PHOSPHORYLATION OF RAT LIVER RIBOSOMAL SUBUNITS: PARTIAL PURIFICATION OF TWO CYCLIC AMP ACTIVATED PROTEIN KINASES

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SUMMARY

Two protein kinases can be prepared from rat liver supernatant. The enzymes catalyze phosphorylation of three proteins when the 40S ribosome subunit is the substrate; nine when the 60S subunit is the substrate. Phosphorylation of ribosomal proteins is stimulated by cyclic AMP.

Rat liver (1) and rabbit reticulocyte ribosomal proteins (2, 3) are phosphorylated if [^{32}P] phosphate is administered to the animals, or if the ribosomes are incubated with [γ-^{32}P] ATP and a crude supernatant fraction. Cyclic AMP stimulated phosphorylation of liver but not reticulocyte ribosomes. We report a partial purification of two protein kinases from rat liver that phosphorylate liver 40S and 60S ribosomal subunits. The enzyme is stimulated by cyclic AMP. At least three 40S and nine 60S ribosomal proteins are phosphorylated.

MATERIALS AND METHODS

Ribosomes and ribosomal subunits were isolated as described before (4, 5); the post-microsomal supernatant was used to prepare protein kinase. The concentration of ribosomes and ribosomal subunits was calculated from the absorption at 260 mμ (8); 1 A_{260} unit was taken to be the equivalent of 45 μg of rRNA. Calf thymus histone (Type II-A, lyophilized) was purchased from Sigma. [γ-^{32}P] ATP was prepared by the method of Glynn and Chappell (6).

Protein kinase assay. Protein kinase catalyzed phosphorylation of protein was measured by a modification of the procedure of Walsh et al. (7). Ribosomal subunits or histone were incubated in 0.1 ml of buffer (40 mM Tris-HCl, pH 7.7; 6 mM $MgCl_2$; 5% glycerol; 4 mM β-mercaptoethanol) containing [γ-^{32}P] ATP. The amount of substrate (ribosomal subunits or histone) and [γ-^{32}P] ATP is given in the tables or the legends to the figures. The concentration of cyclic AMP when present was 10^{-5} M. The reaction was started by adding [γ-^{32}P] ATP. After incubation for 15 min at 37°, 0.5 ml of 10% trichloroacetic acid and bovine serum albumin (1.25 mg in 0.2 ml) were added to the samples and the mixture was heated at 90-95° for 15 min. The precipitate was collected by centrifugation, dissolved in 1 ml of 0.1 N NaOH and reprecipitated by addition of 2 ml of 10% trichloroacetic acid. The precipitate was collected, washed with 3 ml of 5% trichloroacetic acid, drained, and dissolved in 0.5 ml of 88% formic acid. The radioactivity of the protein samples was determined as described before (8); the efficiency of the measurement was 80%.

Gel electrophoresis and radioautography of ribosomal proteins. Ribosomal subunits were phosphorylated in the reaction just described; the proteins were extracted (9) and dialyzed against 7% acetic acid. The concentration of protein was determined (10). The dialysate was lyophilized (to reduce the volume and to remove acetic acid) and the protein was dissolved in 7M urea containing 0.1 M β-mercaptoethanol. The samples were adjusted to pH 8 with solid Tris and incubated for 2 hr at 37° to assure that the proteins were reduced (9). The ribosomal proteins (in 6M urea) were separated by electrophoresis on discontinuous polyacrylamide gels (11). The destained gels were sliced into longitudinal sections and the slices dried on filter paper on a Buchner funnel; drying was speeded by covering the slices on the funnel with Saran wrap and heating with an infra-red lamp. The dried gels on filter paper were put on X-ray film (Kodak, NS-54T) and exposed 1-8 days.

RESULTS AND DISCUSSION

Initially we used unresolved 100,000 g supernatant and ribosomes from rat liver. The results were unsatisfactory, for considerable phosphorylation of the supernatant occurred in the absence of ribosomes, and there was phosphorylation of ribosomes in the absence of supernatant (Table I; see also reference 3). The assay was not improved by washing ribosomes with 0.5 M KCl, for while that decreased phosphorylation of the particles in the

Table I. Phosphorylation of Ribosomal Proteins by Liver Supernatant

Expt.	Substrate	Supernatant	Cyclic AMP	Phosphorylation (cpm)
1.	None	+	-	375
	None	+	+	508
	80S Ribosomes	-	-	3171
	80S Ribosomes	-	+	3426
	80S Ribosomes	+	-	1407
	80S Ribosomes	+	+	1572
	Washed 80S Ribosomes	-	-	595
	Washed 80S Ribosomes	-	+	507
	Washed 80S Ribosomes	+	-	365
	Washed 80S Ribosomes	+	+	605
2.	None	+	-	302
	None	+	+	425
	60S Subunits	-	-	1124
	60S Subunits	-	+	1026
	60S Subunits	+	-	335
	60S Subunits	+	+	1164

The amount of substrate was: 80S ribosomes, 340 μg rRNA; washed 80S ribosomes, 210 μg rRNA; 60S subunits, 290 μg rRNA. Washed 80S ribosomes were prepared by suspending the particles in 50 mM Tris-HCl (pH 7.7), 500 mM KCl, 12.5 mM $MgCl_2$, 1 mM dithiothreitol and incubating for 1 hr at 37°. The ribosomes were reisolated by centrifugation. Caffeine (1 mM) was added with cyclic AMP. The concentration of [γ-^{32}P] ATP was 3.8 μM; the specific activity was 3.4×10^5 cpm/nmole (experiment 1) or 2.4×10^5 cpm/nmole (experiment 2). The amount of rat liver supernatant when present was 90 μg. Each 1000 cpm incorporated into ribosomal protein is equivalent to 2.94 pmoles of phosphate (experiment 1) or 4.16 pmoles (experiment 2).

absence of supernatant, it also decreased that which occurred in its presence. Ribosomal subunits were a somewhat better substrate for the supernatant fraction; there was, for example, a definite stimulation of phosphorylation of the 60S subunit (3.5x) by cyclic AMP. One distressing, and still unexplained, finding was that the crude supernatant actually inhibited phosphorylation of ribosomal proteins (Table I). It was clear that meaningful experiments required purification of the protein kinase. The 60S subunit was generally used to follow the course of purification since it seemed a better substrate than the 40S subunit for the supernatant---especially with cyclic AMP.

A protein kinase that phosphorylates proteins of the 60S subunit was purified from liver 100,000 g postmicrosomal supernatant by a modification of the procedure Gill and Garren (12) used to purify a histone kinase from bovine adrenal supernatant. Kinase activity was found in two peaks (I and II) of the DE-52 eluate (Fig. 1). The phosphorylation of the proteins of the 60S ribosomal subunit is maximal when 6-10 μg of protein kinase (peak I) is used in the assay (Fig. 2). Phosphorylation is increased four- to five-fold by cyclic AMP. The ribosomal subunits have no appreciable protein kinase activity since there was little phosphorylation in the absence of enzyme; nor was there significant phosphorylation without substrate. Apparently the protein kinase activity found on ribosomes (3, 13; also Table I) is removed by washing the particles in 0.88 M KCl during the preparation of subunits. We had found that unresolved cystosol supernatant (such as had been used by Loeb and Blat (1)) inhibited phosphorylation of ribosomal proteins (Table I and also reference 3). The inhibition is eliminated when the protein kinases are purified by chromatography on DEAE-cellulose (Fig. 2).

The two protein kinases (peak I and II) will phosphorylate 40S as well as 60S ribosomal subunits (Table II), although the peak II enzyme has a lower specific activity. The activity of both enzymes is increased by cyclic AMP. Since the peak I and II enzymes phosphorylate calf thymus histone in addition to ribosomal

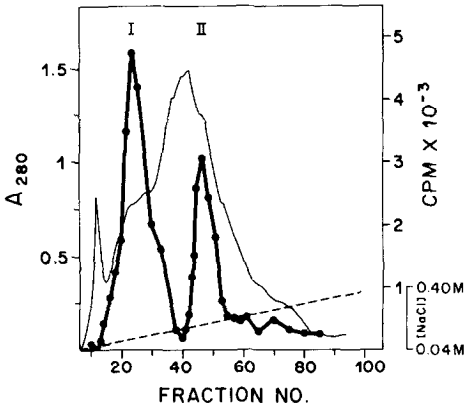

Figure 1. The separation of protein kinases by DEAE-cellulose chromatography. Purification of protein kinase was carried out at 4°. The 100,000 x g post-microsomal supernatant from rat liver (prepared as in reference 4) was made 6 mM in β-mercaptoethanol (MSH). The pH was adjusted to 5.0 with glacial acetic acid and the precipitate removed by centrifugation; the pH of the supernatant was made 7.0 with 0.5 N KOH and brought to 45% saturation by slow addition of solid ammonium sulfate. The precipitate was collected by centrifugation and dissolved in TM buffer (10 mM Tris, pH 7.7; 6 mM MSH) and dialyzed against TM for 16 hours. Aged calcium phosphate gel (Sigma), 3% (dry weight) in TM buffer, was added slowly to the protein solution (12-13 mg/ml) in a ratio of gel to protein of 1:1 and stirred for 35 minutes. The gel was collected by low speed centrifugation, washed twice with TM and the protein kinase activity eluted with 0.25 M KPO_4 (pH 8.1); the elution with 0.25 M KPO_4 was repeated twice. The combined eluates were dialyzed against TM containing 10% glycerol (TMG) for 16 hours. Approximately 550 mg of dialyzed protein were applied to a DEAE-cellulose column (DE-52 Whatman; 22 x 3 cm) that had been equilibrated with TMG. The column was washed with 2 column volumes of TMG and the protein kinase eluted with a 1 liter linear gradient of 0.04 to 0.40 M NaCl in TMG. The absorbance at 280 mμ of the eluate was determined and 0.025 ml of selected fractions were assayed for protein kinase activity using 60S ribosomal subunits (96 μg rRNA) as substrate and 5.3 μM [γ-^{32}P] ATP (1.1×10^5 cpm/nmole). The activity of 60S ribosomes without added protein fractions was 375 cpm. Each 1000 cpm incorporated into ribosomal protein is equivalent to 9.1 pmoles of phosphate. The continuous thin line is the A_{280} of the eluate; the thicker line is the protein kinase activity of selected fractions; the interrupted line is the concentration of NaCl.

Table II. Phosphorylation of Ribosomal Proteins and Histones by Partially Purified Protein Kinases.

Substrate	Enzyme	Cyclic AMP	Phosphorylation (cpm)	Stimulation (fold)
		Peak I (3.8 μg)		
None	+	-	85	
None	+	+	213	2.6
60S	-	+	284	
60S	+	-	1478	
60S	+	+	8388	6.7
40S	-	+	47	
40S	+	-	252	
40S	+	+	2491	11.9
Histone	-	+	28	
Histone	+	-	13138	
Histone	+	+	16834	1.3
		Peak II (8.52 μg)		
None	+	-	184	
None	+	+	446	2.4
60S	-	+	284	
60S	+	-	864	
60S	+	+	5769	8.6
40S	-	+	47	
40S	+	-	234	
40S	+	+	1415	7.3
Histone	-	+	28	
Histone	+	-	4527	
Histone	+	+	7597	1.7

The amount of substrate was: 60S ribosomal subunits, 81 μg rRNA; 40S ribosomal subunits, 26 μg rRNA; calf thymus histone, 40 μg. The concentration of the [γ-^{32}P] ATP was 7.7 μM; the specific activity was 7.9×10^4 cpm/nmole. Each 1000 cpm incorporated into protein is equivalent to 12.7 pmoles of phosphate. In calculating the stimulation due to cyclic AMP, phosphorylation in the absence of enzyme was substracted.

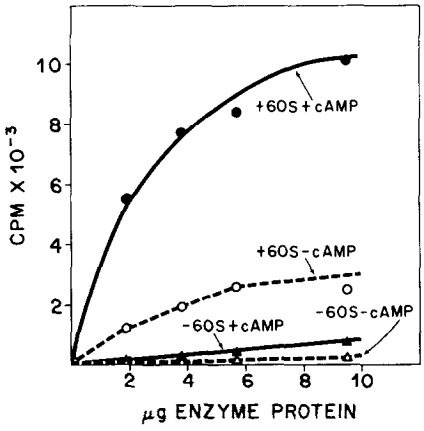

Figure 2. Effect of amount of protein kinase on phosphorylation of 60S ribosomal proteins. The amount of 60S ribosomal subunits when present was 96 μg rRNA; the concentration of [γ-^{32}P] ATP was 7.8 μM (9.3 x 10^4 cpm/nmole). Each 1000 cpm incorporated into ribosomal protein is equivalent to 10.8 pmoles of phosphate.

proteins, they either have not been resolved from other protein kinases or lack substrate specificity (Table II).

Proteins were extracted from ribosomal subunits phosphorylated by peak I protein kinase and radioautographed after separation by electrophoresis on polyacrylamide gels. Three protein bands (one intense, two faint) from the 40S subunit were radioactive; nine bands from the 60S subunit were radioactive (Fig. 3). The actual number of proteins phosphorylated may be greater since gel electrophoresis does not resolve all the ribosomal proteins (11). Phosphorylated ribosomal proteins were hydrolyzed with HCl (2), and analyzed by paper electrophoresis (14). The hydrolysates contained radioactivity that co-electrophoresed with o-phosphoserine and o-phosphothreonine as well as a good deal of radioactive orthophosphate (results not shown)---the latter probably arose from hydrolysis of phosphoester bonds (2).

There are indications that the function of ribosomes may be susceptible to alteration and hence control (15). One can imagine a number of ways in which the structure and function of ribosomes might be changed: by addition or deletion of proteins

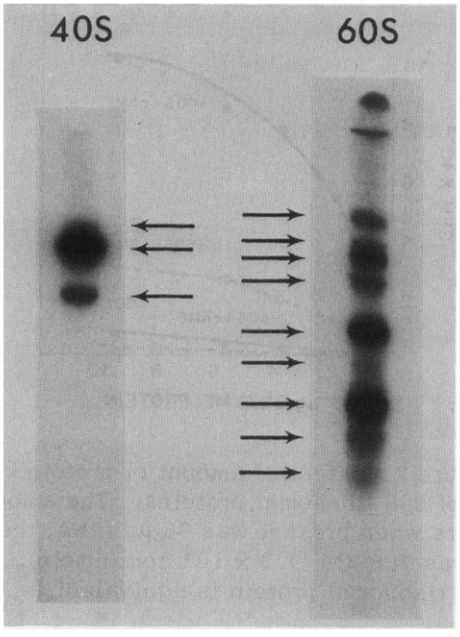

Figure 3. Radioautographs of phosphorylated ribosomal proteins. 40S ribosomes (450 μg rRNA) or 60S ribosomes (945 μg rRNA) were incubated for 30 minutes at 37° with 91.2 μg protein kinase I in 1.6 ml of medium containing 10^{-5} M cyclic AMP and 58 μM [γ-^{32}P] ATP (8.0×10^3 cpm/nmole). The reaction was stopped by the addition of 0.18 ml of ice-cold 1 M magnesium acetate (to precipitate ribosomal subunits) and chilled 30 minutes. Ribosomes were collected by centrifugation at 10,000 x g for 10 minutes. They were resuspended in 50 mM Tris-HCl (pH 7.5), 80 mM KCl, 12.5 mM $MgCl_2$, and centrifuged again. This process was repeated. Protein extraction, polyacrylamide gel electrophoresis, and radioautography were performed as described in MATERIALS AND METHODS.

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which are not essential for ribosome function but amplify ribosome activity (15); by a change in the conformation of a ribosomal protein or of the ribosome itself; or by chemical modification (phosphorylation, acetylation, etc.) of ribosomal proteins. Whether the function of ribosomes can be regulated by reversible phosphorylation of their proteins is an important unresolved problem.

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