 25</td>
<td>100</td>
</tr>
<tr>
<td>Phosvitin</td>
<td>333 ± 33</td>
<td>86</td>
</tr>
<tr>
<td>BSA</td>
<td>7 ± 0.5</td>
<td>2</td>
</tr>
<tr>
<td>Histone (calf thymus)</td>
<td>3 ± 0.5</td>
<td>1</td>
</tr>
<tr>
<td>None</td>
<td>1 ± 0.5</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>

DISCUSSION

The physical and enzymic properties of cauliflower casein kinase suggest that it is analogous to the chromatin-associated casein kinase in soybean (15) and the casein kinases isolated from nuclei in several other systems (1, 3, 4, 19). All of these protein kinases require ATP as the phosphate donor and the presence of Mg2+. The mol wt estimate of 39,000 for cauliflower casein kinase compares with an estimate of 35,000 for the comparable enzyme from soybean (15). None of these casein kinases displays significant activity toward histones nor do they require the presence of cyclic nucleotides for the expression of maximal activity. To date, no unequivocal evidence for the existence of CAMP or of cyclic nucleotide-regulated protein kinases in higher plants has been presented (11).

The biological significance of cauliflower casein kinase as well as those detected in other systems is poorly understood. Nuclear proteins are highly phosphorylated in transcriptionally active tissue with over half of the total cellular phosphoprotein found in the nucleus (20). The correlation between high protein kinase activity, increased chromosomal protein phosphorylation, and the onset of increased RNA synthesis suggests that protein phosphorylation is involved in the regulation of transcription (8, 9, 16, 20). While the addition of phosphorylated nonhistone proteins often gives increase in in vitro transcription [refs. in 20], it has not been possible to prove that chromosomal protein phosphorylation is the primary cause of increased transcription. Extensive phosphorylation of protein moieties of ribonuclear protein particles suggests that much of the protein phosphorylation observed in the nucleus may be required for posttranscriptional processing of RNA transcripts (5, 20). Observations that RNA polymerase is subject to phosphorylation and that protein kinase activities often co-associate with RNA polymerase have led to speculation that the activity of RNA polymerase may be directly affected by phosphorylation (3, 10). While phosphorylation does appear to alter in vitro transcription, the effect may be indirect (20). Bell et al. (1) have been unable to demonstrate that yeast RNA polymerase I and II activities are affected by phosphorylation in spite of the fact that these enzymes are phosphorylated in vivo and may be phosphorylated in vitro by a protein kinase similar to that reported here. Under the standard casein kinase assay conditions, we were unable to detect any phosphorylation of cauliflower RNA polymerase I and II by purified casein kinase (data not shown).

Understanding the function of protein phosphorylation in the nucleus will require basic knowledge of the protein kinases involved from a variety of different organisms. In addition, purified protein kinase preparations may be useful tools in evaluating the possible role of nuclear protein phosphorylation in the regulation of RNA synthesis.

LITERATURE CITED


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