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Adenosine 3′,5′-Monophosphate and Protein Kinase Dependent Phosphorylation of Ribosomal Protein†

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ABSTRACT: Cyclic adenosine 3′,5′-monophosphate (cAMP) stimulated phosphorylation of adrenal cortical ribosomal protein *in vitro*. The phosphorylation reaction is catalyzed by cAMP-dependent protein kinase (EC 2.7.1.37) purified from the cytosol or by endogenous activity remaining on 0.5 M KCl washed ribosomes. cAMP-dependent phosphorylation of the same ribosomal protein bands is observed using either exogenous or endogenous protein kinase activity. When salt washing is carried out prior to phosphorylation, 53% of the total ribosomal sites for ^{32}P incorporation remain with the ribosome. When phosphorylation is carried out prior to salt washing, only 22% of the total phosphorylated sites remain with the ribosome suggesting that phosphorylation has altered the affinity of these substrate proteins for the ribosomal structure. Phosphorylated proteins from isolated 60S and 40S subunits have been resolved by gel electrophoresis into six–eight labeled protein bands from the large subunit and a single radioactive band from the small subunit. Complete phosphorylation of the subunits resulted in a total of 10 and 2 mol of phosphate incorporated/mol of 60S and 40S subunits, respectively, in the *in vitro* catalyzed reaction. It is postulated that ribosomal protein phosphorylation is regulated through cAMP-dependent protein kinase; this provides one mechanism for hormonal control of ribosome function.

Mammalian ribosomes contain phosphoproteins. The phosphate groups esterified to serine and threonine residues turn over in reticulocyte ribosomes at a rate of approximately 3%/min (Kabat, 1972). The phosphate groups are present on only a limited number of ribosomal proteins (Kabat, 1970, 1971; Eil and Wool, 1971; Blat and Loeb, 1971; Bitte and Kabat, 1972; Traugh *et al.*, 1973). Because different methodology has been used for ribosomal protein separation, it is uncertain whether the same ribosomal proteins are phosphorylated in different tissues. However, when the same gel system was used the patterns of ribosomal protein phosphorylation in mouse sarcoma 180 cells and rabbit reticulocytes were found to be identical (Bitte and Kabat, 1972). The hormonal state appears to influence the phosphorylation of ribosomal protein. Treatment of rats with glucagon resulted in increased phosphorylation of ribosomal proteins (Blat and Loeb, 1971). Phosphorylation of one ribosomal protein band was increased two- to threefold (Blat and Loeb, 1971). Thyroidectomy resulted in a 35% decrease in the phosphate content of ribosomal proteins; 3,5,3′-triiodothyrionine administration restored the phosphate content to control levels (Correze *et al.*, 1972).

One mechanism for hormonal control of ribosomal protein phosphorylation is through cAMP[1]-dependent protein kinase. cAMP-dependent protein kinase (EC 2.7.1.37) originally described in skeletal muscle by Walsh *et al.* (1968) is the major effector of cAMP action in eukaryotic cells presently identified. cAMP-dependent protein kinase exists as a molecular complex consisting of regulatory receptor and catalytic kinase subunits; binding of cAMP to receptor results in dissociation of the inhibitory receptor and full activation of the catalytic kinase subunit (Gill and Garren, 1970, 1971; Tao *et al.*, 1970; Kuman *et al.*, 1970; Reimann *et al.*, 1971; Erlichman *et al.*, 1971).

A number of substrates for cAMP-dependent protein kinase have been identified. cAMP-dependent protein kinase catalyzed phosphorylation of phosphorylase kinase and of glycogen synthetase results in marked alteration of enzyme activity with activation of the first and inactivation of the second enzyme (Soderling *et al.*, 1970). Similar cAMP-depen-

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[1] cAMP, cyclic adenosine 3′,5′-monophosphate; ACTH, adrenocorticotropic hormone; TKM buffer, 50 mM Tris-HCl (pH 7.5)–25 mM KCl–5 mM $MgCl_2$; TMD buffer, 2 mM Tris-HCl (pH 7.5)–0.5 mM $MgCl_2$–0.1 mM dithiothreitol; TM buffer, 10 mM Tris-HCl (pH 7.5)–2 mM $MgCl_2$.

dent phosphorylation of hormone-sensitive lipase results in activation of that enzyme (Huttunen *et al.*, 1970; Corbin *et al.*, 1972). Phosphorylation of histone, protamine, microtubules, and synaptic membranes also occurs (Langan, 1969a; Jergil and Dixon, 1970; Goodman *et al.*, 1970; Johnson *et al.*, 1971).

Loeb and Blat initially demonstrated that cAMP stimulated phosphorylation of rat liver ribosomes incubated with microsomal supernatant (Loeb and Blat, 1970). We found that cAMP-dependent protein kinase from adrenal cortical cytosol catalyzed the phosphorylation of ribosome associated protein (Walton *et al.*, 1971). A half-maximal saturation concentration of 4×10^{-8} M cAMP was observed which resembled that found with other substrates for this enzyme. Studies of subcellular distribution have demonstrated the presence of cAMP-dependent protein kinase in the endoplasmic reticulum of adrenal cortical microsomes (Walton *et al.*, 1971). Some specificity for ribosomal protein phosphorylation may result from this subcellular localization. Using rat liver Eil and Wool reported that *in vitro* phosphorylation of proteins on each of the ribosomal subunits was catalyzed by cAMP-dependent protein kinase (Eil and Wool, 1971). At least three proteins on the 40S and nine proteins on the 60S ribosomal subunit were phosphorylated in the *in vitro* reaction.

The functional importance of the ribosomal phosphorylation has not been defined. In the adrenal cortex, ACTH[1] control of steroidogenesis requires protein synthesis (Ferguson, 1963; Garren *et al.*, 1965). Because cAMP, generated in response to ACTH, regulates steroid production, the nucleotide has been implicated in the control of protein synthesis (Garren *et al.*, 1971). Regulation of protein synthesis at a posttranscriptional level by cAMP has also been implicated in parotid, pituitary, and liver tissues (Grand and Gross, 1970; Labrie *et al.*, 1971; Wicks, 1971).

Initial studies of the phosphorylation of adrenal cortical ribosomes demonstrated cAMP-stimulated, protein kinase dependent ^{32}P incorporation into protein which remained with the 80S ribosome on sucrose gradient sedimentation (Walton *et al.*, 1971). Phosphorylation of serine and threonine residues of ribosomal protein occurred. The present studies examine the cAMP-dependent phosphorylation of ribosomal protein in greater detail. cAMP-dependent protein kinase has been identified on isolated ribosomes. The phosphorylation reaction catalyzed by the enzyme present on the ribosome has been compared to that catalyzed by cAMP-dependent protein kinase from the cytosol. Complete phosphorylation of the same ribosomal proteins has been demonstrated using both exogenous and endogenous cAMP-dependent protein kinase activity. cAMP-dependent protein kinase catalyzed phosphorylation of proteins on each of the ribosomal subunits has been demonstrated. The phosphoproteins from each subunit have been characterized by polyacrylamide gel electrophoresis and the extent of phosphorylation of each subunit resulting from the *in vitro* reaction has been calculated. After phosphorylation increased ribosomal protein is removed from the ribosome in response to increasing ionic strength.

Materials and Methods

[γ-^{32}P]ATP was prepared (Glynn and Chappell, 1964) using carrier-free [^{32}P]orthophosphate obtained from Schwarz/Mann and purified according to Walsh *et al.* (1971); the purity (93–97%) was determined by descending paper chromatography in isobutyric acid–ammonia–water (66:1:33) and the concentration was determined with absorbance at 259 nm. It was used as prepared or diluted to desired specific activity with unlabeled ATP. ATP and cAMP were from P-L Biochemicals, Tris and calf thymus histone were from Sigma Chemical Co., dithiothreitol and Triton X-100 were from Calbiochem, sodium deoxycholate was from Fisher Scientific Co., and Liquifluor was from New England Nuclear.

Protein Kinase Assay. The method of DeLange *et al.* (1968) was modified as follows. Duplicate assays were carried out in reaction mixtures at 30° as described in the figure legends. Reactions were stopped with the addition of 1.2 mg of bovine serum albumin and 1 ml of 15% trichloroacetic acid. The precipitate was centrifuged, dissolved in 0.5 ml of 0.1 N NaOH, and immediately precipitated with 1 ml of 15% trichloroacetic acid. This procedure was repeated a second time when the specific activity of ATP was greater than 1.2×10^4 cpm/nmol. The precipitate was washed three times in 1.5 ml of 5% trichloroacetic acid; the second wash was incubated at 90° for 15 min to solubilize RNA and minimize nonspecific absorption of radioactivity. The acid-insoluble precipitate was dissolved in 1 ml of 97% formic acid and counted in 10 ml of Bray's solution (Bray, 1960). Background, generally less than 120 cpm, was subtracted for each experiment.

Preparation of cAMP-Dependent Protein Kinase. Enzyme was purified from the cytosol of bovine adrenal cortices as previously described (Gill and Garren, 1970). Two peaks of cAMP-dependent protein kinase were resolved on DEAE-cellulose chromatography which have been designated I and III in order of elution with a linear NaCl gradient. As previously reported (Gill and Garren, 1970, 1971) each peak of activity contains both cAMP receptor and cAMP-activated protein kinase in a complex which sediments at 7 S in sucrose gradients and migrates in polyacrylamide gels with a mol wt of ~144,000. Both kinase activities incorporate ^{32}P from [γ-^{32}P]ATP into ribosomal protein. The incorporation catalyzed by each activity is stimulated by cAMP.

Because DEAE peak I activity was relatively less abundant and less stable during storage than DEAE peak III, the latter was used for this study. The specific activity of DEAE peak III was 2.5 nmol of ^{32}P incorporated/min per mg of enzyme with histone as substrate. Further purification of DEAE peak III activity was accomplished with a sucrose gradient (SG) centrifugation step (Gill and Garren, 1970) to provide an enzyme preparation (SG peak III) with a specific activity of 5.6 nmol of ^{32}P incorporated/min per mg of enzyme-protein. This latter preparation was used for the incorporation experiments with ribosomal subunits. The SG peak III activity represents a 52-fold increase in specific activity compared to cytosol. The endogenous ^{32}P incorporation for each enzyme preparation was determined in the absence of substrate under the reaction conditions described. These values are reported and subtracted from total incorporation observed with substrate.

Preparation of Ribosomes and Ribosomal Subunits. All procedures were performed at 5° unless stated otherwise. Freshly obtained bovine adrenal cortices were homogenized in 2 vol (w/v) of 0.25 M sucrose in 50 mM Tris-HCl (pH 7.5), 25 mM KCl, and 5 mM $MgCl_2$ (TKM) buffer and microsomes were isolated by centrifugation as previously described (Gill and Garren, 1969). The microsomal pellet was resuspended in the same volume of homogenizing buffer and adjusted to 4% Triton X-100 and 1% sodium deoxycholate and centrifuged at 12,000*g* for 10 min. Five milliliters of supernatant was layered onto 7 ml of 0.5 M sucrose in TKM and centrifuged at 164,000*g* for 3 hr. The ribosomal pellet was purified further by precipitation with $MgCl_2$ as described by Takanami (1960) ex-

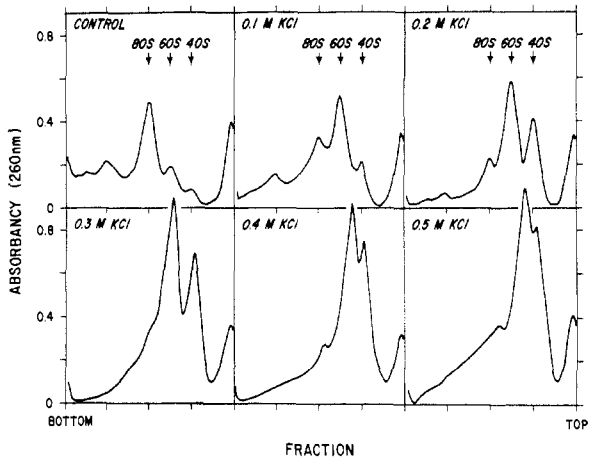

FIGURE 1: Sucrose gradient analysis of the effects of KCl concentrations on the dissociation of adrenal cortical ribosomes. An aliquot of ribosomes (3.6 A_{260} units) was combined with a volume of 1 M KCl to give the final concentration indicated. Samples were layered on a 10–30% linear sucrose gradient containing 10 mM Tris-HCl (pH 7.5), 2mM $MgCl_2$, and the KCl concentration indicated. Centrifugation was at 40,000 rpm for 3 hr at 5° in a SW-40 rotor. Gradients were monitored with a 1-cm flow-through cell at A_{260} nm.

cept that the ribosomes were resuspended and dialyzed with 2 mM Tris-HCl (pH 7.5), 0.5 mM $MgCl_2$, and 0.1 mM dithiothreitol (TMD) buffer. Ribosomes, at 75–100 A_{260} units/ml, were stored at −75° and centrifuged free of aggregates at 12,000*g* for 10 min immediately prior to use.

For the preparation of subunits, the postmitochondrial supernatant was treated with 4% Triton X-100 and 0.5% sodium deoxycholate and ribosomes were isolated by centrifugation at 105,000*g* for 2 hr. Magnesium chloride precipitation was performed as described above. Since dissociation of ribosomes into 60S and 40S subunits is dependent on the levels of Mg^{2+} as well as K^+ (Martin *et al.*, 1969; Nolan and Arnstein, 1969; Falvey and Staehelin, 1970; Blobel and Sabatini, 1971; Faust and Matthaei, 1972), optimal conditions for the isolation of adrenal cortical ribosomal subunits were determined. Following magnesium chloride precipitation, the ribosomal preparations were dialyzed against low concentrations of magnesium chloride (0.5 mM) in TMD buffer. Under these conditions of preparation, the structural integrity of the ribosome was preserved in the absence of KCl as determined by sucrose gradient analysis (Figure 1, control). Nearly complete dissociation into subunits was observed at KCl concentrations between 0.25 and 0.30 M (Figure 1). Below these levels dissociation into subunits was incomplete, while above 0.3 M KCl the larger subunit shows a significant reduction in sedimentation suggesting the loss of substantial amounts of protein.

For subunit isolation an aliquot of ribosomes was combined with an equal volume of TMD buffer and adjusted to 0.3 M KCl with a 1 M solution. A_{260} units (42–55) were layered onto 38 ml of a 15–35% linear sucrose gradient in 10 mM Tris-HCl (pH 7.5), 250 mM KCl, and 2 mM $MgCl_2$ and sedimented at 25,000 rpm for 15 hr in a SW-27 rotor. Peaks of 60S and 40S subunits were collected and diluted with 0.5 vol of 10 mM Tris-HCl (pH 7.5)–2 mM $MgCl_2$ (TM) buffer and concentrated by centrifugation at 113,000g for 18 hr. Pellets were resuspended in 0.25 M sucrose in TM buffer at about 40 A_{260} units/ml, adjusted to 0.3 M KCl, and centrifuged a second time at 20–25 A_{260} units/tube as described above. The 40S peak was concentrated and resuspended as above. The 60S peak was dialyzed against TM buffer and concentrated to about 50 A_{260} units/ml in a pressure cell (Amicon PM-30 membrane) and finally adjusted to 0.25 M sucrose with a 2 M solution. The extent to which the subunits were purified was determined by analysis of the ribosomal RNA in each species following sodium dodecyl sulfate treatment and sucrose gradient centrifugation (Noll and Stutz, 1968). Because of the ability of each species to cross-contaminate the other through dimerization of 40S subunits and stripping of proteins from the 60S species, the analysis of RNA provided strict criteria for purity. The 60S subunit preparations contained 7–14% 18S RNA; the 40S subunit preparations were contaminated with 3–8% 29S RNA.

Disc Gel Electrophoresis. To separate ribosomes from other reaction components, entire reaction mixtures were layered on 7 ml of 0.5 M sucrose in TM buffer and centrifuged at 39,000 rpm for 18 hr at 5° in a SW-40 rotor. Ribosomal pellets were resuspended in 0.5 ml of 10 mM sodium phosphate (pH 7.6), 10 mM 2-mercaptoethanol, and protein extracted with 6 M LiCl and 8 M urea (Leboy *et al.*, 1964). Protein was dialyzed against three changes of 6 M urea containing 10 mM 2-mercaptoethanol. Recovery of radioactivity as hot trichloroacetic acid insoluble protein was 86–95%. Disc gel electrophoresis was performed with stacking gels on 0.6 × 10 cm separating gels in 8 M urea in a β-alanine–acetic acid buffer (pH 4.5) system or Tris–glycine buffer (pH 8.3) system (Low and Wool, 1967). Gels contained 6.67% acrylamide and 0.18% *N,N'*-methylenebisacrylamide. Electrophoresis was carried out at 3 mA/tube at room temperature. Protein bands were stained for 30 min with 0.5% Aniline Blue Black in 7% acetic acid, and destained in excess 7% acetic acid for 15 hr followed by electrophoresis for 1 hr. Gels were scanned in a linear transport system with absorbance at 540 nm to record protein bands prior to slicing into 1.5-mm disks. Each disk was counted directly in 1 ml of toluene–Liquifluor scintillator.

Protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as a standard. The phosphate content of ribosomal protein was determined by the method of Ames and Dubin (1960). Hot trichloroacetic acid insoluble ribosomal protein was treated with 1 N NaOH at 37° for 18 hr to hydrolyze alkali-labile phosphate bonds; protein was precipitated with 2 vol of 20% trichloroacetic acid and removed. The supernatant was lyophilized, the residue solubilized in 0.5 N HCl, and total phosphate quantitated.

Results

cAMP-Dependent Phosphorylation of Ribosomal Protein. Isolated adrenal cortical ribosomes contain phosphoproteins. After ribosome isolation, ribosomal protein was extracted and the phosphoprotein content determined by the method of Ames and Dubin (1960). As isolated, ribosomes contain 3.5 nmol of phosphate/mg of protein. Assuming a mol wt of 5.2×10^6 (Vournakis and Rich, 1971) and $E^{1\%}_{1\,cm}$ (Tashiro and Siekevitz, 1965) this represents 18 mol of phosphate/mol of ribosome.

Protein kinase activity is also associated with adrenal cortical ribosomes. The method of ribosome isolation involved sedimentation of microsomes in buffered sucrose at low ionic strength (0.025 M KCl), detergent solubilization of membranes, and resedimentation through discontinuous sucrose gradients. Further purification was performed by precipitation with $MgCl_2$. This procedure resulted in adrenal cortical ribosomes with an average A_{260}:A_{235} ratio of 1.34 indicating approximately 20% more associated protein than

TABLE I: Effect of Buffer and pH on the Activation of Protein Kinase by cAMP.

Buffer (pH)	Enzyme	^{32}P incorpn[a] (nmol/min per mg) −cAMP	+cAMP
Glycerol phosphate (6.0)	−	0.08	0.16
	+	0.08	0.52
Tris-HCl (7.5)	−	0.35	0.56
	+	0.85	1.13

[a] Reaction mixtures of 0.25 ml contained 50 mM buffer, as indicated, 1 mM dithiothreitol, 20 mM NaF, 5 mM $MgCl_2$, 5 μM cAMP, 2.7 A_{260} units of 0.5 M KCl washed ribosomes, 0.6 mM [^{32}P]ATP (9.27 × 10^3 cpm/nmol), and 46 μg of enzyme (DEAE peak III) when added. Incubation time was 10 min. Incorporation was determined as described under Materials and Methods. Value given was corrected for endogenous incorporation of enzyme, when added, and was 0.09 nmol/min per mg.

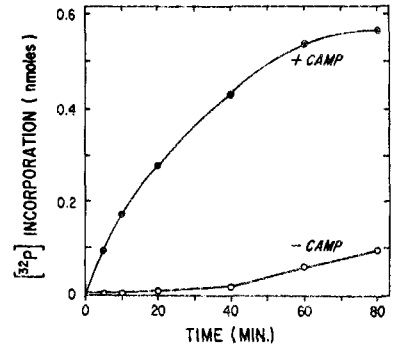

FIGURE 2: Cytosol protein kinase catalyzed phosphorylation o ribosomal protein. Reaction mixtures of 0.25 ml contained 50 mM glycerol phosphate (pH 6.0), 1 mM dithiothreitol, 20 mM NaF, 5 mM $MgCl_2$, 5 μM cAMP when added, 2.7 A_{260} units of 0.5 M KCl washed ribosomes, 0.6 mM [^{32}P]ATP (9.27 × 10^3 cpm/nmol), and 46 μg of enzyme (DEAE peak III). Incorporation was determined as described under Materials and Methods and was corrected for endogenous incorporation into enzyme (2.5 and 2.9 pmol/min) and into ribosomes (3.8 and 7.8 pmol/min) in the absence and presence of cAMP, respectively.

that present in isolated ribosomal subunits with an A_{260}:A_{235} ratio of 1.6 (Petermann, 1964). The amount of endogenous protein kinase activity residing with the ribosome can be substantially reduced by washing the ribosomes in solutions of high ionic strength (Kabat, 1971; Traugh *et al.*, 1973). Endogenous protein kinase activity is reduced as preparations with higher A_{260}:A_{235} ratios are obtained. Because the high level of endogenous phosphorylation observed with the unwashed ribosomes obscured the effects of added cAMP and protein kinase, 0.5 M KCl washed ribosomes were utilized to study the cAMP-dependent reaction catalyzed by endogenous and exogenous protein kinase. The enzyme and substrate removed by the 0.5 M KCl extraction have not been studied further, but Traugh *et al.* (1973) have indicated that a non-cAMP-dependent protein kinase with altered substrate and nucleotide specificity is present in this fraction in reticulocytes.

The effect of cAMP on the rate of ^{32}P incorporation into ribosomal protein catalyzed by exogenous protein kinase (DEAE peak III prepared from the cytosol) is shown in Figure 2. In the absence of cAMP only minimal phosphorylation was observed in response to exogenous enzyme. The apparent K_m for cAMP was 4 × 10^{-8} M (Walton *et al.*, 1971). The effect of cAMP on the rate of ^{32}P incorporation, as catalyzed by the cytosol protein kinase, was influenced by buffer and pH (Table I). The strong dependency on cAMP observed in glycerol phosphate at pH 6.0 was less pronounced with Tris-HCl, pH 7.5. The rate of phosphorylation, however, was increased at pH 7.5. A similar effect of the endogenous enzymic activity associated with the ribosomes was observed (Table I). The cAMP dependency was again more marked at pH 6.0 while the rate was increased at pH 7.5.

Though added exogenous cAMP-dependent protein kinase increases the rate of ribosomal protein phosphorylation, complete phosphorylation of available sites occurs without added enzyme. The rate of ^{32}P incorporation catalyzed by exogenous and endogenous protein kinase is shown in Figure 3. The reaction was performed in Tris-HCl buffer at pH 7.5 to maximize incorporation. At 80 min complete phosphorylation of available sites has occurred in the absence of added enzyme. Excess levels of ATP were maintained throughout the reaction.

The ribosomal protein phosphorylation resulting from endogenous protein kinase activity was compared to that obtained with added enzyme. KCl (0.5 M) washed ribosomes were incubated for 2 hr in the presence and absence of added enzyme and cAMP. Following phosphorylation, the ribosomes were separated from exoogenous enzyme by sedimentation through discontinuous sucrose gradients, the ribosomal protein was extracted, and the patterns of protein phosphorylation were compared in polyacrylamide gel electrophoresis. The endogenous phosphorylation observed with the enzyme preparation does not adhere to the ribosomes as isolated from the reactions by discontinuous sucrose gradient centrifugation. The phosphorylation of a number of ribosomal proteins is stimulated by cAMP (Figure 4). The electrophoretic pattern

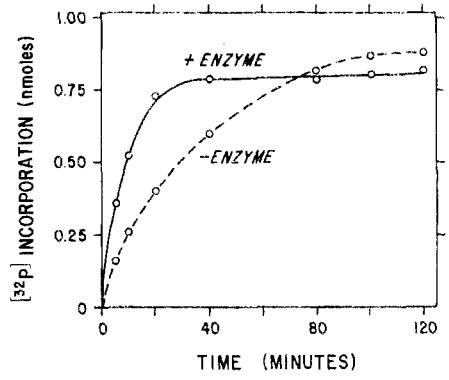

FIGURE 3: The phosphorylation of salt-washed ribosomes as a function of time. Ribosomes were washed in 50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, and 10 mM 2-mercaptoethanol containing 0.5 M KCl at 40 A_{260} units/ml and then layered on 7 ml of 0.5 M sucrose in the same buffer and centrifuged at 164,000*g* for 3 hr at 5°. Pellets were resuspended in their original volume of 20 mM Tris-HCl (pH 7.5), 1 mM $MgCl_2$, and 10 mM 2-mercaptoethanol buffer, dialyzed against the same buffer, and used directly. Reaction mixtures of 0.25 ml contained 50 mM Tris-HCl (pH 7.5), 5 mM dithiothreitol, 5 mM $MgCl_2$ 5 μM cAMP, 2.7 A_{260} units of ribosomes, 0.6 mM [^{32}P]ATP (5.44 × 10^3 cpm/nmol) and 46 μg of enzyme (DEAE peak III) when added. Incorporation was determined as described under Materials and Methods and was corrected for endogenous incorporation into the enzyme preparation which ranged from 0.05 nmol at 5 min to 0.19 nmol at 120 min. Excess levels of ^{32}P are maintained throughout the reaction as twice the amount of ribosomes under the same conditions doubled the total ^{32}P incorporation.

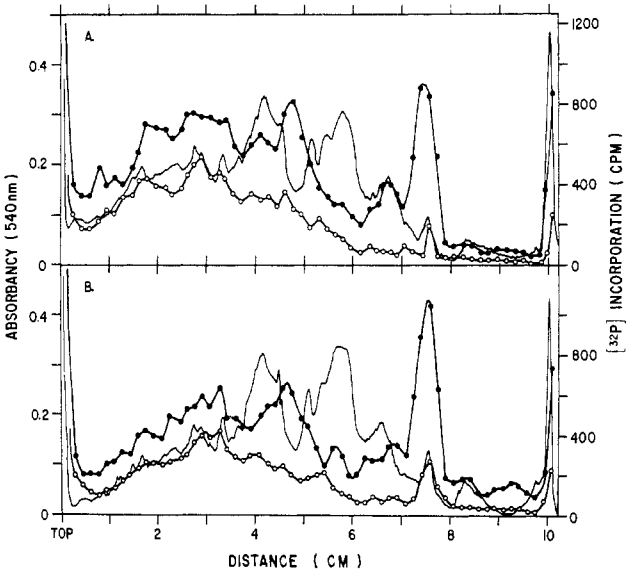

FIGURE 4: Polyacrylamide gel electrophoresis of phosphorylated ribosomal protein. Phosphorylation was performed in a volume of 5.0 ml containing 50 mM Tris-HCl (pH 7.5), 1 mM dithiothreitol, 20 mM NaF, 5 mM $MgCl_2$, 5 μM cAMP when added, 60 A_{260} units of 0.5 M KCl washed ribosomes, 0.3 mM [^{32}P]ATP (3.53 $\times$ 10^5 cpm/nmol), and (A) 1.37 mg of enzyme (DEAE peak III) or (B) no enzyme. Incubation time was 120 min. Reactions were cooled, and discontinuous sucrose gradient sedimentation, protein extraction, and gel electrophoresis were performed as described under Materials and Methods (●) plus cAMP; (○) minus cAMP.

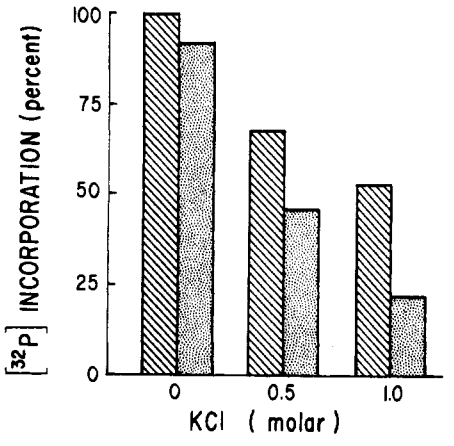

FIGURE 5: Effects of high salt washing on the removal of substrate and phosphoprotein from ribosomes. Left columns: Before phosphorylation, ribosomes were washed, centrifuged, and resuspended as described in Figure 3 with the concentration of KCl indicated. Phosphorylation was performed in the presence of exogenous enzyme under conditions described in Figure 3 except 3.0 A_{260} units of ribosomes was used and the incubation time was 80 min. Incorporation was determined as described under Materials and Methods (100% was 1.00 nmol) and was corrected for endogenous incorporation into enzyme (0.17 nmol). Right columns: phosphorylation of unwashed ribosomes was performed before high salt wash. Phosphorylation was performed in a volume of 2.5 ml as described in Figure 3 except 28 A_{260} units of ribosomes was used. After incubation, reactions were stopped with an equal volume of ice-cold buffer (50 mM Tris-HCl (pH 7.5)–5 mM $MgCl_2$–10 mM 2-mercaptoethanol) containing twice the KCl concentration indicated. Ribosomal pellets were collected by sedimentation through discontinuous sucrose gradients and resuspended in 1 ml of buffer. Incorporation was determined as described under Materials and Methods on an aliquot of the resuspended pellet and on the salt-washed extract and corrected for an enzyme blank. A total of 32,400 $\pm$ 900 cpm (mean $\pm$ standard deviation) represents 100% recovery calculated from each experiment.

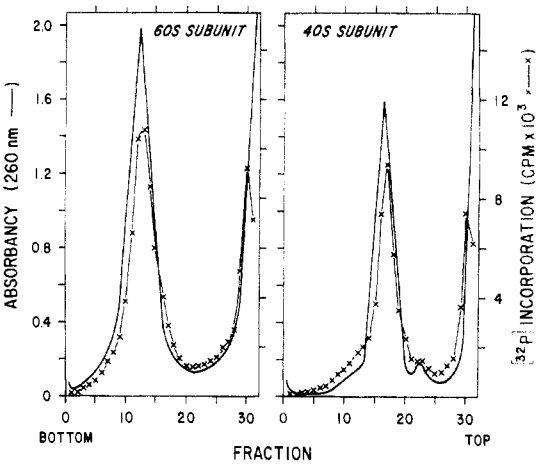

FIGURE 6: Sucrose gradient analysis of phosphorylated 60S and 40S subunits. Phosphorylation was performed in reaction mixtures of 0.50 ml containing 50 mM Tris-HCl (pH 7.5), 5 mM dithiothreitol, 2 mM $MgCl_2$, 5 μM cAMP, 0.29 mM [^{32}P]ATP (1.26 $\times$ 10^5 cpm/nmol), 68 μg of SG peak III enzyme, and 3.9 A_{260} units of subunit. Incubation time was 60 min. Reactions were cooled, adjusted to 0.25 M KCl, and centrifuged in a 10–30% sucrose gradient in 10 mM Tris-HCl (pH 7.5), 250 mM KCl, and 2 mM $MgCl_2$. Centrifugation was at 40,000 rpm for 5 hr at 5° in a SW-40 rotor. Fractions were collected and incorporation into hot trichloroacetic acid insoluble protein was determined as described under Materials and Methods.

of protein bands and of ^{32}P incorporation observed using exogenous enzyme in the presence and absence of cAMP is shown in the upper panel (Figure 4A). A similar pattern of ^{32}P incorporation was observed when ribosomes were phosphorylated in the absence of added protein kinase (Figure 4B). Stimulation by cAMP is evident using both exogenous and endogenous protein kinase activity. The exogenous enzyme does not appear to phosphorylate additional ribosomal sites not available to the endogenous activity. It is, therefore, pos-

TABLE II: Complete Phosphorylation of Ribosomal Protein.[a]

Addition	^{32}P Incorpn[b] (nmol)
Phosphorylated ribosomes (1 M KCl, washed)	0.69
plus enzyme	0.71
plus [^{32}P]ATP	0.72
plus enzyme; plus [^{32}P]ATP	0.68

[a] Initial phosphorylation of ribosomes was performed in a volume of 2.5 ml, as described in Figure 3, using [^{32}P]ATP of sp act. 1.06 $\times$ 10^4 cpm/nmol and 35 A_{260} units of ribosomes with incubation for 80 min. The reaction was stopped with an equal volume of cold 2 M KCl and centrifuged through 0.5 M sucrose at 5°. Total ^{32}P incorporation was 3.14 $\times$ 10^5 cpm, and represented 28.8 and 71.2% recovered in the ribosomal pellet and 1 M KCl extract, respectively. This represents 0.68 nmol of ^{32}P incorporation per 2.9 A_{260} units of washed ribosomes. [b] Phosphorylation of the 1 M KCl washed pellet (2.9 A_{260} units) was performed as described above in a volume of 0.25 ml for 15 min with 58 μg of enzyme and [^{32}P]ATP added as indicated. ^{32}P incorporation was determined as described under Materials and Methods. When appropriate, values given are corrected for endogenous incorporation into enzyme which was 0.15 nmol.

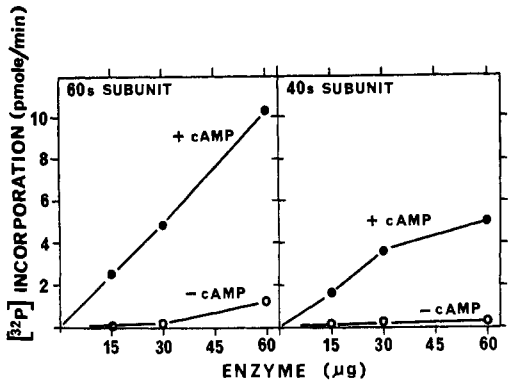

FIGURE 7: Phosphorylation of isolated 60S and 40S ribosomal subunits as a function of added protein kinase. Phosphorylation was performed in reaction mixtures of 0.25 ml containing 50 mM glycerol phosphate (pH 6.0), 5 mM dithiothreitol, 2 mM $MgCl_2$, 5 μM cAMP when added, 0.7 mM [^{32}P]ATP (2.85×10^4 cpm/nmol), 2.6 A_{260} units of 60S or 2.8 A_{260} units of 40S, and SG peak III enzyme as indicated. Incubation time was 10 min. Incorporation into hot trichloroacetic acid insoluble protein was determined as described under Materials and Methods. Values are corrected for endogenous incorporation into enzyme (0.3 and 0.5 pmol/min per 10 μg of enzyme) and for endogenous incorporation into the 60S subunit (0.81 and 0.95 pmol/min) and into the 40S subunit (1.51 and 2.46 pmol/min) in the absence and presence of cAMP, respectively.

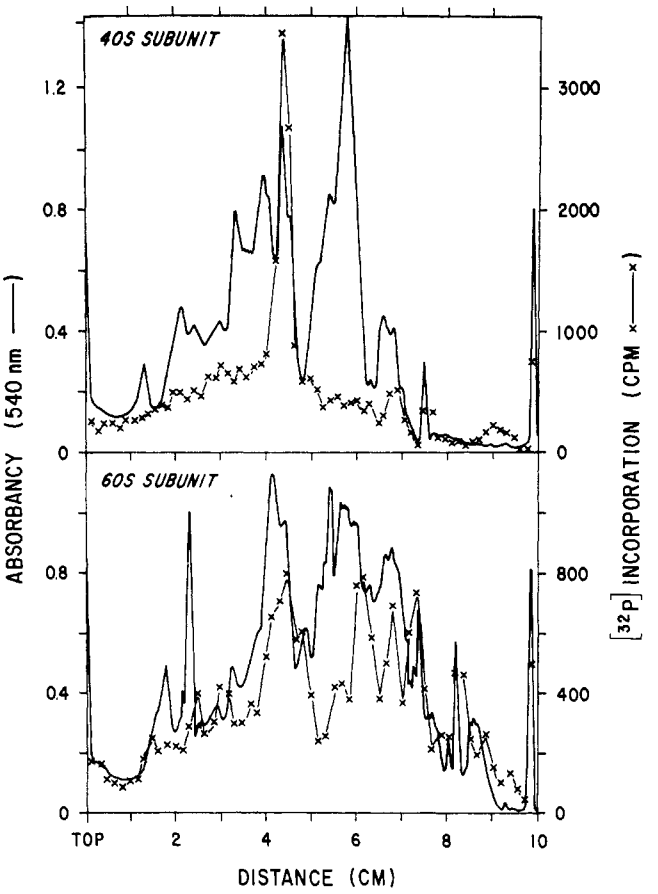

FIGURE 8: Polyacrylamide gel electrophoresis of protein extracted from 60S and 40S subunits. Phosphorylation was performed for 80 min in a volume of 2.5 ml containing 50 mM Tris-HCl (pH 7.5), 1 mM dithiothreitol, 2 mM $MgCl_2$, 5 μM cAMP, 0.3 mM [^{32}P]ATP (9.2×10^4 cpm/nmol), 450 μg of DEAE peak III enzyme, and 52 A_{260} units of 40S subunit or 49 A_{260} units of 60 S subunit. Reactions were stopped with an equal volume of 14 mM ATP (pH 7.5). Discontinuous sucrose gradient sedimentation and protein extraction was carried out as described under Materials and Methods. Protein (300 and 260 μg in 0.2 ml from 40S and 60S subunits, respectively) was used for electrophoresis.

sible to completely phosphorylate available ribosomal protein sites using endogenous cAMP-dependent protein kinase activity.

Because of the incomplete resolution of every individual protein by one-dimensional electrophoretic techniques, these patterns represent the minimum number of labeled proteins present. Phosphorylation resulted in no observable change in the migration of individual protein bands as identified by staining. Gel electrophoresis at pH 8.3, of protein extracted from similarly treated ribosomes, revealed no migration of radioactive bands. This does not eliminate the possibility of minor acidic proteins but confirms the previous finding that most ribosomal proteins have an isoelectric point greater than pH 8.3 (Sherton and Wool, 1972).

Effect of Phosphorylation on the Association of Protein with the Ribosome. Phosphorylation of basic ribosomal proteins may alter their association with the ribosomal structure and with RNA. Ionic elution of protein from the ribosome was used as a measure of the protein–ribosome interaction. Ribosomes were washed with 0.5 and 1.0 M KCl as described in the legend to Figure 3. These ribosomal pellets were then incubated in complete reaction mixtures. After salt washing the total ^{32}P incorporation into available sites was reduced to approximately 68 and 53% for the 0.5 and 1.0 M KCl washed ribosomes (Figure 5). Higher salt resulted in no further reduction in available phosphorylation sites. When complete phosphorylation was carried out prior to salt elution, there was a greater reduction in phosphoprotein remaining with the ribosome (Figure 5). Only 22% of the phosphoprotein remains with the ribosome after washing with 1.0 M KCl or higher. The completeness of ribosomal phosphorylation was confirmed by incubating the previously phosphorylated salt washed ribosomal pellet in a fresh protein kinase reaction mixture containing both enzyme and cAMP. As shown in Table II no additional sites were available for phosphorylation. No significant reduction of incorporated ^{32}P was observed when the ribosomal pellet was incubated with additional enzyme indicating a lack of significant phosphatase activity. The decreased phosphoprotein content of the ribosome when complete phosphorylation occurred prior to salt elution suggested that phosphorylation has decreased the affinity of protein for the ribosome.

cAMP and Protein Kinase Dependent Phosphorylation of Ribosomal Subunits. The presence of substrate for cAMP-dependent protein kinase on high salt treated ribosomes suggested phosphorylation of structural proteins. To define this further, isolated ribosomal subunits were tested as a substrate for the cAMP-dependent protein kinase. The 60S and 40S subunits were incubated in complete protein kinase assay mixtures and the reaction mixtures were layered onto sucrose gradients. Figure 6 demonstrated the incorporation of ^{32}P into proteins of each of the isolated subunits. Phosphorylation of each subunit was increased in response to the addition of enzyme and was markedly stimulated by cAMP (Figure 7). Endogenous phosphorylation of the isolated subunits was minimal. The absence of protein kinase from subunits has been noted by others (Traugh *et al.*, 1973).

The proteins associated with each subunit can be resolved by gel electrophoresis with an indication of the minimum number of proteins which provides sites for phosphorylation. The 60S subunit reveals six–eight distinct peaks of labeled protein (Figure 8); only a single protein band from the 40S subunit is significantly labeled with ^{32}P.

TABLE III: Complete Phosphorylation of 60S Subunit and 40S Subunit in the Protein Kinase Catalyzed Reaction.

Substrate	Protein Content (mg/A_{260} Unit)	Absorbance Ratio (A_{260}/A_{235})	^{32}P Incorpn[a] (nmol/A_{260} Unit)	^{32}P Incorpn[a] (mol of ^{32}P/mol of Subunit)[b]
60S subunit	0.030 ± 0.002 (3)	1.67 ± 0.03 (3)	0.213 ± 0.053 (6)	10
40S subunit	0.051 ± 0.005 (3)	1.61 ± 0.02 (3)	0.158 ± 0.016 (6)	2

[a] Phosphorylation was performed in reaction mixtures as described in Figure 7 with cAMP and 6.8–11.6 μg of enzyme (SG peak III) and [^{32}P]ATP of sp act. of 2.6–8.5 × 10^4 cpm/nmol. Amounts of substrate varied between 0.8 and 3.9 A_{260} units. Incubation time was 2 hr. Values are means ± standard deviations with the number of determinations in parentheses and incorporation corrected for 0.015 nmol of endogenous incorporation per 10 μg of enzyme. [b] Molecular weights of 3.0 × 10^6 and 1.5 × 10^6 and protein contents of 43 and 55% for the 60S and 40S subunits, respectively, were used for molar incorporation calculations (Hamilton *et al.*, 1971).

A closer approximation of the total number of sites on each of the subunits available to the *in vitro* reaction was examined. Each species was completely phosphorylated in the protein kinase catalyzed reaction with the highly purified SG peak III enzyme which contained a minimum of endogenous incorporation and phosphatase activity. Results of these studies are summarized in Table III. The 60S and 40S species were calculated to contain 10 and 2 mol of ^{32}P/mol of subunits, respectively. These figures represent a minimum estimate of the extent of total ribosomal protein phosphorylation because ribosomes as isolated contain phosphoprotein prior to use in the cAMP-dependent reaction. The absence of significant levels of phosphatase activity in either the enzyme or ribosomal subunits indicated total incorporation into available sites rather than phosphate exchange. Because of the heterogeneity of the ribosomal proteins and the possibility of multiple phosphorylation sites on individual proteins, the calculations do not reflect the extent of phosphorylation of the individual proteins. The observation that the majority of the adrenal 40S subunit phosphorylation is present in a single peak indicates significant phosphorylation of that protein band.

Discussion

In the *in vitro* reaction a significant amount of cAMP-dependent phosphorylation of ribosomal protein was observed. In addition to the localization of cAMP-dependent protein kinase in the endoplasmic reticulum of adrenal cortical microsomes, as reported previously (Walton *et al.*, 1971), catalytic amounts of enzyme are associated with ribosomes themselves. The apparent K_m (4 × 10^{-8} M) for cAMP activation of ribosomal phosphorylation using endogenous and exogenous enzymes was similar to that found using other substrates. The addition of cAMP-dependent protein kinase increased the initial phosphorylation reaction rates but eventual complete phosphorylation of the same ribosomal proteins occurred in response to cAMP without the addition of enzyme. Because of the broad range of substrates phosphorylated by the cAMP-dependent protein kinase, the cellular localization of enzyme and available substrate may be an important determinant of specificity.

Phosphorylation appears to alter the association of protein with the ribosome. Washing with high ionic strength removes 47% of the total ^{32}P incorporation sites. In contrast, complete phosphorylation of ribosomes and subsequent ionic elution removed 78% of the ^{32}P incorporation sites. No additional phosphorylation sites were identified. These studies suggest that phosphorylation of ribosomal protein decreases the affinity of these proteins for the ribosomal structure. Phosphorylation of these basic proteins may be predicted to alter the protein–nucleic acid interaction; alteration of protein–protein interactions may also occur. Phosphorylation of histone and protamine results in an alteration of the association of these basic proteins with deoxyribonucleic acid and subsequent changes in the function of the protein–nucleic acid complex (Kleinsmith *et al.*, 1966; Langan, 1969b; Sung and Dixon, 1970; Louie and Dixon, 1972). Further studies are required to define any functional alterations which may result from the phosphorylation of ribosomal protein.

Previous studies demonstrated only minimal phosphorylation of ribosomal subunits (Walton *et al.*, 1971). In those studies, initial reaction rate conditions and lower specific activity [γ-^{32}P]ATP limited the sensitivity of detection of phosphorylation. It is evident from the present studies that the 60S and 40S ribosomal subunits each contain proteins which are phosphorylated in response to cAMP-dependent protein kinase. One to two proteins of the 40S subunit and eight–ten proteins of the 60S subunit were phosphorylated. These findings are in agreement with the observations of Eil and Wool (1971), Kabat (1972), and Traugh *et al.* (1973). The present studies indicate very significant phosphorylation of ribosomal protein. In the *in vitro* reaction 2 mol of phosphate is incorporated per mol of 40S subunit and 10 mol of phosphate is incorporated per mol of 60S subunit.

The functional role of ribosomal protein phosphorylation is as yet unknown. Phosphorylation of ribosomal proteins may be important in the assembly of the ribosomal subunits or in ribosomal function. Phosphorylation could alter the activity of proteins important in protein synthesis or could alter function by altering its structure. cAMP regulation of protein kinase provides one mechanism for hormonal control of ribosomal protein phosphorylation. The removal of phosphate groups by a phosphatase enzyme system must also exist but has not been defined in the present studies.

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