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Histone Kinase from Soybean Hypocotyls

PURIFICATION, PROPERTIES, AND SUBSTRATE SPECIFICITIES[1]

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PAUL P.-C. LIN[2] AND JOE L. KEY
Departments of Biochemistry and Botany, University of Georgia, Athens, Georgia 30601

ABSTRACT

A histone-type protein kinase (EC 2.7.1.37) has been partially purified (320-fold) from the crude extracts of soybean hypocotyls by means of a combination of gel filtration and anion exchange procedures. The purified enzyme fraction is devoid of the activities of phosphoprotein phosphatase (EC 3.1.3.16), histone protease, and casein (or phosvitin)-type kinase. The soybean histone kinase uses ATP to phosphorylate specifically lysine-rich histone H1 from either pea seedlings or calf thymus.

The histone kinase requires free sulfhydryl group(s) for activity, but not stability. The pH optimum is around 9 to 10. The apparent K_m values for histone H1 of pea seedlings and calf thymus are 0.4 and 0.9 micromolar, respectively. The K_m values for ATP are 40 nanomolar with the optimal concentration of Mn^{2+} (50 nanomolar) and 0.4 micromolar with that of Mg^{2+} (5 millimolar). The estimated molecular weight of the kinase is 52,000 by gel filtration or 48,600 by sedimentation constant (3.2 S). cAMP does not alter the sedimentation velocity of the kinase. The enzyme activity is unaffected by cyclic nucleoside monophosphates and plant growth substances. Like arginine-rich histones, a variety of divalent cations and polycations (polyamines) are inhibitory.

This cAMP-independent soybean histone kinase is not associated with the isolated ribosomes but shows highest specific activity in the nuclear-chromatin fraction, suggesting that it may function in the regulation of histone H1 phosphorylation in the soybean hypocotyl.

Like other types of reversible covalent modification of protein structure, such as oxidation-reduction of sulfhydryl groups and adenylation-deadenylation, protein phosphorylation-dephosphorylation has been implicated as an important mechanism in controlling the activities of a variety of key metabolic enzymes and the action of many regulatory proteins in mammalian cells (10, 18, 31, 32, 34). Additionally, phosphorylation-dephosphorylation of histones and nonhistone chromosomal acidic proteins may be crucial in regulating the structure and function of chromatin (8, 13, 16). Recently, Randall and his colleagues (30) have shown that the activity of pyruvate dehydrogenase complex from broccoli mitochondria can be modulated by phosphorylation as it is in mammalian cells. A number of investigators have reported that treatments with plant growth substances affect *in vivo* protein phosphorylation (5, 25, 29, 39) and that protein phosphorylation occurs in various subcellular fractions in higher plants (5, 36, 37, 39, 40), including chloroplast thylakoid membrane proteins (2, 3). Although all of these results seem to suggest a major role of protein phosphorylation in the control of metabolic processes and cellular functions in higher plants, research in this potentially important area is still at a primitive stage. We do not know much about the complexities and properties of the enzymes involved in catalyzing the protein phosphorylation-dephosphorylation reaction, nor do we know anything about the mechanisms which regulate the activities of these enzymes in higher plants.

The protein phosphorylation-dephosphorylation reaction is catalyzed by the two enzymes phosphoprotein kinase (EC 2.7.1.37) and phosphoprotein phosphatase (EC 3.1.3.16). Based on *in vitro* protein substrate specificity, phosphoprotein kinases can be generally classified into two major types: histone-type kinase and casein (or phosvitin)-type kinase. The histone-type kinases can be further divided into two general groups: cAMP (or cGMP)-dependent kinase and cAMP-independent kinase. The activities of casein (or phosvitin)-type protein kinases in general are refractory to cyclic nucleotides. All these classifications are primarily based on the enzymes so far characterized from animal tissues (18, 32). In higher plants, a number of investigators have demonstrated the presence of casein-type protein kinase activities which are also unaffected by cyclic nucleotides (4, 14, 15, 26). There apparently has been no characterization of histone-type protein kinase from higher plants. The study of protein phosphorylation, particularly histone-type protein kinase, may also yield evidence for or against a functional role of cAMP in higher plants.

Here, we describe the purification and properties of an activity of protein kinase extracted from the soybean hypocotyl, which phosphorylates specifically lysine-rich histone H1. The activity of soybean histone kinase is unaffected by cyclic nucleotides but is inhibited by polycations such as arginine-rich histones and polyamines. A preliminary account of parts of the present results has been published (20), which clearly indicates that the apparent difficulty of demonstration of this particular histone kinase activity in the soybean hypocotyl is primarily due to the strongly inhibitory effects of arginine-rich histones (or histone-like compounds) present in the histone substrates generally used for assay of the enzyme activity.

EXPERIMENTAL PROCEDURES

MATERIALS

Soybean seeds (*Glycine max* var. Wayne) were obtained from Noble Brothers Seeds Co., Chicago. Cyclic nucleoside monophosphates, nucleotides, casein, and phosvitin were purchased from Sigma Chemical Co. Total histone mixtures and histone subfractions enriched in certain histone species from calf thymus were purchased from Sigma and Worthington; Sephadex products from Pharmacia; ammonium sulfate (enzyme-grade) and sucrose (RNase-free) were from Schwarz/Mann; Bio-Gel products, Bio-Rax 70, electrophoretic pure acrylamide, and bisacrylamide were from Bio-Rad; carrier-free [^{32}P]orthophosphoric acid was from

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[2] Present address: Department of Agronomy, University of Kentucky, Lexington, Ky. 40546. To whom correspondence should be addressed.

New England Nuclear. [γ-^{32}P]ATP was prepared as described under "Methods."

METHODS

Plant Tissue. After pretreatment with 10% NaOCl for 25 min and soaking in H_2O, soybean seeds were germinated under darkness in moist Vermiculite at 29 C for 3 days and then sprayed with 2.5 mM 2,4-D (pH 6.0). Mature hypocotyl tissue was harvested 36 h after 2,4-D treatment and chilled in an ice bath for enzyme preparation.

Assay of Protein Kinase Activity. Unless otherwise stated, the activity of histone kinase was routinely determined in a 0.2-ml reaction mixture containing 5 mM $MgCl_2$, 5 μM [γ-^{32}P]ATP (125–150 cpm/pmol), and 20 μg histone in buffer A (20 mM Tris-Cl [pH 8.0] and 5 mM 2-mercaptoethanol). The activity of phosvitin (or casein)-type kinase was assayed in the reaction mixture (0.2 ml) which contained 10 mM $MgCl_2$, 25 μM [γ-^{32}P]ATP, and 50 μg protein substrate in addition to buffer A. The reactions were initiated by the addition of 0.02 ml enzyme solution. After incubation at 28 C for 20 min, the reactions were terminated by the addition of 6 ml chilled trichloroacetic acid containing 10 mM Na-pyrophosphate. Trichloroacetic acid (20%) was used to precipitate histone substrates and protamine, whereas the acid (10%) was used for precipitation of casein, phosvitin, and other acidic protein substrates. Each precipitate was collected on a Whatman GF/A filter, washed with five aliquots (3 ml each) of 5% trichloroacetic acid, and then washed with 3 ml 95% ethanol. The filter was completely dried under a heat lamp and then put into a scintillation vial. To each vial, 5 ml of a toluene-based scintillation fluid were added and radioactivity was determined by liquid scintillation spectrometry. Under the standard assay conditions, a background of less than 70 cpm/filter was generally obtained in the assay with or without addition of heat-denatured enzyme preparation. One unit of activity of protein kinase was defined as that amount of enzyme which catalyzes the transfer of 1 pmol/min of the terminal phosphoryl group from ATP to a protein substrate. Specific activity was defined as units/mg protein.

Purification of Histone Kinase. All purification procedures were carried out at 0 to 4 C. Routinely, 100 g mature soybean hypocotyls were homogenized in a Waring Blendor at top speed for 1 min with 100 ml buffer A containing 0.25 M NaCl and 0.25 M $(NH_4)_2SO_4$. The homogenate was squeezed through four layers of cheesecloth and subsequently filtered through Miracloth. The filtrate was centrifuged at 10,000g for 10 min. The resulting supernatant (134 ml) was brought to 50% saturation with ammonium sulfate and stirred for at least 2 h prior to centrifugation at 10,000g for 20 min to recover the pellet. The pellet, containing most of the activity of histone kinase, was dissolved in 38 ml buffer A and centrifuged at 105,000g for 1 h to remove the bulk of aggregated material. The resulting high speed supernatant (7 ml each) then was loaded onto a buffer A-equilibrated Sephadex G-150 column (2.5 × 105 cm) and eluted with the same buffer in 5-ml fractions. The fractions were assayed for activities of protein kinases on casein, histone H1, and total histone mixture. The peak fractions containing the activity of histone kinase were pooled and loaded directly onto a DEAE-Sephadex A-25 column (1.5 × 15 cm) equilibrated with buffer A. The column was washed with 75 ml buffer A and then eluted in 5-ml fractions with a linear salt gradient of 0 to 0.5 M NaCl in the same buffer. Fractions having the activity of histone kinase eluted in a single peak were combined and reduced in volume to approximately 7 ml by ultrafiltration with an Amicon PM-10 membrane. The concentrated enzyme solution then was applied to a Sephadex G-100 column (1.5 × 100 cm) that had been equilibrated with buffer A. The activity of histone kinase was eluted in 5-ml fractions with the same buffer. The resulting peak fractions of histone kinase activity were pooled and concentrated to approximately 5 ml by the Amicon membrane filter for study of the properties of the kinase.

General Methods. Carrier-free [γ-^{32}P]ATP was synthesized enzymically and purified as described (33). The purity of [^{32}P]ATP was confirmed by polyethyleneimine-cellulose TLC; contamination of [^{32}P]Pi was less than 6%. The concentrations of histone were determined by A at 230 nm, according to 3.5 O.D. units for 1 mg/ml histone (22). Quantitations of proteins other than histone were conducted according to the Lowry method (23) with BSA as standard. Purifications of histone subfractions from pea seedlings and calf thymus were accomplished by the methods described (12, 22). The purity of each histone subfraction was assured by urea/acetic acid-polyacrylamide gel electrophoresis (27). Total nonhistone chromosomal acidic protein was extracted from the chromatin isolated from soybean hypocotyls and separated from histones by Bio-Rad 70 (22). From rat liver cytosol, the cAMP-dependent histone kinase was partially purified according to Chen and Walsh (7) with minor modification: the enzyme was fractionated by ammonium sulfate prior to chromatography on a DEAE-Sephadex A-25 column. Phosphoprotein phosphatase was isolated from the soybean hypocotyl and purified as described in the following paper (21). The determination of the sedimentation constant of histone kinase was done by sucrose density gradient centrifugation according to Martin and Ames (24).

RESULTS

SUBCELLULAR DISTRIBUTION OF THE ACTIVITY OF HISTONE KINASE

All of the subcellular fractions of soybean hypocotyl tissue contain the activity of histone kinase toward histone H1 (Table I). Nearly half of the total enzyme activity is present in the cytosolic fraction (105,000g supernatant) as a soluble form, whereas the specific activity of the enzyme in the nuclear fraction is about 3 times that observed in the cytosolic fraction. A similar distribution of the enzyme activities in the subcellular fractions is observed for the total histone mixture as substrate, although the phosphorylating activity is much lower. In addition, the activity of this particular histone kinase, which prefers histone H1 as substrate, is also associated with the chromatin isolated from the soybean hypocotyl (data not shown). The activity of this histone kinase is not detectable in highly purified ribosomes from the soybean hypocotyl; the ribosome preparation apparently contains a distinct activity of protein kinase which phosphorylates acid-extracted basic ribosomal proteins, not histones (J. Hammet, personal communication).

Table I. *Subcellular Distribution of the Activity of Soybean Histone Kinase*

One hundred g mature soybean hypocotyls were homogenized with 100 ml 20 mM Tris-Cl (pH 8.0) containing 5 mM DTT. Phosphorylating activity with endogenous substrates was subtracted from the total kinase activity assayed with purified calf thymus histone H1 (0.1 mg/ml) as substrate. Activity expressed as pmol phosphate incorporated/min.

Fraction	Total Protein	Enzyme		
		Total Activity	Specific Activity	Yield
	mg	*units*	*units/mg*	*%*
Crude homogenate (low salt)	680	10,200	15	100
600g (pellet)	36	1,130	31	11
10,000g (pellet)	63	1,550	25	15
105,000g (pellet)	160	1,630	10	16
105,000g (supernatant)	412	4,560	11	45

PURIFICATION OF SOYBEAN HISTONE KINASE

Table II is a summary of the overall purification of the activity of histone kinase (histone H1 as substrate) from 100 g soybean hypocotyls. The enzyme is purified over 300-fold to a specific activity of 7.7 nmol ^{32}P incorporated from [γ-^{32}P]ATP into histone H1/min·mg protein at 28 C.

Solubilization and Ammonium Sulfate Fractionation. Including high salt in the homogenization buffer results in about 60% increase in both total and specific activities of histone kinase in the crude extracts. Evidently, the activity of histone kinase can be effectively solubilized from the particulate fractions (Table I) by 0.2 M NaCl and 0.2 M $(NH_4)_2SO_4$ in buffer A. Most of the solubilized kinase activity on either histone H1 or total histone mixture can be precipitated by 50% $(NH_4)_2SO_4$ and subsequently recovered by redissolving in low-salt buffer A. Further purification by centrifugation at 105,000*g* for 1 h recovers 84% of the total activity of histone kinase in the supernatant and represents a 1.5-fold purification.

Gel Filtration on Sephadex G-150. Chromatography of the above 105,000*g* supernatant on a Sephadex G-150 column in low-salt buffer A yields a clear separation of the major activity of histone-type protein kinase from that of phosvitin (or casein)-type protein kinase, as well as from the majority of protein (Fig. 1A). Essentially, an identical elution profile of the activity of histone kinase is observed irrespective of the type of histone (histone H1 or total histone mixture) used as protein substrate. The activity of histone kinase on the total histone mixture (Sigma type II) is only about 15% of that on purified histone H1. The minor peak (high mol wt) of the activity of histone kinase appears to be the aggregate form of the major peak (low mol wt), as judged by the fact that both fractions behave similarly with respect to substrate specificity, insensitivity to cyclic nucleotides, and binding characteristics to DEAE-Sephadex A-25 (will be described later). The pooled fractions (No. 45-55) of the major peak of the activity of histone kinase still contain phosphoprotein phosphatase activity which is predominately eluted in the fractions of 55 to 65 and which is able to dephosphorylate phosphohistones (21). Nevertheless, the phosphatase activity is removed in the later stages of purification. Chromatography on Sephadex G-150 recovers 72% of the total input activity of histone kinase in the major peak and results in a 10-fold increase in the enzyme specific activity.

Fractionation on DEAE-Sephadex A-25. The major peak activity of histone kinase pooled from Sephadex G-150 was fractionated on a DEAE-Sephadex A-25 column with a linear salt gradient. The activity of histone kinase is mainly eluted with 0.2 to 0.3 M NaCl in a single peak and the enzyme activity on total histone mixture is eluted coincidentally with that on histone H1 (Fig. 1B). This step gives an 80% yield and a 6-fold increase in the specific activity of histone kinase. Any original contamination with activity of casein (or phosvitin)-type protein kinase in the histone kinase preparation from Sephadex G-150 would be eluted in the run-off fractions because it does not bind to DEAE-Sephadex A-25 under the experimental conditions. Most contaminating activity of phosphoprotein phosphatase from Sephadex G-150 is eluted by 0.32 M NaCl and, therefore, is almost removed from the peak activity of histone kinase eluted from DEAE-Sephadex. Additionally, there is no detectable activity of histone kinase in the run-off fractions. Concentration by ultrafiltration with Amicon PM-10 membrane filter recovers more than 90% of the pooled activity of histone kinase eluted from DEAE-Sephadex. An identical elution pattern from DEAE-Sephadex columns is observed for both the major and minor pooled peaks of histone kinase activity (Fig. 1A).

Chromatography on Sephadex G-100 and Sucrose Density Gradient Centrifugation. Chromatography of the concentrated activity of histone kinase from DEAE-Sephadex on a Sephadex G-100 column results in a single symmetrical peak of enzyme activity on both histone H1 and total histone mixture (Fig. 2A). The enzyme corresponds to a protein mol wt of about 55,000. There is a 2-fold purification of the enzyme but only 30% recovery of the activity of histone kinase from Sephadex G-100 column chromatography. The loss of the enzyme activity may be due to protein dilution during the chromatography. However, we have not checked whether addition of 30 to 50% glycerol will prevent such an inactivation. Further concentration by ultrafiltration recovers most of the activity of histone kinase pooled from the Sephadex G-100 fractions. The preparation of histone kinase purified up to this step contains no detectable activity of phosphoprotein phosphatase or protease toward histone H1. Sucrose density gradient centrifugation of the final enzyme preparation reveals a single symmetrical peak of activity of histone kinase which has a sedimentation constant of 3.2 S and an estimated mol wt of 48,600 (Fig. 2B). Again, the enzyme activities on both histone H1 and total histone mixture coincidentally sedimented in an identical pattern.

PROPERTIES OF SOYBEAN HISTONE KINASE

Stability. The results in our preliminary report (20) have indicated that freezing and thawing totally inactivated the partially purified histone kinase, even at early stages of purification. The final concentrated histone kinase preparation, however, remained nearly stable at 4 C for more than 1 month. Removal of the reducing agent from the kinase solution immediately lowers the enzyme activity, which can be restored effectively (85% in 20 min) by adding 2 to 5 mM 2-mercaptoethanol or DTT to the enzyme solution. Iodoacetate and *N*-ethylmaleimide at 5 mM reduce the

Table II. *Purification of Histone Kinase from Soybean Hypocotyls*

Histone kinase was partially purified from 100 g 36-h 2,4-D-treated soybean hypocotyls in a high salt buffer as described. Purified calf thymus histone H1 (0.1 mg/ml) was used as substrate. Activity expressed as pmol phosphate incorporated/min.

Fraction	Total Protein	Enzyme			
		Total Activity	Yield	Specific Activity	Purification
	mg	*units*	*%*	*units/mg*	*-fold*
Crude homogenate (high salt)	697	16,700	100	24	1
50% $(NH_4)_2SO_4$	376	16,900	100	45	2
105,000*g* (supernatant)	210	14,100	84	67	3
Sephadex G-150	15	10,100	61	675	28
DEAE-Sephadex A-25	2	8,430	50	4,210	176
Sephadex G-100	0.30	2,200	13	7,450	310
Amicon PM-10 membrane (concn.)	0.28	2,170	13	7,760	324

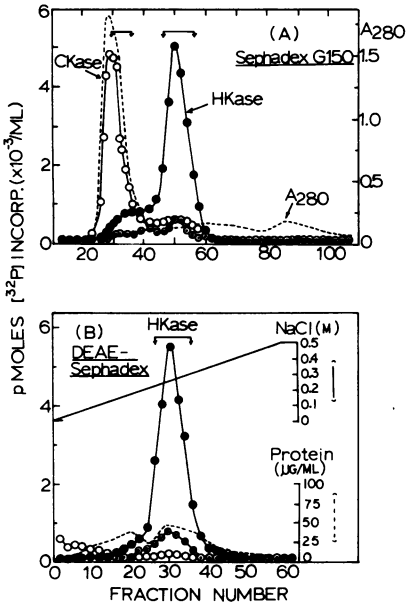

FIG. 1. Fractionation of the activity of soybean histone kinase on Sephadex G-150 and DEAE-Sephadex A-25. A: the 105,000*g* supernatant fraction of soybean histone kinase was subjected to chromatography on Sephadex G-150. Fractions of 0.02 ml each were assayed for the activities of protein kinases toward purified histone H1 (●——●), total histone mixture (⊙——⊙), and casein (○——○). The bars indicate the fractions pooled as the minor peak (No. 29-36) and the major peak (No. 45-55) of the activity of histone kinase (HKase). CKase: casein-type protein kinase. B: the major peak activity of histone kinase from Sephadex G-150 was pooled and fractionated on DEAE-Sephadex A-25 by a linear NaCl gradient. Assays of the enzyme activity are described under A. The bar indicates the fractions of the enzyme activity pooled for further purification.

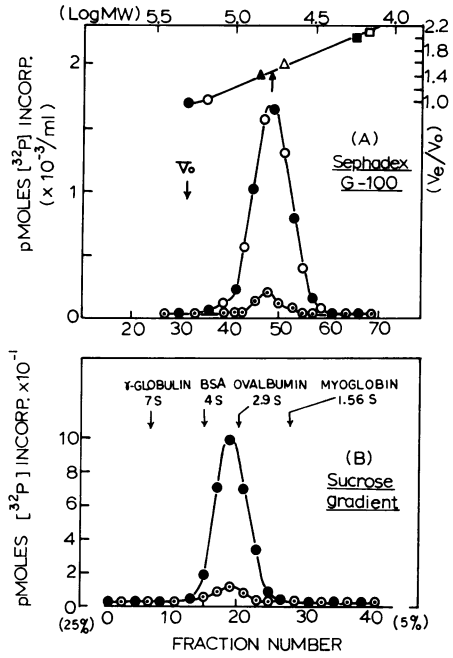

FIG. 2. Gel filtration on Sephadex G-100 and sucrose density gradient centrifugation of soybean histone kinase. A: the activity of histone kinase pooled from DEAE-Sephadex A-25 was concentrated by Amicon PM-10 membrane and chromatographed on Sephadex G-100. Assays of the enzyme activity are described in Fig. 1A. The protein substrates: histone H1 without (●——●) or with (○——○) the presence of 1 µM cAMP; total histone mixture (○——○). Protein mol wt markers: γ-globulin, 160,000 (○); BSA, 68,000 (▲); ovalbumin, 45,000 (△); myoglobin, 17,800 (■); and Cyt *c*, 12,400 (□). Blue dextran (mol wt, 2,000) (●) was used for estimation of void volume (V_o). Ve is elution volume. B: the elution profile of the activity of soybean histone kinase from sucrose density gradient centrifugation. The concentrated enzyme preparation (0.15 ml) from Sephadex G-100 was layered over a 5 to 25% sucrose density gradient (4.8 ml buffer A as described in the text). Centrifugation was performed with a Beckman SW 50.1 rotor in a Beckman L5-75 ultracentrifuge at 48,000 rpm for 18 h at 4 C. After centrifugation, 10-drop fractions (total of 41 fractions) were collected as described previously (22) and assayed for the activity of histone kinase toward histone H1 (●——●) and total histone mixture (⊙——⊙). The sedimentation positions of marker proteins as noted were determined separately.

enzyme activity by 50 and 75%, respectively, indicating that the histone kinase requires free sulfhydryl group(s) for activity.

Reaction Rate and pH Optimum. The extent of protein phosphorylation is essentially linear with respect to the incubation time over a period of 30 min at 28 C (Fig. 3A). The addition of 1.0 mM unlabeled ATP during the time course only levels off but does not reduce the total amount of phosphorylation, indicating that the exchange reaction of phosphate, if any, is minimal under the assay conditions. Additionally, there is an approximately linear relationship between phosphorylating activity and enzyme concentration over a range of 0 to 1.3 µg protein/0.2 ml of reaction mixture. Further increases in enzyme concentration result in deviation from linearity. Soybean histone kinase, like many histone kinases so far characterized from animal tissues (18, 32), also exhibits maximal activity at alkaline pH; the phosphorylating activity of the enzyme increases exponentionally from pH 6.5 to 9.0 and levels off from pH 9.0 to 10.5 (Fig. 3B). A similar pH effect on the phosphorylating activity occurs with either histone H1 or total histone mixture as substrate. The amounts of phosphorylation of the total histone mixture is 15 to 20% of that of histone H1, irrespective of pH from 6.5 to 10.5.

Effects of Metal Ions. A variety of metal ions can satisfy the requirement for a divalent cation. The concentration of cation is critical to the enzymic activities exhibited by many kinases because a divalent cation complex of ATP is, in fact, the enzyme substrate. The optimal concentrations of Mg^{2+} and Mn^{2+} are 5 mM and 50 µM, respectively (Fig. 4A). The maximal phosphorylating activity obtained with Mn^{2+} is about 60% of that with Mg^{2+}. At 2.5 and 5.0 mM, Fe^{2+} is equally effective as Mg^{2+}. Additionally, Co^{2+} at 1.0 mM shows a maximal effect as Mn^{2+}. Although Ca^{2+}, Zn^{2+}, or Hg^{2+} alone cannot support the kinase activity, it (K_i, about 5 mM) significantly inhibits the phosphorylating activities assayed in the presence of optimal concentrations of Mg^{2+} and Mn^{2+} (Fig. 4B). Higher concentrations of monovalent cations inhibit the phosphorylating activity; 50% inhibition by NH_4^+ and Na^+ occurs at 50 and 250 mM, respectively (Fig. 4C).

Specificity of Nucleotide Substrate. The histone kinase partially purified from soybean hypocotyls can transfer effectively the terminal phosphoryl group of ATP to the serine and/or threonine moieties of histone H1. The competition experiment on the influence of the concentration of nucleotide upon the phosphotransferring activity clearly demonstrates that GTP, UTP, and CTP are poor substitutes for ATP as phosphoryl donors (Fig. 5A). A separate experiment with [γ-^{32}P]GTP, prepared as described for [γ-^{32}P]ATP under "Methods," as nucleotide substrate indicates that GTP cannot effectively support the kinase to phosphorylate histone H1 and other histone species. Figure 5, B and C show the typical hyperbolic plots for the activity of histone kinase as a function of ATP concentration. The double reciprocal plots of 1/V *versus* 1/S show that apparent K_m values for ATP are 0.4 and 0.04 µM for the assays with the optimal concentrations of Mg^{2+} and Mn^{2+}, respectively.

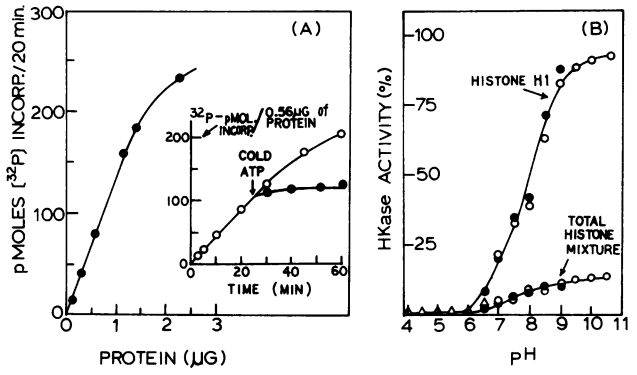

FIG. 3. The activity of soybean histone kinase as a function of protein concentration, time course, and pH. A: the enzyme activity was assayed with various protein concentrations of the kinase preparation. Histone H1 was the substrate. In the time-course experiment, the standard reaction mixture contained 5 μM [γ-^{32}P]ATP, 20 μg histone H1, and 0.56 μg protein of the kinase preparation; 0.02 ml of 10 mM unlabeled ATP was added at 25 min and the enzyme activity was determined for another additional 35 min as noted: plus cold ATP (●——●) and control (○——○). B: the effect of pH on activity of soybean histone kinase (HKase) toward histone H1 (50 μg/ml) and total histone mixture (100 μg/ml). The standard reaction mixture contained 0.45 μg protein of the kinase preparation and 50 mM K-acetate (△), Tris-Mes (●), or Tris-HCl (○).

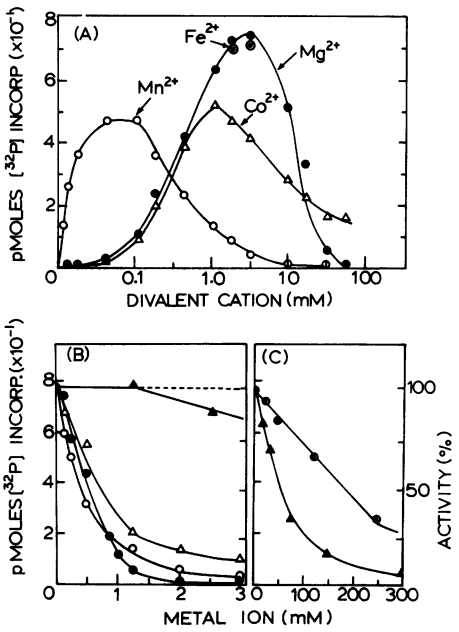

FIG. 4. Influence of metal ion concentrations on the activity of soybean histone kinase. A: requirement of divalent cation for enzyme activity. The standard assay conditions are as described in the text with histone H1 as substrate and 0.50 μg protein of the kinase preparation. Metal ion: Mn^{2+} (○——○), Mg^{2+} (●——●), Co^{2+} (△——△), and Fe^{2+} (⊙). B and C: the inhibitory effects of $ZnSO_4$ (●——●), $CaCl_2$ (○——○), $HgCl_2$ (△——△), $(NH_4)_2SO_4$ (▲——▲), NaCl (○——○), and KCl or Na_2SO_4 (- - -) on the phosphorylation of histone H1 by the soybean kinase. All of the assays contained 5 mM $MgCl_2$ in the standard reaction mixture.

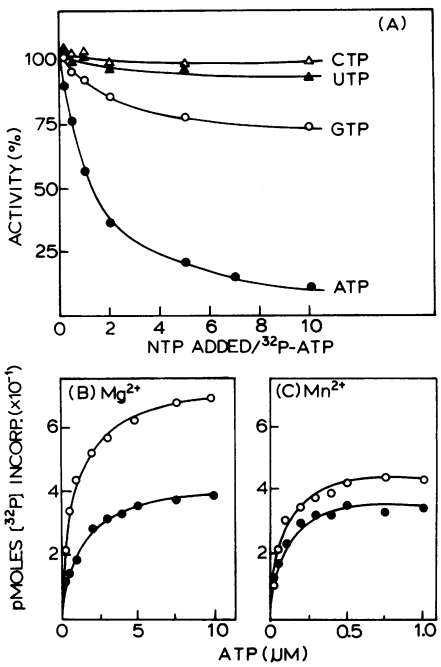

FIG. 5. Nucleotide substrate specificity of soybean histone kinase. A: the standard reaction mixtures contained 10 μg histone H1, 0.35 μg protein of the kinase preparation, 10 μM [γ-^{32}P]ATP (145 cpm/pmol), and various concentrations of unlabeled nucleotides as indicated. The influence of ATP concentration on the phosphorylating activity was as follows: B: assay with Mg^{2+} at 5 mM (○——○) and 0.5 mM (●——●); C: assay with Mn^{2+} at 0.1 mM (○——○) and 0.02 mM (●——●).

Table III. *Protein Substrate Specificities of Soybean Histone Kinase*

The phosphorylating activity was determined as described, with the protein substrates as noted. The amounts of protein substrates used were 0.1 mg/ml for histones and 0.25 mg/ml for nonhistone proteins. The standard reaction mixture contained 1.14 μg soybean kinase preparation, 5 mM $MgCl_2$, and 10 μM [γ-^{32}P]ATP (125 cpm/pmol). All of the histones were from calf thymus. Total histone mixture was Sigma type II preparation.

Protein Substrate	Enzyme Activity	
	pmol ^{32}P incorporated/20 min	%
Total histone mixture	24	13.7
Histone H1	175	100
Histone H1 (pretreated with phosphoprotein phosphatase)	190	108
Histone H2A	3.3	2.0
Histone H2B	14.9	8.5
Histone H3	2.5	1.5
Histone H4	2.0	1.0
Protamine	0	0
Phosvitin	4.2	2.4
Casein	8.5	4.9
Bovine serum albumin	0	0
Nonhistone chromosomal proteins (soybean)	10.2	5.8

Specificity of Protein Substrate. Table III summarizes the relative activities of soybean histone kinase acting on various protein substrates. It is evident that the kinase catalyzes specifically the phosphorylation of the lysine-rich histone H1; other histone subfractions (H2A, H2B, H3, and H4) are poor substrates. Similar results are obtained with histones, whether they are from calf thymus or pea seedlings. No significant phosphorylating activity occurs with phosvitin, casein, protamine, and nonhistone chromosomal acidic proteins of the soybean hypocotyl. Pretreatment of histone H1 with soybean phosphoprotein phosphatase (21) to dephosphorylate endogenous phosphoserine and/or phosphothreonine does not result in any significant increase in the total amount of phosphorylation by the soybean kinase on the histone either from calf thymus or from pea seedlings. Apparently, the

endogenous phosphorylation of histone H1 prepared from pea seedlings is minimum as it is from calf thymus (18).

As observed earlier, the phosphorylating activity with the total histone mixture is much lower, about 10 to 15% of that observed from histone H1. Since arginine-rich histones are inhibitory (20), the amount of phosphorylation from the total histone mixture depends on the ratio of arginine-rich histones to lysine-rich histone H1 in the histone mixture. Analysis of the phosphorylated histones by gel electrophoresis indicates that most of the ^{32}P incorporated in the total histone mixture is associated with histone H1 (Fig. 6A). The histone kinase preparation apparently contains no detectable proteolytic activity on histone H1, as judged by the fact that there are no detectable peptide fragments of ^{32}P-labeled histone H1 formed after incubation of the histone with the kinase under the standard assay conditions (Fig. 6B). The results of the kinetic behavior of histone kinase as a function of histone concentration (5 to 100 μg/ml) further support the premise that, unlike the cAMP-dependent histone kinase from rat liver cytosol, the soybean kinase catalyzes specifically the phosphorylation of histone H1 (Fig. 7A). The apparent K_m values are 0.9 μM for histone H1 (mol wt, 22,000) purified from calf thymus and 0.4 μM for histone H1 (mol wt, 40,000) from pea seedlings. Liver kinase

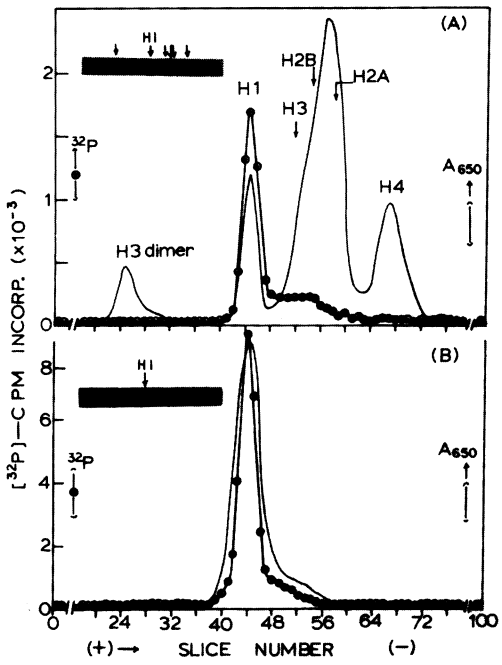

FIG. 6. Urea-acetic acid polyacrylamide gel (15%) electrophoresis (pH 4.3) of total histone mixture and purified histone H1 after phosphorylation with soybean histone kinase. Disc gel electrophoresis was performed at 3 mamp/tube for 3 h according to Panyim and Chalkley (27). The gels were stained with Amido black and scanned at 650 nm and/or sliced into 1-mm segments. The radioactivity of each segment was determined. A: analysis of ^{32}P-labeled total histone mixture. After phosphorylation at 28 C for 45 min with soybean histone kinase in the standard reaction mixture as described in the text. One hundred μg ^{32}P-labeled total calf thymus histone mixture (Sigma, type II) were subjected to electrophoresis. The specific activity of [γ-^{32}P]ATP used was 83 cpm/pmol. The phosphorylated histone was dialyzed against 3 M urea overnight prior to electrophoresis. B: analysis of ^{32}P-labeled histone H1. The reaction mixture (1.0 ml) contained 100 mg purified histone H1, 25 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, 10 μM [γ-^{32}P]ATP (83 cpm/pmol), and 0.3 μg protein of the kinase preparation. After incubation at 28 C for 1 h, 0.5 ml nonradioactive histone H1 (2 mg/ml) was added and the mixture was dialyzed against 3 M urea overnight. After dialysis, 0.07 ml of the sample containing about 77 μg histone H1 was subjected to electrophoresis.

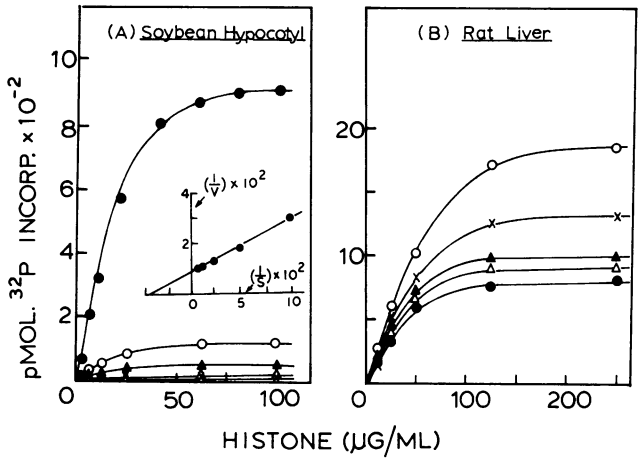

FIG. 7. Influence of histone concentration on the activities of histone kinases from soybean hypocotyls and rat liver. The reaction mixtures contained various concentrations of purified calf thymus histone species H1 (●——●), H2A (▲——▲), H2B (○——○), H3 (△——△), and H4 (×——×). The kinases were purified as described in the text. A: histone kinase from soybean hypocotyls. A double reciprocal plot of 1/V *versus* 1/S for histone H1 is shown. B: cAMP-dependent histone kinase from rat liver cytosol. cAMP (1 μM) was added to the reaction mixtures, which increased the phosphorylating activity by 350%.

Table IV. *Effects of Selected Compounds on the Phosphorylation of Histone H1 by Soybean Histone Kinase*

The standard reaction mixtures contained 20 mM Tris-Cl (pH 8.0), 5 mM $MgCl_2$, 2.5 μM [γ-^{32}P]ATP (125 cpm/pmol), 40 μg calf thymus histone H1, and 1.0 μg soybean histone kinase preparation with additions as noted. The enzyme activity was determined as described.

Addition	Enzyme Activity	
mM	*pmol ^{32}P incorporated/20 min*	*%*
None	140	100
3′:5′-cAMP, 0.5	104	60
3′:5′-cAMP, 0.005	130	93
3′:5′-cGMP, 0.5	136	97
3′:5′-cUMP, 0.5	126	90
3′:5′-cCMP, 0.5	133	95
Putrescine, 5.0	116	83
Spermidine, 5.0	68	49
Spermine, 5.0	52	37

phosphorylates the histone species in the presence of 1 μM cAMP at the following relative rates (V_{max}: H2B (100%), H4 (63%), H3 (50%), H2A (45%) and H1 (40%) (Fig. 7B); this is in agreement with the data reported in the literature (7, 18).

Effects of Selected Compounds. Additions of cyclic nucleoside 3′:5′-monophosphates to the reaction mixtures do not affect the phosphorylating activity of soybean histone kinase on histone H1 (Table IV). Similar results are obtained with other histone species as protein substrate. It is important to note that 1 μM cAMP also has no effect on the activity of crude histone kinase preparation (ammonium sulfate-precipitated fraction). The inhibitory effect of 0.5 mM cAMP appears to be physiologically insignificant because the endogenous level of cAMP, if it exists in higher plants, would be in the picomolar range (1). At the concentrations of 0.005 to 0.5 mM, cyclic purine and pyrimidine nucleoside 2′:3′-monophosphates also have no effect on the phosphorylation. No significant cAMP-binding activity or cAMP (or cGMP)-dependent activity of protein kinase (histone H1 or total histone mixture as substrate) could be detected in any of the eluted fractions from Sephadex G-150 (Fig. 1A). This is in agreement with our previous report (20)

that by means of the Millipore filter technique and/or by gel filtration chromatography on Sephadex G-25, the specific cAMP-binding activity in extracts of soybean hypocotyl tissue is less than 0.1% of that obtained with rat liver cytosol (about 2.8 pmol cAMP bound/mg protein). Including 1 μM cAMP in the sucrose gradient (Fig. 2B) does not change the sedimentation behavior of the kinase. Similar results of the negative effect of cAMP are obtained when the crude and partially purified (Sephadex G-150) preparations of histone kinase are subjected to sucrose density gradient centrifugation (data not shown). No effect is observed from the additions of 1.0 μM GA_3, 20 μM 2,4-D, and 1.0 μM kinetin to the assay mixtures. Polyamines, such as putrescine, spermidine, and spermine, inhibit the phosphorylating activity as do arginine-rich histones (22). The degree of inhibition by polyamines (polycations) seems to be closely and positively related to the number of amino groups (cation) associated with the molecules. Polyamines do not change the apparent K_m values for ATP and histone H1 but reduce the V_{max} value for histone kinase (P. P.-C. Lin, unpublished observation).

DISCUSSION

Previous studies of the soybean hypocotyl have demonstrated a chromatin-associated casein (or phosvitin)-type kinase (26) and a ribosome-associated kinase which phosphorylates basic ribosomal proteins but not histones (J. Hammet, personal communication). Here, we describe the purification of a third type of protein kinase (ATP: histone H1 phosphotransferase) from soybean hypocotyls, which shows high specificity for lysine-rich histone H1 isolated from either pea seedlings or calf thymus.

By means of a combination of gel filtration and anion exchange procedures, a 320-fold purification of the histone H1 protein kinase was obtained. The purified enzyme fraction is devoid of the activities of phosphoprotein phosphatase, histone protease and casein (or phosvitin) kinase that were detectable during the early stages of purification. In the purification, advantage was taken of the fact that the histone kinase was soluble in buffers of low ionic strength but that the casein (or phosvitin)-type protein kinase tended to form aggregates as reported (26). Accordingly, a low ionic strength buffer was employed for separation of the histone kinase from casein kinase during gel filtration on Sephadex G-150 (Fig. 1A).

The purified histone kinase from soybean hypocotyls, like histone kinases characterized from animal tissues (18, 32, 41), requires free sulfhydryl group(s) for activity but apparently not for stability. In contrast to histone kinases derived from several animal tissues (18, 32), the soybean histone kinase is unaffected by cAMP or other cyclic nucleotides. This has been demonstrated by several procedures: (*a*) the activity of the soybean enzyme with a number of protein substrates is independent of added cyclic nucleotides over a concentration range of 5 to 500 μM; (*b*) no significant cAMP binding to the soybean enzyme preparation can be detected; and (*c*) cAMP does not alter the sedimentation velocity of the soybean enzyme as has been reported for many animal cAMP-dependent histone kinases which exist as complexes of regulatory catalytic subunits (18, 41). The independence of activity of the soybean enzyme on cAMP and the inability to detect a cAMP-dependent protein kinase with casein, phosvitin, and various histones as substrates at various stages of purification argue strongly for the absence of cAMP-regulated protein kinases in soybean hypocotyl tissue. These results, along with previous observations (1, 19), indicate that cAMP, if it exists in higher plants, may function differently from its known action in animals (32, 38).

The results in this study do not rule out the existence of other types of histone kinases in the soybean hypocotyl, which may phosphorylate the lysine, arginine, and/or histidine moieties of histones. Unlike the acid-stable P-O phosphoryl linkages formed from the phosphorylation of serine, threonine, and hydroxyproline moieties of proteins (11, 18), phosphorylation of the basic amino acids would form P-N phosphoryl linkages which are acid-labile and, therefore, are not detectable under the experimental conditions. In animal cells, two histone kinases, which catalyze the formation of acid-labile histone phosphates, have been reported to be cAMP-independent (6, 35).

The function of the soybean histone H1 protein kinase studied here remains unresolved. It seems clear, from these *in vitro* studies that the soybean histone kinase is specific for lysine-rich histone H1 and that the soybean enzyme appears to share some properties with the chromatin-associated protein kinase of mitotic Chinese hamster cells (17). Both kinases are cAMP-independent and both are specific for histone H1. The activity of the enzyme from hamster cells has been shown to vary depending on specific phases of the cell cycle (17).

The previously demonstrated occurrence of *in vivo* phosphorylation of histone H1 in higher plants (39, 40) coupled with the association (highest specific activity) of the soybean enzyme with the nuclear-chromatin fraction suggests that the cAMP-independent histone H1 kinase may function in the control of growth and development in the soybean hypocotyl. Since endogenous phosphorylation of histone H1 does not seem to correlate positively with DNA synthesis (39), the presumed function of the histone H1 kinase of the soybean hypocotyl may be in processes leading to chromatin decondensation. In animal cells, such a function of histone H1 phosphorylation has been described (9, 28).

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