LYSINE-RICH HISTONE H1 KINASE FROM SOYBEAN HYPOCOTYL

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SUMMARY: A protein kinase (ATP: histone phosphotransferase) with high specificity for the phosphorylation of the very lysine-rich histone H1 has been partially purified and characterized from soybean hypocotyl. The enzyme has a molecular weight of about 48,500. Its activity and sedimentation behavior are refractory to cyclic nucleoside monophosphates. No significant amount of cyclic AMP or cyclic GMP binding activity could be detected in the crude or partially purified enzyme preparations. Km for ATP and histone H1 are 0.4 μM and 0.7 μM, respectively. The enzyme requires Mg^{2+} or Mn^{2+} for activity, while addition of 0.5 mM Ca^{2+}, Zn^{2+} or Hg^{2+} results in 50% inhibition. Arginine-rich histones H3 and H4 are inhibitory to histone H1 phosphorylation; these histones affect the Vmax of the enzyme, but not the Km for histone H1.

INTRODUCTION

Histones are subject to postsynthetic modification via phosphorylation and dephosphorylation reactions which are catalyzed by histone kinases and histone phosphatases, respectively. Such alternation in histone structure may be relevant to changes in chromatin structure during gene activation and to the organization of chromatin during DNA replication (1). In mammalian systems the phosphorylation reaction is often regulated by hormones acting through cyclic AMP and/or cyclic GMP (2). Many protein kinases, including some which prefer histone as substrate, have been characterized as cyclic mucleotide-dependent (3,4). No similar enzymes or regulatory mechanisms, however, have been demonstrated in higher plants. Despite several works on protein kinase (5-7), there apparently are no reports of *in vitro* studies on histone kinase from higher plants. Furthermore, several lines of evidence obtained in the past few years strongly argue against the existence of cyclic nucleotides in higher plants (8,9). Thus, it would be interesting to know the mechanism(s), operative in the regulation of protein (enzyme) phosphorylation in general and histone phosphorylation in particular in higher plants. Studies on

histone kinase and cyclic nucleotide binding activity may assist in obtaining new insights into the controversy of whether cyclic nucleotides exist in higher plants. In this communication we demonstrate the presence of a major histone kinase form soybean hypocotyl, which specifically phosphorylates histone H1 and which is cyclic nucleotide-independent.

MATERIALS AND METHODS

Purification of Histone Kinase: Mature hypocotyls of 5-day-old, etiolated soybean (Glycine max var. Wayne) seedlings were homogenized in a Waring blender with an equal amount (v/w) of 5 mM 2-mercaptoethanol-20mM Tris-Cl (pH 8.0). 250 mM each of NaCl and $(NH_4)_2SO_4$ was included for the initial homogenization. After centrifugation at 10,000 x g for 10 min, the supernatant was brought to 50% saturation with ammonium sulfate. The resulting pellet was dissolved in the above buffer without additional salts and centrifuged at 105,000 x g for 1 hr to remove the aggregate material. About 7 ml (40 mg protein) of the resulting supernatant were loaded onto a Sephadex G150 column (2.5 x 105 cm) and eluted with the above buffer in 5-ml fractions. Peak fractions of histone kinase activity were pooled and loaded onto to a DEAE-Sephadex A25 column (1.5 x 15 cm). The column was washed with 75 ml of the loading buffer and then eluted with a linear gradient of 0-0.5M NaCl in 5-ml fractions. Fractions of histone kinase activity, as eluted by 0.25M NaCl in a single peak, were pooled and concentrated by Amico membrane filter (PM 10) prior to chromatography on a Sephadex G100 column (1.5 x 100 cm).

Purification of Histone Subfractions: Commercially avaliable calf histone subfractions (from Sigma and Worthington Co.) as prepared by the method of Johns (10) are cross contaminated by all histone species. Partial purification can be achieved by chromatography on BioGel P60 (11). Very lysine-rich histone H1 as eluted is virtually pure as judged by urea-acetic acid acrylamide gel electrophoresis (12). Further purification through BioGel P10 and BioRex 70 (from BioRad Lab.) are necessary to obtain the other histone species (H2A, H3 and H4) at comparable purity (13). Pea histones were isolated by the method described previously (14). As compared to histone H1 from calf thymus, pea H1 appears to have higher molecular weight, about 40,000. The amount of histone was determined by absorbance at 230 nm (3.5 O. D. units for 1 mg/ml histone). The concentration of nonhistone protein was measured by the Lowry method (15).

Assay of Protein Kinase: The activity of histone kinase was assayed in a 0.2 ml reaction mixture, containing 20 mM Tris-Cl (pH 8.0), 5 mM 2-mercaptoethanol, 5 mM $MgCl_2$, 4 μM [γ-^{32}P] -ATP (125-150 cpm/pmole) and 20 μg histone. For the assay of casein-type kinase activity, 25 μM ATP, 10 mM $MgCl_2$ and 100 μg α-casein (Sigma Co.) were used. [γ-^{32}P]-ATP was prepared enzymatically (16) and purified by fractionation on Dowex-1. All the assays were initiated by the addition of 5 to 35 μl of enzyme preparation, incubated at 28°C for 20 min and terminated with 20% cold trichloroacetic acid (10% used for precipitation of nonhistone proteins), containing 10 mM Na-pyrophosphate. Precipitates were collected on Whatman GF/A glass fiber filters and dried. The radioactivity was determinated in a toluene-based scintillation fluid. Typically, 70$\pm$ 20 cpm/assay is obtained for the reaction without enzyme or with heat-denatured enzyme preparation.

RESULTS AND DISCUSSION

Extracts of soybean hypocotyl have been shown to contain at least two types

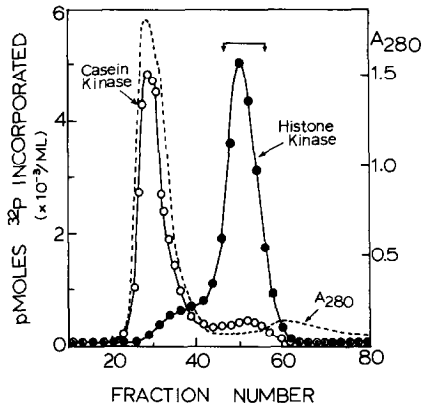

Fig. 1. Resolution of protein kinase form soybean hypocotyl by chromatography on Sephadex G150. As described in the Methods, histone kinase (●——●) and casein-type kinase (○——○) activities were assayed with pure calf histone H1 and α-casein as substrate, respectively. Dash line represents absorbance at 280 nm.

of protein kinase activity, namely histone kinase in this study and casein kinase (Murray, Guilfoyle and Key, in preparation), this nomenclature being based on their in vitro substrate specificity. Unlike the casein kinase, histone kinase once solubilized (originally about 50% activity found in particulate form) by high salt tends not to form aggregates even in the crude extracts at low salt. As shown in Fig. 1, the histone kinase activity is clearly separated from the casein kinase activity and the majority of protein by chromatography on Sephadex G 150. The same elution pattern for histone kinase is obtained using either pure calf histone H1 or total calf histone mixture (type II from Sigma Co.) as substrate, although when the total histone mixture is used, a much lower phosphorylating activity is observed.

Addition of 2 μM cyclic AMP or cyclic GMP does not alter the elution pattern of histone kinase from Sephadex, nor does the nucleotide, when added directly to the enzyme assay mixture, affect histone kinase activity. Cyclic AMP also has no effect on the sedimentation behavior of this histone kinase in sucrose density gradient centrifugation, indicating that its activity is not regulated by the nucleotide via the mechanism demonstrated in animal systems (1,2). Furthermore,

no significant amount of [^{3}H]-labeled cyclic AMP or cyclic GMP binding activity could be detected in the crude or partially purified enzyme preparations by the Millipore filter technique (17) and/or by gel filtration chromatography on Sephadex G25. The specific cyclic AMP binding activity in extracts of soybean hypocotyl is less than 0.1% of that obtained, using the above methods, with rat liver cyotsol (about 2.8 $\pm$ 0.2 pmoles of cyclic AMP bound/mg of rat liver protein). This value for the soybean preparation is not corrected for nonspecific binding, or the binding due to degraded products derived from the [^{3}H]-labeled nucleotide, which may occur under the experimental conditions and is therefore a maximum estimate.

Further purification of the histone kinase through DEAE-Sephadex A25 and Sephadex G100 results in an enzyme specific activity of 7.5 nmoles of [^{32}P] incorporated into histone H1 per min per mg protein. This preparation of histone kinase is free of histone phosphatase and histone protease activities. The histone kinase, with a molecular weight of about 48,500 as judged by sucrose density gradient centrifugation, exhibits maximal activity at pH 8.0 - 9.0 with either pure histone H1 or total histone mixture as substrate. A divalent cation (Mg^{2+}, Mn^{2+}, or Co^{2+}) is required for the enzyme activity; the optimal concentration of Mg^{2+} or Co^{2+} is 5 mM, while that of Mn^{2+} is 0.06 mM. Addition of 0.5 mM Ca^{2+}, Zn^{2+} or Hg^{2+} to the reaction mixture, containing optimal concentrations of the required divalent cation, results in a 50% inhibition of histone kinase activity. The enzyme ultilizes ATP, but not GTP, CTP or UTP as phosphodonor. It is not clear whether the enzyme can catalyze the reaction in the reversible direction by using phosphohistone H1 and ADP as substrate. The apparent Km for ATP is 0.4 µM and 0.04 µM with optimal concentration of Mg^{2+} and Mn^{2+}, respectively. For the preferred protein substrate, histone H1, the Km is 0.7 µM (about 15 µg/ml). These values are considerably lower than that for the corresponding enzyme from animal cells (18-20). Neither cytokinin nor 3',5' - (or 2',3'-) cyclic nucleoside monophosphates affect the activity of histone kinase from soybean hypocotyl. The partially purified enzyme, even at the very earliest stage of purification, is totally inactivated by freezing and thawing, but is stable at 0-4°C for up to one month. As to substrate specificity,

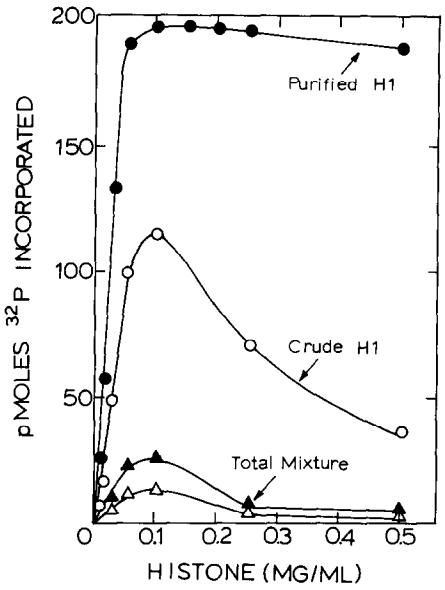

Fig. 2. Correspondence between amount of various calf histone preparations added and incorporation of γ-^{32}P from ATP under standard assay conditions. Histone preparations: total histone mixtures type II (Δ —— Δ) and type IIA ($\blacktriangle$ —— $\blacktriangle$) from Sigma Co.; crude lysine-rich histone fraction (O —— O) as prepared by the method of Johns (10); and histone H1 (● —— ●) as purified by chromatography on BioGel P60.

histone kinase from soybean hypocotyl apparently catalyzes specifically the phosphorylation of very lysine-rich histone H1 as judged by gel electrophoresis. The enzymes shows less than 5% of its maximal activity when nonhistone proteins, such as casein, phosvitin, bovine serum albumin and chromosomal acidic proteins from soybean chromatin are used as substrate. Kinetic data further show that the histone kinase has the following relative activities toward each pure histone species: histone H1 (100%) > H2B (5-10%) > H3 (0-3%) > H2A and H4 (0%). Histone subfractions purified form either calf thymus or pea seedlings are phosphorylated to the same relative amount. Thus, the enzyme, based on its substrate specificity, is appropriately referred to as histone H1 kinase.

Phosphorylation of histone H1 is significantly inhibited at substrate concentration greater than 0.1 mg per ml, whenever impure histone H1 preparations are used as substrate (Fig.2). Less than 15% and 7% phosphorylation is observed for total histone mixtures type II and IIA (from Sigma Co.), respectively, relative

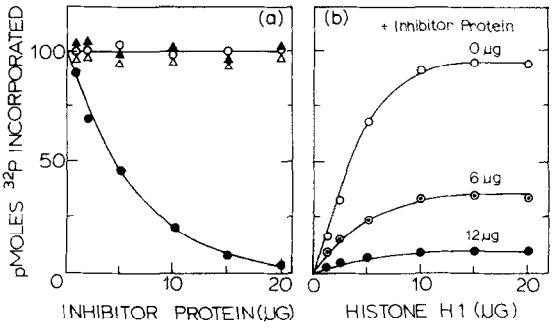

Fig. 3. Inhibition of protein kinase activity by a heat acid stable basic inhibitory protein copurified with arginine-rich histone H3. (a) Varying amounts of the inhibitory protein were added to the standard reaction mixture before addition of histone H1 kinase (●—●) or casein-type kinase (○—○) from soybean hypocotyl; or cyclic AMP-dependent histone kinase (▲—▲) or casein kinase (Δ—Δ) form rat liver. (b) Inhibition of histone H1 phosphorylation by the inhibitor protein. Varying amounts of the "inhibitor" were added as indicated to the standard reaction mixture,containing varying concentrations of pure histone H1, for the assay of histone H1 kinase activity from soybean hypocotyl.

to pure histone H1 in the standard assay condition. The difference in the amount of phosphorylation at these two types of histone preparation is apparently due to a reduced content of histone H1; type II contains about 20% H1, while type IIA contains less than 10% H1. The low content of histone H1 in the above total histone mixtures, however, does not explain the inhibition of histone H1 phosphorylation at higher histone concentrations shown in the figure. Further experiments clearly demonstrate that the inhibition does not result from the higher substrate concentration of histone H1, but that the inhibition depends on the presence of other histone subfractions or nonhistone contaminants which copurify with the histones. Addition of commercially available histone subfractions, enriched in slightly lysine-rich (H2A and H2B) or arginine-rich (H3 and H4) histones, to the reaction mixture inhibits the phosphorylation of histone H1; the amount of phosphorylation observed depends on the ratio of argine-rich to lysine-rich histones. Further purification of these histone subfractions through BioGel P60, BioGel P10 and BioRex 70 shows that the potential inhibitor is an acid and heat stable basic protein(s) which copurifies with arginine-rich histones H3 and H4, but not with slightly lysine-rich histones H2A and H2B. Similar inhibition is also observed

with arginine-rich histone preparations from pea seedlings. Evidently, the "inhibitor" affects the Vmax of histone H1 kinase from soybean phypocotyl, but not the Km for histone H1 (Fig.3b); the "inhibitor" does not compete or interact with histone H1 as substrate. Direct interaction between lysine-rich and arginine-rich histones to form heterotypic complexes is somehow limited under the experimental conditions used,although homotypic dimer and tetramer of H3 and H4 can form (21). The "inhibitor protein" does not affect the activity of cyclic AMP-dependent histone kinase from rat liver cytosol, nor does it affect the activities of casein-type kinases from either rat liver nuclei or soybean hypocotyl chromatin (Fig.3a).

The physiological significance, if any, of inhibition of histone H1 kinase activity by arginine-rich histone fractions remains to be answered. Histone H1 phosphorylation has been implicated in the control of cell division, especially the initiation step of mitosis (18-20,22). Evidently, the histone H1 kinase described here could play a crucial role for the in vivo phosphorylation of histone H1 in higher plants. The possible role of this kinase, as well as other kinases, in the control of the growth and development of soybean hypocotyl by auxin is currently under investigation. Finally, the failure to detect cyclic nucleotide binding activity and the lack of any demonstrable effect of cyclic nucleotides on the activity of histone kinase from soybean hypocotyl, together with the previous data (8), argue against a major functional role of cyclic nucleotides in higher plants.

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