ADENOSINE 3',5'-CYCLIC PHOSPHATE-DEPENDENT AND INDEPENDENT HISTONE KINASES FROM RAT LIVER*

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Summary Two protein kinases which phosphorylate preferentially histone have been partially purified from rat liver soluble fraction. One requires adenosine 3',5'-cyclic phosphate (cyclic AMP) for its maximum activity, whereas the other does not respond to the cyclic nucleotide. Nevertheless, both kinases seem to incorporate the terminal phosphate of ATP into the same predominantly seryl but also threonyl residues in histone molecules, and show closely similar properties except that the cyclic AMP-independent enzyme is inactivated rapidly upon storage and heat treatment.

A protein kinase which phosphorylates preferentially histone has been partially purified from rat liver by Langan and Smith (1967). Subsequent to the work by Walsh et al. (1968) who have shown that cyclic AMP greatly stimulates phosphorylase b kinase kinase obtained from rabbit skeletal muscle, Langan (1968) has found that the rat liver protein kinase is also stimulated by the cyclic nucleotide, and phosphorylates a seryl residue in lysine-rich (f_1) histone in vitro. The phosphorylation of the specific site of the histone has been shown in vivo to be enhanced mark-

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edly by the administration of either insulin, glucagon or cyclic AMP, and a role for the cyclic nucleotide in regulating transcription of genetic information has been implied (Langan, 1969). Cyclic AMP-dependent histone kinases have been found also in rabbit brain (Miyamoto et al., 1969), Escherichia coli (Kuo and Greengard, 1969) and in rainbow trout testis (Jergil and Dixon, 1970). Recently, cyclic GMP-dependent protein kinases have been found in lobster muscle and in bovine brain and bladder (Kuo et al., 1970). This communication describes the resolution of rat liver histone kinase into two fractions, either one of which per se is capable of phosphorylating the same, predominantly seryl but also threonyl, residues in histone molecules. Although these enzymes show rather similar properties, one requires cyclic AMP, whereas the other does not respond to any cyclic nucleotide. Partial purification and properties of these kinases are described briefly here. The cyclic AMP-dependent and independent enzymes will be tentatively referred to as protein kinase B_1 and protein kinase B_2, respectively.

The enzyme activity was assayed using a Millipore filter by measuring the radioactive phosphate of ATP-γ-^{32}P incorporated into an acid-precipitable material. The standard reaction mixture (0.25 ml) contained 100 µg of histone, 2.5 mµmoles of ATP-γ-^{32}P (20,000-45,000 cpm/mµmole), 5 µmoles of magnesium acetate, 5 µmoles of potassium phosphate buffer, pH 7.5, and an enzyme preparation. Where indicated cyclic AMP (3×10^{-6} M) was added. The incubation was carried out for 5 min at 30°, and the reaction was stopped by the addition of 10% trichloroacetic acid. ATP-γ-^{32}P was prepared by photophosphorylation of ADP with spinach chloroplasts by the method of Jagendorf and Avron (1958). Calf thymus histones were prepared by the differential extraction procedure described by

Johns (1964). Histone f_{2a1} and f_{2a2} were separated with a Sephadex G-100 column by the method of Starbuck et al. (1968). Protein was determined by the method of Lowry et al. (1951).

Wistar albino rats, weighing 150-200 g fed ad libitum on CLEA laboratory chow, were employed for the present studies. The liver, 8 g, was quickly removed after decapitation, and homogenized with 5 volumes of 0.25 M sucrose containing 3.3 mM $CaCl_2$ using a Teflon-glass homogenizer. The homogenate was centrifuged for 20 min at 15,000 x g. To the supernatant ammonium sulfate was added to a final concentration of 70%. After centrifugation the precipitate was dissolved in 20 ml of 0.02 M Tris-Cl buffer, pH 7.5, and dialyzed for 15 hours against the same buffer. The enzyme solution (20 ml, 25 mg protein/ml) was chromatographed on a DEAE-sephadex column (A-50; diameter, 3 cm; length, 30 cm) with application of a linear concentration gradient of NaCl in 0.02 M Tris-Cl buffer, pH 7.5 (mixing chamber, 0.05 M; reservoir, 0.5 M; 500 ml each). Protein kinase B_1 and B_2 were eluted together as a single peak. When this fraction was chromatographed further on a hydroxylapatite column two enzyme peaks appeared as shown in Fig. 1. Protein kinase B_1 (first peak) was stimulated greatly by the addition of cyclic AMP, and the maximum stimulation was found at 8×10^{-8} M of the nucleotide. Cyclic IMP was about 15% as active as cyclic AMP, and practically no stimulatory effect was found with any other cyclic nucleotide such as cyclic GMP at this concentration. In contrast, protein kinase B_2 (second peak) did not respond to the nucleotide and essentially identical results were obtained with and without cyclic AMP. Cyclic GMP did not stimulate the latter enzyme.

Both protein kinase B_1 and B_2 were relatively specific for histone and reacted with calf thymus, rat liver as well as chick

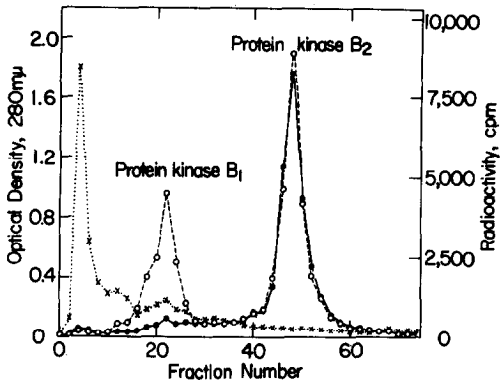

Fig. 1. Separation of protein kinase B_1 and B_2 by hydroxylapatite column chromatography. The column (diameter, 2 cm; length, 5 cm) was eluted by a convex concentration gradient of potassium phosphate buffer. The mixing chamber (150 ml) and reservoir contained 0.05 M and 0.2 M phosphate buffer, pH 7.5, respectively. Fractions (5 ml each) were collected. Each tube was assayed under standard conditions with calf thymus whole histone as substrate. x·····x protein (O.D. at 280 mμ); o------o activity with cyclic AMP; ●——● activity without cyclic AMP.

erythrocyte histones. Among many other proteins thus far tested only salmon sperm protamin could serve as substrate. Egg phosvitin, bovine casein, and rat liver non-histone nuclear proteins were inactive. Table I summerizes the maximum amounts of radioactive phosphate incorporated into limited amounts of various histones. Although neither histone f_{2a1}, f_{2a2} nor f_{2b} has been firmly established to be phosphorylated in vivo (Kleinsmith et al., 1966; Ord and Stocken, 1967), all histones were phosphorylated in in vitro systems by protein kinase B_1 as well as by protein kinase B_2. With any histone species as substrate cyclic AMP stimulated consistently the reaction catalyzed by protein kinase B_1 but not by protein kinase B_2. However, the amount of phosphate incorporated by the simultaneous addition of protein kinase B_1 and B_2 did not exceed the amount by either one of these kinases alone, and the phosphate was saturated at the same level

Table I Maximum Amounts of Phosphate Incorporated into Various Histone Species by Protein Kinase B_1 and B_2

The incubation was carried out under the standard conditions except that 20 μg of each histone and an excess amount of enzyme were employed, and the mixture was incubated for 3 hours. The specific activity of ATP-γ-^{32}P employed was 45 cpm/μμmole.

Histone	Protein kinase B_1		Protein kinase B_2	
	without cyclic AMP	with cyclic AMP	without cyclic AMP	with cyclic AMP
	(cpm)	(cpm)	(cpm)	(cpm)
Whole histones	2,343	14,004	15,579	15,971
F_1	326	8,630	9,420	8,608
F_{2a1}	1,180	5,388	5,875	4,275
F_{2a2}	2,168	8,546	6,956	6,684
F_{2b}	4,629	37,496	40,240	39,911
F_3	1,664	3,929	2,463	1,612

with either one or both kinases as exemplified in Fig. 2. Acid hydrolysis of each histone phosphorylated under various conditions resulted in the formation of phosphoserine and phosphothreonine in the ratio of more than 9.

In order to ascertain whether or not these kinases phosphorylate the same seryl and threonyl residues in each histone molecule, histone f_{2b} was fully phosphorylated by either one or both kinases in the presence and absence of cyclic AMP, and the radioactive histones were subjected to tryptic digestion, followed by high voltage paper electrophoresis and autoradiography. As illustrated in Fig. 3, identical patterns were observed with radioactive histone preparations phosphorylated by protein kinase B_1, B_2 or both in the presence and absence of cyclic AMP.

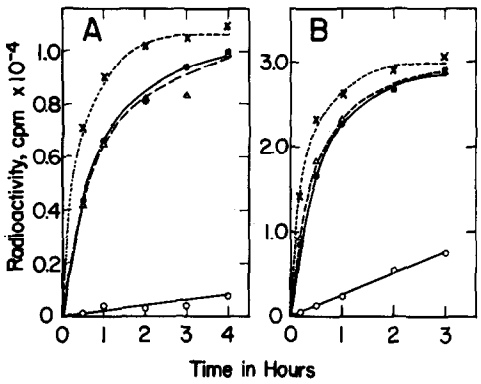

Fig. 2. Enzymic phosphorylation of histone f_1 (A) and f_{2b} (B) with protein kinase B_1 and B_2 in the presence and absence of cyclic AMP. The reaction was carried out under the standard conditions, except that 20 μg of each histone was employed. ●——● B_1 with cyclic AMP; o——o B_1 without cyclic AMP; Δ----Δ B_2 without cyclic AMP; x·····x B_1 plus B_2 with cyclic AMP.

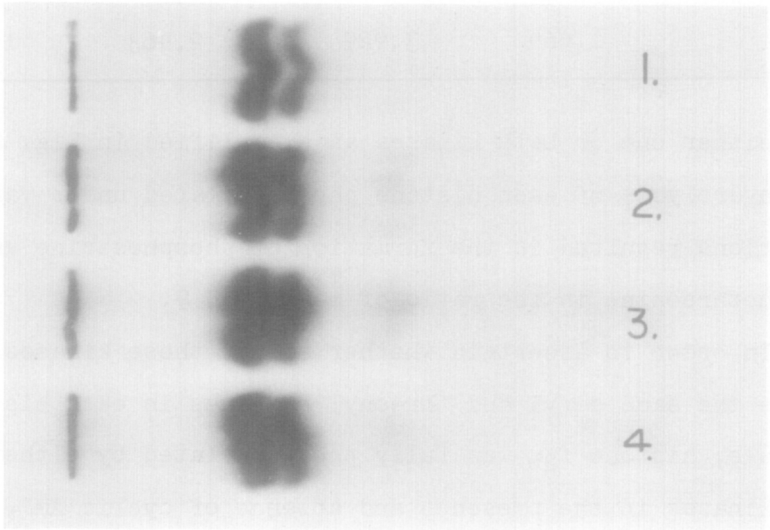

Fig. 3. Autoradiograms of the tryptic digests of histone f_{2b} phosphorylated by protein kinase B_1 and B_2 in the presence and absence of cyclic AMP. Phosphorylated histone f_{2b} was digested at pH 8.0 for 4 hours at 37° with trypsin (trypsin : histone 1 : 100). The digests were subjected to high voltage paper electrophoresis at 4,000V for 90 min with pyridine-acetic acid-water (1 : 10 : 89, pH 3.5) buffer. (1) B_1 without cyclic AMP; (2) B_1 with cyclic AMP; (3) B_2 without cyclic AMP; (4) B_1 plus B_2 with cyclic AMP.

Similar results were obtained with other histones as substrates. These results indicate that both protein kinase B_1 and B_2 incorporate the terminal phosphate of ATP into the same specific seryl and threonyl residues in histone molecules, and that cyclic AMP stimulates only the rate of reaction catalyzed by protein kinase B_1.

Both protein kinase B_1 and B_2 showed maximum activities at pH 7.5 in the presence of 2×10^{-2} M magnesium ion. The enzymes were specific for ATP with Km value of 4×10^{-6} M, and were inhibited about 50% by 4×10^{-5} M ADP, although the reactions were irreversible. Protein kinase B_2 was rather unstable and approximately 50% of the activity was lost upon storage for 2 days at -20°, or heat treatment for 2 min at 50°, while protein kinase B_1 was stable under these conditions. Although these kinases show closely similar properties and phosphorylate the same sites of histone molecules as described above, all attempts to explore the correlation of these kinases have thus far been unsuccessful. Rechromatography of either one of these kinases did not shift one enzyme to the other, even in the presence of both ATP and cyclic AMP. No evidence has been obtained indicating that protein kinase B_1 is capable of binding cyclic AMP or that protein kinase B_2 contains the nucleotide. Further studies are currently underway to elucidate the exact nature as well as the relation of these protein kinases.

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