PHOSPHOPROTEIN KINASES ASSOCIATED WITH RAT LIVER CHROMATIN*

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Summary Two protein kinases associated with rat liver chromatin catalyze the phosphorylation of each specific seryl and threonyl residues of nuclear non-histone proteins. The phosphate acceptor proteins contain a significant quantity of phosphate, and are separated from the enzymes by chromatography on a phosphocellulose column. Casein and phosvitin also serve as phosphate acceptors. The enzymes show closely similar properties, but were clearly distinguished each other by substrate specificities. With casein as a model substrate one phosphorylated mainly seryl residues, whereas the other preferentially threonyl residues.

Langan (1) and Kleinsmith et al. (2) have provided evidence for the nuclear localization of significant amounts of phosphoproteins containing up to 1.3 % phosphorous by weight as mainly phosphoserine and some phosphothreonine. The phosphate groups bound to these proteins have been shown to turn over rapidly, and a role of the phosphoproteins in regulating some nuclear functions, particularly transcription of genetic activities, has been implied (1-5). Although the phosphorylation of such proteins has been studied in in vitro systems (1,2), the phosphoprotein preparations thus far obtained carry along the catalytic activity and no information has been available on the properties of enzyme(s) concerned as well as on the precise nature of phosphoprotein phosphorylation

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reactions. The evidence presented in this paper indicates that the phosphorylation of phosphoproteins are catalyzed by two enzymes associated with chromatin which may be separated from bulk of phosphoproteins. These enzymes incorporate the terminal phosphate of ATP into each specific seryl and threonyl residues of the phosphoproteins.

Wistar albino rats, weighing 150-200 g, maintained ad libitum on CLEA laboratory chow were employed for the present studies. The liver nuclei were isolated by the method of Chauveau et al. (6) using 2.2 M sucrose containing 3.3 mM $CaCl_2$. Nuclear soluble proteins were removed by washing with saline-EDTA by the method of Ueda et al. (7), and chromatin was extracted from the final sediment with 0.05 M Tris-Cl, pH 7.5, containing 1 M NaCl (one ml/nuclei from 2 g liver) by homogenizing in a Teflon-glass homogenizer, and shearing for one min using a 20 kc Tominaga sonic oscillator followed by centrifugation for 30 min at 34,000 x g.

The enzyme activity was assayed by measuring the radioactivity of ATP-γ-^{32}P incorporated into bovine casein (Hammarsten, Merk) which was employed as a model substrate in routine assays. The standard assay conditions are described in the legend to Fig. 1. A blank incubation without casein was run simultaneously and the phosphate incorporated into an enzyme preparation itself was subtracted from the experimental value. ATP-γ-^{32}P was prepared by the method of Jagendorf and Avron (8). Protein was determined by the method of Lowry et al. (9).

A rat was given an intraperitoneal injection of radioactive orthophosphate (1 mCi). One hour after the injection, the rat was sacrificed by decapitation, and the liver chromatin was isolated as described above. Then, the salt concentration of the chromatin preparation was lowered by adding 1.5 volumes of 0.05 M Tris-Cl,

pH 7.5, and the resulting precipitate was removed by centrifugation for 30 min at 34,000 x g. The supernatant thus obtained contained about 60 % of the total alkali-labile phosphate together with more than 90 % of the enzyme activity originally associated with chromatin. When the supernatant was applied to a phospho-cellulose column, most of the phosphoproteins was recovered in the effluent as judged by radioactive alkali-labile phosphate (Fig. 1). In contrast, the enzyme activity was adsorbed on the column, and was eluted with 0.05 M Tris-Cl buffer, pH 8.1, containing 0.6 M NaCl. Practically no alkali-labile phosphate was found in this fraction as judged by radioactivity and by a color reaction of phosphate (10). Although a trace amount of enzyme activity was detected in the effluent, the fractions appeared earlier (fraction No. 5 and 6) were essentially free of enzyme activity as shown in Fig. 1. These fractions contained a significant quantity of alkali-labile phosphate (0.25 % phosphorus by weight), and could accept additional phosphate upon incubation with ATP and the protein kinases described below.

In another set of experiments, phosphoproteins were first enriched from rat liver nuclei by the method of Langan (1). This preparation carried along kinase activity and was actively phosphorylated without addition of exogeneous enzyme as described by previous investigators (1,2). However, when this preparation was chromatographed on a phospho-cellulose column, a phosphoprotein fraction essentially free of kinase was obtained in the effluent. The latter preparation contained a large quantity of phosphate (1.25 % phosphorus by weight), and served as substrate for the enzymes which remained on the column. The phosphate acceptor proteins were heterogeneous and at least 8 distinct bands of phosphoproteins were observed upon electrophoresis on a polyacrylamide gel.

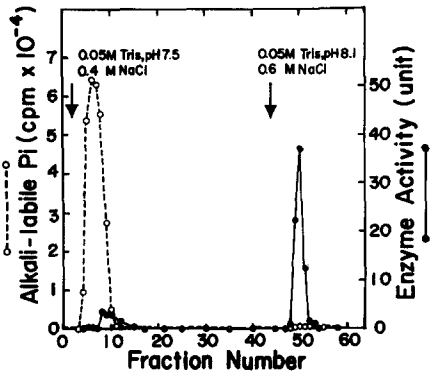

Fig. 1. Separation of phosphoproteins and protein kinase in rat liver chromatin by phospho-cellulose column chromatography. The supernatant (see text, 38 mg protein) was applied to a phospho-cellulose column (1.6 x 12 cm) equilibrated with 0.05 M Tris-Cl, pH 7.5, containing 0.4 M NaCl. Fractions (5 ml each) were collected at a flow rate of 0.4 ml/min. For enzyme assay, the standard reaction mixture (0.25 ml) contained: 200 μg of casein; 2.5 mμmoles of ATP-γ-^{32}P (20,000-45,000 cpm/mμmole); 5 μmoles of magnesium acetate; 25 μmoles of NaCl; 20 μmoles of Tris-Cl, pH 7.5; and an enzyme preparation. The incubation was carried out for 10 min at 30°. The reaction was stopped by adding 10 % trichloracetic acid. Acid-insoluble radioactivity was measured with a Millipore filter using a Nuclear Chicago gas-flow counter. One unit of enzyme was defined as that amount of enzyme which incorporated one mμmole of ^{32}Pi under the conditions described above. The alkali-labile phosphate bound to protein was determined by the method of Martin and Doty (10) modified by Meisler and Langan (11).

The enzyme associated with chromatin was resolved into two fractions by gel filtration on a Sephadex G-200 column (Fig. 2). These fractions are referred to tentatively hereafter as protein kinase A_1 and protein kinase A_2 as indicated in Fig. 2. These kinases were adsorbed separately on a phospho-cellulose column, and were eluted with 0.05 M Tris-Cl, pH 8.1, containing 0.6 M NaCl and 0.05 M acetate, pH 5.2, containing 0.8 M NaCl, respectively. By these procedures protein kinase A_1 and A_2 were purified about 100 and 20 fold, respectively, from chromatin with an overall yield of 50 %, all together, of the original activity. The enzyme preparations were essentially free of proteinase activity, and practically no phosphate was uptaken without acceptor protein added.

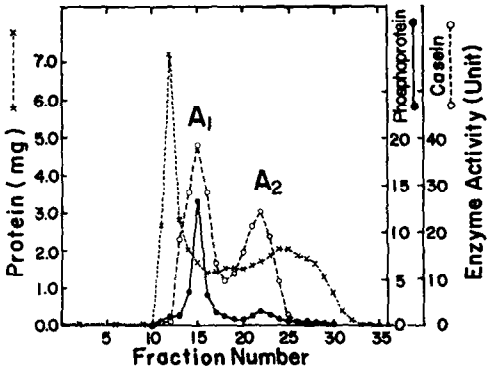

Fig. 2. Separation of protein kinase A_1 and A_2 by Sephadex G-200 gel-filtration. To the supernatant used in Fig. 1, solid ammonium sulfate was added to 75 % saturation. The resulting precipitate was collected by centrifugation and was dissolved in 0.05 M Tris-Cl, pH 7.5, containing 0.4 M NaCl at protein concentration of 15 mg/ml. The solution (45 mg protein) was applied to a Sephadex G-200 column (1.3 x 114 cm). The equilibration and elution of the column were carried out with the same buffer containing 0.4 M NaCl. Fractions (3.8 ml each) were collected at a flow rate of one ml/10 min. Nuclear phosphoprotein (50 μg) or casein (200 μg) was used as substrate. One unit of enzyme was defined as described in the legend to Fig. 1.

In addition to nuclear phosphoproteins described above, casein as well as egg yolk phosvitin (Mann Research Laboratories) served as phosphate acceptors. None of other proteins thus far tested including histone and protamine was active as substrate. With nuclear phosphoproteins as substrates the maximum amount of phosphate additionally incorporated by protein kinase A_1 was approximately 5 times more than that incorporated by protein kinase A_2 (Table I). Upon electrophoresis on polyacrylamide gel the pattern of radioactivity distributed among several phosphoproteins phosphorylated by protein kinase A_1 was not coincided with that phosphorylated by protein kinase A_2. These results may suggest that each protein kinase reacts with specific amino acid residues of different acceptor proteins.

Nuclear phosphoproteins were fully phosphorylated with either protein kinase A_1 or A_2, and then subjected to acid hydrolysis fol-

Table I Maximum Amounts of Phosphate Incorporated and Relative Labeling of Phosphoserine and Phosphothreonine by Protein Kinase A_1 and A_2

The incubation was carried out at 30° for 5 hours under the conditions given in Fig. 1 except that 50 μg of the substrate protein indicated and 17 μg of protein kinase A_1 or A_2 were employed. For measuring the ratio of radioactive phosphoserine to phosphothreonine (^{32}P-Ser/^{32}P-Thr), each reaction mixture was dialyzed overnight against a large volume of water and was hydrolyzed for 15 hours in 2 N HCl at 100°. HCl was removed under vacuum over NaOH and P_2O_5. The residue was subjected to electrophoresis (4,000 V for 2 hours) on Toyo Roshi No. 51 paper at pH 1.9 (12). After autoradiography, the areas corresponding to phosphoserine and phosphothreonine were cut out and the radioactivity was measured (13) using a Kobe Kogyo liquid scintillation spectrometer, Model GSL-160. Taking into account the breakdown of phosphoserine (64 %) and phosphothreonine (43 %) during the hydrolysis mentioned above, the value of ^{32}P-Ser/^{32}P-Thr was corrected.

Substrate	^{32}Pi incorporated mμmole/mg substrate		Ratio ^{32}P-Ser/^{32}P-Thr	
	A1	A2	A1	A2
Nuclear phosphoprotein	6.0	1.3	8.7	7.0
Casein	30.8	8.9	0.5	7.1
Phosvitin	9.4	2.2	1.5	11.1

lowed by high voltage paper electrophoresis. As shown in Table I, both kinases phosphorylated preferentially seryl but some threonyl residues, and the ratios of phosphoserine and phosphothreonine produced by protein kinase A_1 and A_2 were 8.7 and 7.0, respectively. Protein kinase A_1 phosphorylated seryl as well as threonyl residues of phosvitin, and preferentially threonyl residues of casein. Whereas, protein kinase A_2 reacted mainly with seryl residues of all acceptor proteins tested.

Protein kinase A_1 and A_2 were most active at pH 6.0-6.5 and 7.0-7.5, respectively, in the presence of 20 mM Mg ion. The Km values for ATP were 1.1 x 10^{-5} M for protein kinase A_1 and 2.0 x 10^{-5} M for protein kinase A_2. Unlike the protein kinase from

rabbit skeletal muscle described by Walsh et al. (14), protein kinase A_1 and A_2 did not respond to 3',5'-cyclic AMP. Although the enzyme activity to phosphorylate casein was found also in cytoplasm, the specific activity in chromatin was 25 times greater than that in cytoplasm.

The results presented above indicate that the phosphorylation of some nuclear phosphoproteins is not auto-catalytic but is catalyzed by at least two enzymes which are separable from bulk of phosphoproteins. These enzymes show different substrate specificities and phosphorylate seryl and threonyl residues of phosphoproteins in a specific but not random manner. Since the nuclear phosphoprotein preparations thus far available are heterogeneous and composed of at least 8 phosphate acceptor proteins, the precise substrate specificities of enzymes as well as the detailed properties of enzymic phosphorylation reactions will be explored by further investigations.

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