Isolation and Characterization of a Chromatin-associated Protein Kinase from Soybean

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ABSTRACT

A chromatin-associated casein-type protein kinase has been purified 500-fold from soybean (Glycine max, var. Wayne) tissue. The enzyme can be completely dissociated from isolated chromatin in 250 millimolar (NH₄)₂SO₄. After purification, the kinase preparation is stable for at least 6 months at 0 C. The enzyme will phosphorylate casein, phosvitin, and denatured chromatin proteins, but not histones. Only ATP will serve as a phosphate donor with an apparent Kₘ of 8 micromolar. Five millimolar Mg²⁺ is required for maximal activity, but Mn²⁺ will support phosphorylation at a lower level. The average molecular weight as determined by sucrose gradient sedimentation and gel filtration is approximately 55,000. Under conditions of low ionic strength (less than 250 millimolar (NH₄)₂SO₄) soybean casein kinase forms higher molecular weight aggregates with other chromosomal proteins in the preparation. The enzyme activity is not affected by cyclic AMP. Casein kinase shows a broad optimum between 7 and 8 and the isoelectric point is approximately 9. Preliminary data indicate that soybean casein kinase will not phosphorylate soybean RNA polymerases I or II, nor does it have any obvious effect on in vitro chromatin transcription by endogenous RNA polymerases.

Kinases (EC 2.7.1.37) catalyzing the transfer of the γ-phosphate from ATP to serine or threonine residues in protein substrates occur in a wide variety of organisms (14, 22). Evidence that chromosomal protein phosphorylation may be involved in the regulation of eukaryotic gene transcription has stimulated interest in nuclear protein kinases (13). Correlations exist between protein kinase activity and the activation of RNA synthesis, and high levels of protein kinase activity can be isolated with transcriptionally active chromatin (10, 20; refs. in 26). Modifications of RNA polymerase activity by protein kinases have been reported in several systems including Ascites tumors (5) and calf thymus (9).

Protein kinase activities described in animal systems may be divided into two classes according to substrate specificity and the requirement for cAMP or cGMP for expression of maximal activity. Cyclic nucleotide-dependent protein kinases are especially active on histones but generally will not phosphorylate acidic proteins. Because the majority of the cyclic nucleotide-dependent protein kinase activity is found in the cytosol and/or membrane fractions, they appear to be primarily extranuclear (10, 14, 22). However, histones in the nucleus are subject to cAMP-dependent phosphorylation (14). The second class of protein kinases phosphorylates acidic substrates such as casein, phosvitin, and nuclear nonhistone protein. These protein kinases are operationally defined as casein kinases throughout this paper. Casein kinases are generally cyclic nucleotide-independent and will not phosphorylate histone (6, 10, 14, 22). The high levels of casein kinase typically detected in nuclei and chromatin suggest a role in nuclear nonhistone protein phosphorylation in vivo (10, 14, 22, 26).

While an extensive literature on chromosomal protein phosphorylation and protein kinases exists for animal systems, less work has been done with higher plants. Chromatin protein phosphorylation has been studied in a number of systems including wheat, Lemna, tobacco, and cabbage (refs. in 26). In addition, protein kinase activities have been detected in nuclei, ribosomes, and chloroplasts from a number of higher plants (refs. in 26). However, no casein kinase has been extensively purified or characterized from nuclei or chromatin of higher plants.

Concomitant with auxin-enhanced RNA synthesis in soybean hypocotyls (11) there is an increase in nuclear protein phosphorylation and in nuclear casein kinase activity (21). Moreover, RNA polymerase preparations from soybean were found to co-purify with a protein kinase activity during the early stages of purification (unpublished). Purification of this enzyme was undertaken in order to compare its activity to that of the casein kinases described in animal systems and to test for a possible role of protein phosphorylation in the regulation of RNA synthesis in soybean hypocotyls. Soybean hypocotyl tissue contains a histone kinase (16) in addition to the chromatin-associated casein-type kinase which will be described here. This study shows that the properties of soybean casein kinase are similar to casein-type kinases found in animal systems.

METHODS

Protein Kinase Assays. Carrier-free [γ-³²P]ATP was prepared according to Schendel and Wells (23). Standard protein 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 μg/ml of α-casein (Sigma). Assays were initiated with 5 μl of protein kinase preparation, incubated for 30 min at 28 C, and terminated with 3 ml of cold 10% (w/v) trichloroacetic acid containing 10 mM sodium pyrophosphate. Precipitates were collected on GF/A glass fiber discs (Whatman), washed with 15 ml of cold 5% 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 radioactivity trapped nonspecifically during precipitation (typically 80–150 cpm/assay). A unit of kinase activity is defined as the amount of enzyme that catalyzes the transfer of 1 pmol of ³²Pi from [γ-³²P]ATP to substrate/min at 28 C.

Buffers. Buffer A contained 25 mM MES (pH 6), 250 mM sucrose, 10 mM 2-mercaptoethanol, 0.5 mM phenylmethylsulfonylfluoride (to inhibit protease activity), and 1% (v/v) dimethylsulfoxide-buffer B contained 50 mM Tris-HCl (pH 8), 10 mM

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1 This research was supported by National Institutes of Health Grant CA11624.
DTT, 0.5 mM phenylmethylsulfonylfluoride, and 1% dimethylsulfoxide. Because of its low solubility in water, phenylmethylsulfonylfluoride was dissolved in dimethylsulfoxide before addition to buffers.

**Isolation of Chromatin and Nuclei.** Thirty-six-hr auxin-treated soybean (Glycine max, var. Wayne) seedlings were grown as described previously (8). Mature hypocotyl tissue (that portion from 1 cm below the cotyledon to 1 cm above the root) was harvested onto ice and all subsequent operations were done at 0 to 4°C. Chromatin was isolated from 1- to 1.5-kg lots of hypocotyls at pH 7 according to Lin et al. (15) and stored in buffer A containing 1 mM MgCl₂ and 30% (v/v) glycerol at −70°C until use. For analysis of protein kinase localization, nuclei were isolated at pH 6 according to Chen et al. (4) and purified by pelleting through a pad of buffer A containing 1.2 M sucrose, 20 mM MgCl₂, 20 mM KCl and 30% (v/v) glycerol by centrifugation at 16,000g for 30 min. The nuclear pellet consisted primarily of intact nuclei although some nucleoli and starch grains were evident. Nuclei isolated as described maintain sufficient structural and functional integrity that they do contain both RNA polymerase I and II and carry out RNA chain elongation at high rates (4). That material that did not pellet through the 1.2 M sucrose pad was combined with the postnuclear supernatant and centrifuged at 6,000g for 30 min. The 6,000g pellet is referred to hereafter as chromatin. The 6,000g supernatant was centrifuged at 100,000g for 3 hr, and both the supernatant (cytosol) and 100,000g pellet were retained for protein kinase assays.

**Preparation of Exchange Resins.** DEAE-cellulose (DE32, Whatman) was prepared according to Whatman (Laboratory Manual 1E3) and phosphocellulose (P-11, Whatman) according to Schwarz and Roeder (24). Both were equilibrated in buffer B containing 30% (v/v) glycerol and 25 mM (NH₄)₂SO₄. Ultrogel AC-44 (LKB) was equilibrated in buffer B containing 250 mM (NH₄)₂SO₄.

**Protein Kinase Solubilization.** Chromatin isolated from 7 to 10 kg of hypocotyl was thawed and solid (NH₄)₂SO₄ (enzyme grade, Schwarz/Mann) was added to a final concentration of 250 mM. The suspension was stirred for 3 hr at 4°C followed by the gradual addition of 0.2 ml/ml of 2% (w/v) protamine sulfate (Calbiochem) and additional stirring for 1 hr. The supernatant containing essentially all of the protein kinase activity was collected after centrifugation at 300,000g for 1 hr. Solid (NH₄)₂SO₄ (0.35 g/ml) was added slowly and the precipitate was collected after centrifugation at 300,000g for 1 hr. The pellet was dissolved in 50 ml of buffer B containing 250 mM (NH₄)₂SO₄.

**Ultrogel AC-44 Gel Filtration.** The crude protein kinase preparation was loaded onto an Ultrogel AC-44 column (90 × 2.5 cm) and eluted with buffer B containing 250 mM (NH₄)₂SO₄ at a flow rate of 1 ml/min. Four-ml fractions were collected and 5-μl aliquots of each were assayed for protein kinase activity using casein as a substrate. Peak casein kinase activity fractions were pooled, precipitated with (NH₄)₂SO₄ (0.35 g/ml), and the precipitates were collected by centrifugation at 300,000g for 1 hr.

**DEAE-Cellulose Chromatography.** The (NH₄)₂SO₄ precipitate from the Ultrogel column was dissolved in buffer B containing 30% glycerol and adjusted to 25 mM (NH₄)₂SO₄ by dialysis. The sample was loaded onto a DEAE-cellulose column (1.5 × 30 cm) and washed with 1 column volume of buffer B containing 25 mM (NH₄)₂SO₄ and 30% glycerol. The column was step-eluted with buffer B containing 250 mM (NH₄)₂SO₄ and 30% glycerol. Two-ml fractions were collected and 5-μl aliquots of each assayed for protein kinase activity using casein as a substrate.

**Phosphocellulose Chromatography.** The DEAE-cellulose flow-through and 25 mM (NH₄)₂SO₄ wash contained most of the casein kinase activity. These fractions were loaded directly onto a phosphocellulose column (1.5 × 30 cm) and washed with 1 column volume of buffer B containing 25 mM (NH₄)₂SO₄ and 30% glycerol. The column was step eluted with buffer B containing 250 mM (NH₄)₂SO₄ and 30% glycerol. Two-ml fractions were collected and 5-μl aliquots of each assayed for casein kinase activity.

**Isoelectric Focusing.** Two ml (4,500 units) of the enzyme preparation from phosphocellulose dialyzed extensively against 1% (w/v) glycerol, 10 mM 2-mercaptoethanol, 0.5 mM phenylmethylsulfonylfluoride, and 1% dimethylsulfoxide. The sample was introduced into a 110-ml isoelectric focusing column (LKB) after the pH gradient had been formed as described in the LKB instruction manual. The gradient was stabilized with a 2 to 50% (w/v) sorbitol gradient and contained 0.25% (v/v) pH 9–11 Ampholine (LKB) and 0.25% (v/v) pH 7–5 Bio-Lyte (Bio-Rad). The column reached equilibrium at 0.8 mamp (1,000-v constant potential) after 35 hr. Fractions of 1.5 ml were collected and 5-μl aliquots assayed for kinase activity. pH was measured directly at 4°C.

**Sucrose Gradient Sedimentation Analysis.** Five-ml 5 to 25% (w/v) linear sucrose gradients (containing 50 mM Tris-HCl [pH 7.5], 10 mM 2-mercaptoethanol, 0.5 mM phenylmethylsulfonylfluoride, 1% dimethylsulfoxide, and variable concentrations of (NH₄)₂SO₄) were prepared. One-tenth-ml samples were layered on top of the gradients and centrifuged at 48,000 rpm in a Beckman SW 50.1 rotor for 16 hr. Ten-drop fractions were collected after tubes were punctured and 25-μl aliquots assayed for kinase activity. BSA was included as an internal standard, and mol wt was calculated according to Martin and Ames (18).

**RNA Polymerase Assays.** Chromatin-bound RNA polymerase activity was assayed according to Guilfoyle et al. (8). Assays contained (in a final volume of 0.4 ml) 50 mM Tris-HCl (pH 8), 10 mM DTT, 10 mM MgCl₂, 50 mM (NH₄)₂SO₄, 50% (v/v) glycerol, 0.4 mM unlabeled nucleotide triphosphates, 0.02 mM [3H]UTP (1–2 μCi). Reactions were initiated by the addition of 0.5 to 1 A₅₂₈₀ units of chromatin. Where specified, 10 units of soybean casein kinase were included. Assays were incubated for 30 min at 28°C and terminated with 3 ml of cold 10% trichloroacetic acid containing 10 mM sodium pyrophosphate. Precipitates were collected on GF/A glass fiber discs and processed for counting as described for protein kinase assays.

**Other Procedures.** (NH₄)₂SO₄ concentrations were determined with a Bausch & Lomb refractometer. Protein was determined according to Lowry et al. (17) and DNA was determined according to Burton (3).

**RESULTS**

**Cellular Distribution and Numbers of Casein Kinase Species.** The subcellular distribution of casein kinase activity is shown in Table I. About 60% of the total casein kinase activity is observed in the particulate fractions which contain all of the DNA. Because the nuclear isolation procedure includes a Triton X-114 wash, the pelletable activity cannot be considered to be membrane-associated. Consequently, these data demonstrate the difficulty in obtaining quantitative recovery of intact plant nuclei. Moreover, the high Mg²⁺ concentrations needed to isolate nuclei favor DNase activity. This activity, coupled with mechanical shearing of chromatin released from broken nuclei, may explain the large fraction (43%) of the total DNA pelletable only at 100,000g. The tendency of casein kinase activity to aggregate at low ionic strength (see below) further complicates analysis of subcellular distribution. Nevertheless, the highest casein kinase-specific activities whether expressed per mg of protein or per mg of DNA occur in the nuclear and chromatin fractions. Because nonspecific aggregation of casein kinase with other proteins would be expected to decrease the specific activity in the pelletable fractions, we conclude that much of the casein kinase in soybean is nuclear or chromatin-associated. In agreement with the results of Lin and Key (16) 56% of the histone kinase was found in the 100,000g supernatant and the highest specific activity was in the 100,000g pellet (19).

Crude casein kinase solubilized from particulate fractions was assayed after precipitation with casein as the substrate. The casein kinase activity was assayed using casein as the substrate. The casein kinase activity was assayed using casein as the substrate.
kinases in soybean (Fig. 1). Extracts of the nuclear, chromatin, and 100,000g pellets all show major casein kinase activity peaks at about 4S with minor peaks at about 6S, when analyzed in sucrose gradients containing 100 mM (NH₄)₂SO₄. The similarities in the profiles suggest that at least in terms of sedimentation behavior at 100 mM (NH₄)₂SO₄ concentration, each subcellular fraction contains similar casein kinase activities. The above extracts showed several other high mol wt casein kinase activities in addition to the 4S and 6S components in sucrose gradients containing no (NH₄)₂SO₄ (19). In addition, about 40% of the crude casein kinase activity from these extracts applied to the gradients pelleted through the gradients in the absence of salt. In contrast, at 100 mM (NH₄)₂SO₄ only about 3% of the applied activity pelleted through the gradient. Hence, crude casein kinase is subject to severe aggregation at low ionic strength.

To assess whether the two activities detected in the nuclear extract might still be artifacts of aggregation at 100 mM (NH₄)₂SO₄, samples of casein kinase solubilized from another nuclear preparation were analyzed on sucrose gradients in the presence of 0, 100, or 250 mM (NH₄)₂SO₄ (Fig. 2). Gradients containing 250 mM (NH₄)₂SO₄ show one casein kinase activity at about 4S while at 0 or 100 mM (NH₄)₂SO₄ faster sedimenting species appear. With 250 mM (NH₄)₂SO₄ 75% of the activity applied was recovered in the gradient and 0.5% pelleted through the gradient. With 0 or 100 mM (NH₄)₂SO₄, 50 or 26% of the applied activity was recovered in the gradient and 2 or 31% pelleted through the gradient, respectively. We interpret these faster sedimenting species to be aggregates which form at low ionic strength. As judged by sedimentation velocity in sucrose gradients at high ionic strength [250 mM (NH₄)₂SO₄], soybean nuclei contain one casein kinase activity with an average S value of 3.9 ± 0.4S corresponding to a mol wt of 54,000 ± 2,000 (average and standard deviation of 18 measurements on various preparations).

Casein Kinase Purification. Chromatin served as the starting material for large scale casein kinase purification because sufficient quantities of nuclei were not readily available. Chromatin from auxin-treated hypocotyl contains higher levels of casein kinase than control chromatin (20) and was thus routinely used for isolation. Chromatin was stored frozen until sufficient quantities could be collected. More than 90% of the casein kinase activity remained in frozen chromatin after up to 2 weeks storage. Moreover, the gel exclusion and ion exchange column elution profiles were similar whether the casein kinase was solubilized from 2,4-D-treated or untreated, freshly prepared or frozen chromatin. Hence, freezing and/or the use of auxin-treated tissue did not give selective enrichment for one casein kinase species.

Because soybean casein kinase was first detected during purification of RNA polymerase, the purification scheme is based on the methods of Guilfoyle et al. (8) for the isolation of soybean RNA polymerase I. Two hundred fifty mM (NH₄)₂SO₄ was found to be sufficient to remove casein kinase from chromatin quantitatively. Proteamine sulfate precipitation removed substantial amounts of nucleic acid and acidic protein. The proteamine sulfate supernatant containing virtually all of the casein kinase activity could then be concentrated sufficiently to permit gel filtration with Ultrogel AC-44. Ultrogel AC-44 gives better resolution than

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Table I. Subcellular Distribution of Protein Kinase in Soybean.

Cell fractions prepared as described under Methods were extracted with buffer B containing 250 mM (NH₄)₂SO₄ and 5 mM 25% Tris [1-32P]ATP to substrate/min at 28 C. Means of three replications are shown.

<table>
<thead>
<tr>
<th>Cell Subfraction</th>
<th>% Total Activity</th>
<th>Units/mg protein</th>
<th>% Total Activity</th>
<th>Units/mg DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact nuclei</td>
<td>26.3</td>
<td>82.6</td>
<td>38.0</td>
<td>208</td>
</tr>
<tr>
<td>6,000 g chromatin</td>
<td>6.9</td>
<td>63.6</td>
<td>18.2</td>
<td>121</td>
</tr>
<tr>
<td>100,000 g pellet</td>
<td>26.7</td>
<td>49.3</td>
<td>42.9</td>
<td>108</td>
</tr>
<tr>
<td>100,000 g supernatant</td>
<td>39.4</td>
<td>12.0</td>
<td>0.0</td>
<td>---</td>
</tr>
</tbody>
</table>

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FIG. 1. Sucrose gradient sedimentation analysis of casein kinase solubilized from nuclei, chromatin, or 100,000g pellet. Subcellular fractions were extracted with buffer B containing 250 mM (NH₄)₂SO₄. After centrifugation (300,000g, 1 hr) the supernatant was dialyzed versus buffer B containing 100 mM (NH₄)₂SO₄. Samples containing 120, 48, or 24 units of casein kinase activity from the nuclear, chromatin, or 100,000g pellet, respectively, were analyzed on 5-ml 5 to 25% linear sucrose gradients containing 100 mM (NH₄)₂SO₄ (48,000 rpm, 16 hr, Beckman SW 50.1 rotor). After fractionation, samples (25 μl) were assayed in the standard reaction mixture (0.2 ml). The bottom of each tube was washed with buffer B containing 250 mM (NH₄)₂SO₄ and aliquots were assayed to assess pelleted casein kinase activity. Recoveries of applied enzyme activity were: nuclear extract, 97% in the gradient, 2% pelleted; chromatin extract, 95% in the gradient, 3% pelleted; 100,000g pellet, 83% in the gradient, 4% pelleted. When the same extracts were analyzed on sucrose gradients in the absence of salt, 44%, 28%, and 59% of the applied casein kinase activity from nuclei, chromatin, and 100,000g pellet, respectively, pelleted through the gradient (data not shown). BSA (4.5S) was included on all gradients as an internal standard.

FIG. 2. Effect of salt concentration on sedimentation of casein kinase. Nuclei were extracted with buffer B containing 250 mM (NH₄)₂SO₄ and, after centrifugation (300,000g, 1 hr), aliquots of the supernatant were dialyzed versus buffer B containing either 0, 100, or 250 mM (NH₄)₂SO₄. Samples containing 30, 27, and 51 units of casein kinase activity were analyzed on 5-ml 5 to 25% linear sucrose gradients containing 0, 100, or 250 mM (NH₄)₂SO₄, respectively. Details of centrifugation, fractionation, and casein kinase assays are given in the Figure 1 legend. Sedimentation profiles have been normalized so that the standards (BSA) coincide to correct for slight changes in density resulting from the varying (NH₄)₂SO₄ concentration. Recoveries of applied enzyme activity were: 250 mM (NH₄)₂SO₄ 75% in the gradient, 1% pelleted; 100 mM (NH₄)₂SO₄, 50% in the gradient, 2% pelleted; 0 mM (NH₄)₂SO₄, 26% in the gradient, 36% pelleted.
agarose A-1.5m over this mol wt range (Fig. 3a). The use of high salt [250 mM (NH₄)₂SO₄] during gel filtration reduced aggregation as judged by the elimination of casein kinase activity at the exclusion volume (exclusion limit 120,000 daltons). From the elution volumes of known mol wt standards, a mol wt of 55,000 ± 5,000 SD was calculated (average and standard deviation of four different preparations). Although most (92 ± 4%, average and standard deviation of eight different preparations) of the soybean casein kinase does not bind to DEAE-cellulose in 25 mM (NH₄)₂SO₄ large quantities of contaminating protein are bound, thus permitting substantial purification (Table II). In 25 mM (NH₄)₂SO₄, 93 ± 5% (average and standard deviation of seven different preparations) of the casein kinase does bind to phosphocellulose and can be step-eluted quantitatively with 250 mM (NH₄)₂SO₄. Because elution of phosphocellulose columns with linear 25 to 500 mM (NH₄)₂SO₄ gradients did not resolve more than one casein kinase activity (Fig. 3b), step elution was used routinely for convenience and to maintain high enzyme concentrations.

The small fraction (8 ± 4%) of the casein kinase activity bound to DEAE-cellulose in 25 mM (NH₄)₂SO₄ would not bind to phosphocellulose in 25 mM (NH₄)₂SO₄. The DEAE-bound fraction phosphorylates soybean and calf thymus histones about three times more effectively than casein (19). Soybean histone kinase does bind to DEAE-cellulose (P. P. C. Lin, personal communication), and it is possible that much of the "casein kinase activity" observed in the DEAE-cellulose-bound fraction is attributable to histone kinase. In addition, the DEAE-cellulose-bound casein kinase fraction will support substantial phosphorylation in the absence of any additional substrate, indicating that this fraction is enriched for protein kinase substrates (19).

The purification scheme is summarized in Table II. About 14% of the total casein kinase activity present in the tissue was recovered after phosphocellulose chromatography. Purification at this stage was better than 500-fold, relative to the crude homogenate. The specific activity of 9,800 units/mg of protein is comparable to those attained in rat liver (6, 7, 24) and yeast (2). However, electrophoretic analysis on SDS-polyacrylamide gels indicated a heterogeneous array of polypeptides in the preparation and thus the preparation was not homogeneous (19). After phosphocellulose chromatography, casein kinase is stable for at least 6 months at 0 or -70°C if stored in the presence of phenylmethylsulfonylfluoride and 30% glycerol. All enzyme characterization was done on phosphocellulose-purified enzyme.

In the process of enzyme characterization it was later found that further purification to a specific activity of 28,000 units/mg of protein was possible with preparative isoelectric focusing. After the focusing step the casein kinase was less stable, perhaps due to the low protein concentration in the more highly purified preparation. However, casein kinase purified by isoelectric focusing was tested for effects on chromatin transcription.

**Casein Kinase Characterization.** Casein kinase shows a broad pH response with the maximal activity between pH 7 and 7.5; however, enzyme activity increases again above pH 8 (Fig. 4). Broad, bimodal pH curves have been observed in other systems and are believed to be caused by the increased solubility of casein at higher pH (2, 25). Five mM MgCl₂ is required for maximal activity, but 5 mM MnCl₂ will support about 30% of this (Fig. 5a). In contrast to the nuclear casein kinases described in Ascites tumors (5) and rat liver (7), soybean casein kinase is inhibited by monovalent cations when present along with optimal MgCl₂ concentrations (Fig. 5b). Because casein kinase assays involved a 40-fold dilution of the enzyme preparation, the (NH₄)₂SO₄ concentration had negligible effect on the analysis of column elution profiles. All final yields and specific activities were determined in assays containing less than 1 mM (NH₄)₂SO₄. Calculated recoveries from sucrose gradients containing (NH₄)₂SO₄ are based on the enzyme activity applied when assayed at the same salt concentrations present during analysis of the gradient fractions.

Substrate specificities are shown in Table III. Casein kinase activity is greater with casein, phosvitin, and heat-denatured chromatin than with histone or BSA as phosphate acceptors. Because casein kinase preparations incorporate no detectable ³²Pi in the absence of added substrate, they are free of endogenous protein kinase substrates. Under the standard assay conditions, no significant in vitro phosphorylation of purified soybean RNA polymerases I and II by casein kinase could be demonstrated. Phosphate donor specificity was assayed by attempting to compete out incorporation of [γ-³²P]ATP with excess unlabeled nucleotide triphosphates (Table IV). Addition of a 200-fold excess of unlabeled ATP lowers count incorporation proportionally, but excess GTP, CTP, or UTP has no effect. Thus, this casein kinase requires ATP as the phosphate donor. Cyclic AMP at 5 × 10⁻⁶ M had no effect on casein kinase activity, but was slightly inhibitory at 5 × 10⁻⁴ M.

**Table II. Summary of Soybean Casein Kinase Purification.** Chromatin was isolated from 7 kg of mucina-treated soybean hypocotyl.

<table>
<thead>
<tr>
<th>Purification Step</th>
<th>Total Units</th>
<th>% Recovery</th>
<th>Units/mg Protein</th>
<th>Purification Factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial homogenate</td>
<td>246,430</td>
<td>100</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>Chromatin</td>
<td>81,333</td>
<td>30</td>
<td>42</td>
<td></td>
</tr>
<tr>
<td>Proteasein sulfate</td>
<td>105,494</td>
<td>39</td>
<td>230</td>
<td></td>
</tr>
<tr>
<td>Ultrogel AC-44</td>
<td>78,333</td>
<td>29</td>
<td>960</td>
<td></td>
</tr>
<tr>
<td>DEAE-cellulose</td>
<td>67,333</td>
<td>25</td>
<td>6960</td>
<td></td>
</tr>
<tr>
<td>Phosphocellulose</td>
<td>37,400</td>
<td>14</td>
<td>9800</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 3. Gel filtration and phosphocellulose ion exchange chromatography of casein kinase solubilized from soybean chromatin. (a) Procedures for solubilization and Ultrogel AC-44 gel filtration are described under "Methods." Five µl of each fraction were assayed in the standard reaction mixture (●). In a separate experiment, the column was calibrated with the indicated proteins of known mol wt. After gel filtration, casein kinase was passed over a DEAE-cellulose column in buffer B containing 25 mM (NH₄)₂SO₄. Under these conditions greater than 90% of the activity was recovered in the DEAE flow-through fraction; (b) DEAE column flow-through was passed directly over a phosphocellulose column. At 25 mM (NH₄)₂SO₄ more than 90% of the activity bound. Casein kinase eluted quantitatively starting at 100 mM (NH₄)₂SO₄ when the phosphocellulose column was eluted with a 25 to 400 mM (NH₄)₂SO₄ gradient. In subsequent experiments, casein kinase activity was step-eluted from phosphocellulose with 250 mM (NH₄)₂SO₄.
SOYBEAN PROTEIN KINASE

FIG. 4. Casein kinase activity as a function of pH. Standard reaction mixtures (0.2 ml) were buffered with 50 mM Tris-maleate buffer at the indicated pH. Assays were initiated with 2.5 units of purified casein kinase. Values given are the means of three replicate assays at each point in three separate experiments. Values have been corrected for nonenzymatic incorporation at each point.

The standard reaction using 100 μg of casein and 7 units of casein kinase/0.2-ml reaction is linear with time up to 10 min and begins to plateau by about 60 min (Fig. 6). Incorporation ceases when a 50-fold excess of cold ATP is added after the reaction has proceeded for 30 min but the amount of 32Pi incorporated does not decrease. Maintenance of a stable level of 32Pi incorporation after isotope dilution indicates the absence of significant phosphate turnover. Hence, the kinase preparation is low in phosphoprotein phosphatase activity. Casein kinase gives linear Lineweaver-Burk plots when assayed with ATP concentrations varying from 5 to 100 μM (Fig. 7). The apparent $K_m$ for ATP under these conditions was 7.9 μM. Similar analysis gave an apparent $K_m$ for casein of 2.4 mg/ml (19).

 Isoelectric Focusing. Soybean casein kinase is resolved into two main activity peaks (pI 8.6 and 9.2) by preparative isoelectric focusing (Fig. 8). Since crude casein kinase preparations aggregate at the low ionic strengths required for isoelectric focusing, the two

Table III. Soybean Casein Kinase Substrate Specificity. Assays contained 37 μg of each of the listed substrates and 5 units purified casein kinase. RNA polymerases were assayed in smaller volumes (10 μl in 0.5 ml with 1.25 units casein kinase). Means and standard deviations of three replications are shown.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>pmol [32P] incorp.</th>
<th>% Casein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein</td>
<td>550 ± 63</td>
<td>100</td>
</tr>
<tr>
<td>Phosvitin</td>
<td>1345 ± 59</td>
<td>244</td>
</tr>
<tr>
<td>Soybean histone</td>
<td>57 ± 2</td>
<td>10</td>
</tr>
<tr>
<td>Calf histone</td>
<td>47 ± 2</td>
<td>8</td>
</tr>
<tr>
<td>Denatured chromatin</td>
<td>257 ± 15</td>
<td>47</td>
</tr>
<tr>
<td>Bovine serum albumin</td>
<td>14 ± 1</td>
<td>1</td>
</tr>
<tr>
<td>Soybean RNA polymerase I</td>
<td>&lt; 1</td>
<td>&lt; 0.1</td>
</tr>
<tr>
<td>Soybean RNA polymerase II</td>
<td>&lt; 1</td>
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</tr>
<tr>
<td>None</td>
<td>&lt; 1</td>
<td>&lt; 0.1</td>
</tr>
</tbody>
</table>

Table IV. Effect of Various Additions on Soybean Casein Kinase Activity. Standard assay mixtures with 25 μM [γ-32P]ATP were modified to contain the indicated additions and incubated for 30 min with 3 units of purified casein kinase. Means and standard deviations of three replications are shown.

<table>
<thead>
<tr>
<th>Addition</th>
<th>Conc. (M)</th>
<th>cpm [32P] incorp.</th>
<th>% control</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>-</td>
<td>5327 ± 116</td>
<td>100</td>
</tr>
<tr>
<td>3', 5' -cAMP</td>
<td>$5 \times 10^{-4}$</td>
<td>4331 ± 320</td>
<td>80.9</td>
</tr>
<tr>
<td>3', 5' -cAMP</td>
<td>$5 \times 10^{-6}$</td>
<td>3408 ± 398</td>
<td>101.0</td>
</tr>
<tr>
<td>ATP</td>
<td>$5 \times 10^{-3}$</td>
<td>26 ± 7</td>
<td>0.5</td>
</tr>
<tr>
<td>GTP</td>
<td>$5 \times 10^{-3}$</td>
<td>4568 ± 429</td>
<td>85.6</td>
</tr>
<tr>
<td>CTP</td>
<td>$5 \times 10^{-3}$</td>
<td>4628 ± 246</td>
<td>90.6</td>
</tr>
<tr>
<td>UTP</td>
<td>$5 \times 10^{-3}$</td>
<td>4923 ± 195</td>
<td>92.4</td>
</tr>
</tbody>
</table>

FIG. 5. Effect of divalent and monovalent cations on soybean casein kinase activity. (a) Purified casein kinase (2.5 units) was assayed in the standard reaction mixture containing casein and the indicated concentrations of M₂Cl₂ or MnCl₂; or (b) constant 10 mM MgCl₂ and, in addition, the indicated salt concentrations. Means of three replications are shown.

FIG. 6. Time course for the phosphorylation of casein by casein kinase. Seven units of purified casein kinase were incubated in the standard reaction mixture for the indicated times. After 30-min incubation, either a 100-fold excess unlabeled ATP or an equal volume of H₂O was added. Values have been corrected for dilution and the means of three replications are shown.
peak fractions were compared on sucrose gradients in the presence and absence of 250 mM (NH₄)₂SO₄. Both the pI 8.6 and 9.2 activity peaks from the focusing column resolve into several activity peaks under low salt conditions (Fig. 9a) and only 57% of the pI 8.6 and 76% of the pI 9.2 fractions applied to the gradients could be accounted for in the gradient. In the presence of 250 mM (NH₄)₂SO₄ both fractions show casein kinase activity at about 3.7S and all of the applied activity could be accounted for in the gradient (Fig. 9b). Analysis of samples taken across the focusing gradient by SDS-polyacrylamide electrophoresis indicated that three proteins with mol wt of 56,000, 66,000, and 67,000 occurred in every fraction taken (19). While isoelectric focusing did give substantial purification, it is probable that the observed isoelectric points reflect the focusing of aggregates of casein kinase and various protein contaminants. Attempts to prevent aggregation by the inclusion of urea during the focusing step lead to loss of activity.
Effect of Casein Kinase on Chromatin Transcription. In view of reports that increased phosphorylation of chromatin results in higher template transcription (refs. 13 and 26) and that the activity of RNA polymerase is modified by phosphorylation (5, 9), soybean casein kinase was tested for its effect on in vitro chromatin transcription. In vivo transcription of chromatin isolated from auxin-treated or untreated tissue is enhanced by addition of casein kinase purified through phosphocellulose chromatography or through isoelectric focusing (Table V). However, heat-denatured casein kinase preparations also stimulate in vitro chromatin transcription by endogenous RNA polymerase. Hence, the observed stimulation of transcription probably is artificial and not attributable to phosphorylation of chromatin protein(s) by casein kinase. Exogenous casein kinase also failed to produce a significant increase in the endogenous level of chromatin protein phosphorylation.

DISCUSSION

Soybean contains at least two distinct protein kinase activities: a histone kinase with a specificity for lysine-rich histone H1 (16), and a casein kinase activity described here. While casein kinase activities were detected in all subcellular fractions only partial purification and characterization of the chromatin-associated casein kinase activity were undertaken. Chromatin isolated from auxin-treated hypocotyls provides a good source for the purification of the presumptive nuclear casein kinase. The purification scheme using gel filtration and ion exchange chromatography on DEAE-cellulose and phosphocellulose results in a 500-fold purification of soybean casein kinase over the initial extract. The phosphocellulose-purified casein kinase is free of casein kinase substrates and phosphoprotein phosphatase activity although the enzyme was not homogeneous. The specific activity of 9800 units/mg of protein is comparable to that achieved for comparable enzymes from yeast (2) and rat liver (7, 25). These procedures have also been used to isolate a similar casein kinase from cauliflower (20).

Because of their tendency to aggregate under conditions of low ionic strength, chromatin proteins are difficult to work with. Aggregation of soybean casein kinase makes purification on ion exchange resins difficult and complicates interpretation of the possibility of multiple casein kinase activities. Gel filtration at high ionic strength [250 mM (NH₄)₂SO₄] permitted preliminary fractionation under conditions unfavorable for aggregation. Aggregation was less serious after gel filtration, presumably because much of the protein that coaggregates with the casein kinase had been removed in the voided fractions. Ion exchange chromatography on DEAE-cellulose and phosphocellulose required conditions of low ionic strength which may have limited efficacy by permitting aggregation, but these procedures did give substantial purification. Further purification by preparative isoelectric focusing resulted in an additional 3-fold purification and resolved two casein kinase activities with isoelectric points of 8.6 and 9.2. The low ionic strength required for focusing may have favored aggregation and sedimentation on sucrose gradients in the presence of 250 mM (NH₄)₂SO₄ revealed primarily one casein kinase activity at about 3.7S. Similar results were obtained when crude casein kinase extracts from various subcellular fractions were analyzed.

Some confusion exists as to the number of protein kinase activities present in nuclei. As many as 12 such activities have been reported in beef liver nuclei (12). However, the latter study did not consider the possible presence of endogenous protein kinase substrates in the fractions, nor did it rule out the possibility of enzyme aggregation. Careful studies by Desjardins et al. (6) indicated that the two cAMP-independent nuclear casein kinases in rat liver nuclei aggregate at low ionic strength to produce additional artificial activities. Aggregation of rat liver casein kinases as well as soybean casein kinase is probably due to nonspecific aggregation with contaminating proteins rather than the formation of casein kinase multimers. Similarly, only two nuclear casein kinases have been reported in chick oviduct nuclei (10). Whether the faster sedimenting species of soybean casein kinase observed at low ionic strength may include regulatory subunits or otherwise have functional significance is unknown. Taking aggregation into account, no argument for the presence of more than one chromatin-associated casein kinase activity in soybean can be made at present. However, the possibility that more protein kinase activities would be detected using other substrates cannot be excluded. The mol wt estimate of about 55,000 is within the range reported for nuclear casein kinases in rat liver (6, 25), Ascites tumors (5), yeast (2), and chick oviduct (10).

Soybean casein kinase shows many similarities to nuclear casein-type kinases described in a variety of other systems (2, 5, 6, 10, 25). First, based on the high specific activities observed in intact nuclei and chromatin, the enzyme appears to be chromatin-associated, but it may be removed by relatively mild conditions [250 mM (NH₄)₂SO₄]. Second, soybean casein kinase has a specific requirement for ATP as the phosphate donor. However, the observed Kₘ of 7.9 μM with the optimal Mg²⁺ concentration is lower than for nuclear casein kinases from rat liver (6, 25), or chick oviduct (10). Third, soybean casein kinase will phosphorylate casein, phosvitin, and soybean chromosomal protein but not histone. Finally, as generally observed for animal nuclear casein kinases, phosphorylation by soybean casein kinase is not affected by cAMP. To date, no unequivocal evidence for the existence of cAMP or cAMP-dependent protein kinases has been found in higher plants (1).

Detailed studies on soybean chromatin protein phosphorylation indicate that increases in chromatin protein phosphorylation and chromatin-associated casein kinase activity are correlated with auxin-enhanced RNA synthesis (21). Data in other systems suggest that chromatin protein phosphorylation may alter chromatin template availability for transcription (10, 13; refs. in 26). Results presented here indicate that addition of exogenous casein kinase to chromatin from either auxin-treated or untreated tissue resulted in little or no increase in chromatin protein phosphorylation over that supported by the endogenous kinase coisolated with the chromatin. Since the level of chromatin-bound casein kinase activity is not limiting in either preparation, other mechanisms such as differential susceptibility of chromosomal proteins to phosphorylation may explain the higher levels of phosphate incorporation in chromatin from auxin-treated tissue.

Reports that protein kinase stimulates in vitro transcription by RNA polymerases I and II suggest that RNA polymerase activity in vivo may be regulated by phosphorylation (8, 9; refs. in 26). The soybean in vitro transcription data presented here are subject to several limitations. First, because the system will not reinitiate in vitro, only the level of elongation of RNA chains by endogenous RNA polymerase is measurable. Second, the assay system was optimal for RNA polymerase I activity. In vitro chromatin transcription was stimulated by some factor present in the purified casein kinase preparations. However, the observed enhancement of RNA synthesis cannot be attributed to casein kinase activity because heat-inactivated casein kinase also stimulated RNA synthesis. Bell et al. (2) have rigorously shown that yeast RNA polymerases I and III are phosphorylated in vivo and can be phosphorylated in vitro by a yeast protein kinase having properties similar to those of soybean casein kinase. However, they were unable to detect any effect of phosphorylation on any of the RNA polymerase activities on purified DNA templates.

In conclusion, a casein kinase bearing similar properties to those isolated in animal systems can be isolated from soybean chromatin. Higher levels of casein kinase activity are associated with transcriptionally active (auxin-treated) chromatin than from untreated chromatin. However, the purified casein kinase prepara-
tion could not be shown to affect directly in vitro chromatin transcription.

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