A Comparison of Ribosomal Proteins from Rabbit Reticulocytes Phosphorylated in Situ and in Vitro†

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ABSTRACT: A comparison has been made between the ribosomal proteins phosphorylated in intact cells and proteins isolated from ribosomal subunits after modification in vitro by purified protein kinases and [γ-^{32}P]ATP. When intact reticulocytes were incubated for 2 h in a nutritional medium containing radioactive inorganic phosphate, one phosphorylated protein was identified as a 40S ribosomal component using two-dimensional polyacrylamide gel electrophoresis followed by electrophoresis in a third step containing sodium dodecyl sulfate. This protein, containing 99% of the total radioactivity associated with ribosomal proteins as observed by two-dimensional electrophoresis, is found in a nonphosphorylated form in addition to several phosphorylated states. These states differ by the number of phosphoryl groups attached to the protein. The same 40S protein is modified in vitro by the three cAMP-regulated protein kinases from rabbit reticulocytes. Two additional proteins associated with the 40S subunit are phosphorylated in situ. These proteins migrate as a symmetrical doublet, and contain less than 1% of the radioactive phosphate in the 40S subunit. A number of phosphorylated proteins associated with 60S subunits are observed by disc gel electrophoresis after incubation of whole cells with labeled phosphate. These proteins do not migrate with previously identified ribosomal proteins and are not present in sufficient amounts to be identified as ribosomal structural proteins. Proteins in the large subunit are modified in vitro by cAMP-regulated protein kinases and ATP, and these modified proteins migrate with known ribosomal proteins. However, this phosphorylation has not been shown to occur in intact cells.

Initial observations on the phosphorylation of ribosomal proteins were made in rabbit reticulocytes incubated in a nutritional medium with radioactive inorganic phosphate (Kabat, 1970), and in rat liver after injection of labeled orthophosphate into whole animals (Loeb and Blat, 1970). The phosphate was attached to ribosomal proteins via serine or threonine ester linkages, and the labeled proteins were identified by disc gel electrophoresis. Recently ribosomal phosphoproteins have been observed in whole cell studies with several animal tissues (Bitte and Kabat, 1972; Barden and Labrie, 1973; Gressner and Wool, 1974a,b; Prestayko et al., 1974; Hill and Trachewsky, 1974; Ashby and Roberts, 1975; Rankine and Leader, 1975). Gressner and Wool have shown by two-dimensional electrophoresis, that a single 40S ribosomal protein is phosphorylated in regenerating rat liver. cAMP has been shown to specifically stimulate phosphate incorporation into a single protein band associated with the 40S ribosomal subunit in rabbit reticulocytes (Cawthon et al., 1974). Stimulation of the phosphorylation of one protein band has also been observed in rat liver with glucagon (Blat and Loeb, 1971) and in pituitary slices with dibutyryl cAMP (Barden and Labrie, 1973).

When protein kinases partially purified from the ribosome-free supernatant fraction are added to purified ribosomal subunits, proteins in both the 40S and the 60S subunits are phosphorylated (Kabat, 1971; Eil and Wool, 1971, 1973; Stahl et al., 1972; Barden and Labrie, 1973; Traugh et al., 1973; Walton and Gill, 1973; Martini and Gould, 1973; Ventimiglia and Wool, 1974; Delaunay et al., 1973; Azhar and Menon, 1975). However, large discrepancies exist in the literature as to the number of proteins modified by these enzymes.

In order to determine the physiological significance of phosphorylation of ribosomal proteins on a protein-synthesizing system in vitro, it is essential to compare the ribosomal proteins modified by purified protein kinases and [γ-^{32}P]ATP with proteins phosphorylated in the intact cell. Thus, we have analyzed ribosomal proteins from rabbit reticulocytes phosphorylated both in situ and in vitro by two-dimensional polyacrylamide gel electrophoresis followed by autoradiography. Phosphorylated proteins were identified according to established protein patterns (Traugh and Traut, 1973; Howard et al., 1975), and defined further by electrophoresis in sodium dodecyl sulfate.

Methods

Incubation of Reticulocytes with Radioactive Phosphate and Isolation of Ribosomal Subunits. Reticulocytes were prepared from phenylhydrazine-treated New Zealand doe rabbits as described previously (Traugh and Traut, 1974). Reticulocytes were incubated in the nutritional medium described by Bitte and Kabat (1974) with 0.1 mCi/ml of carrier-free [^{32}P]orthophosphoric acid (ICN). After incubation for 2 hr at 37 °C, the culture was rapidly cooled to 4 °C by the addition of 3 volumes of ice-cold physiological salt solution. All subsequent operations were carried out at 4 °C. Cells were harvested by centrifugation, washed once with physiological salt solution, and lysed by the addition of 2 volumes of 2 mM $MgCl_2$. After exactly 2 min, 0.5 volume of 1.5 M sucrose and 50 mM potassium phosphate (pH 7.4) was added, and the lysate was clarified by centrifugation at 15 000g for 20 min. The supernatant fraction was removed and the ribosomes were isolated by sedimentation through 10 ml of buffer A (40% sucrose, 50 mM potassium phosphate (pH 7.4), 1.5 mM $MgCl_2$, and 0.5 mM dithiothreitol)

† From the Department of Biochemistry, University of California, Riverside, California 92502. *Received August 12, 1975.* This research was supported by a grant from the U.S. Public Health Service (GM 21424) to J.A.T. A preliminary report of a portion of this work was presented to the Federation of American Societies for Experimental Biology, Atlantic City, N.J., April 15, 1975.

at 50 000 rpm for 5 h in a Spinco Ti 60 rotor. The ribosomal pellets were rinsed three times with buffer A minus sucrose, and allowed to resuspend in a small volume of the same buffer overnight at 4 °C. The ribosome solution was made 0.5 M KCl and 1.5 mM $MgCl_2$ and incubated for 10 min at 30 °C. Eighty A_{260} units of ribosomes was layered on 15–40% linear sucrose gradients in buffer B (20 mM Tris-HCl (pH 7.4), 500 mM KCl, 2 mM $MgCl_2$, and 0.5 mM dithiothreitol), and centrifuged at 24 000 rpm for 17 h in a Spinco SW 27 rotor; 1-ml fractions were collected. In early studies the magnesium chloride concentration was 5 mM. The ribosomal subunit regions of the gradients were pooled, concentrated by centrifugation, and resuspended in reticulocyte buffer (10 mM Tris-HCl (pH 7.4), 25 mM KCl, 1.5 mM $MgCl_2$, and 1 mM dithiothreitol).

Polyacrylamide Gel Electrophoresis in Sodium Dodecyl Sulfate of Phosphoproteins in the Protein-Synthesizing Complex. One-milliliter fractions from two identical sucrose gradients containing 0.5 M KCl were pooled and precipitated with 10% trichloroacetic acid. The precipitates were collected by centrifugation at 7000 rpm for 20 min, washed twice with ethanol–ether (2:1) and once with ethanol, and resuspended in 1% sodium dodecyl sulfate. The samples were applied to 10% polyacrylamide disc gels (0.5 × 11.5 cm) containing sodium dodecyl sulfate (Weber and Osborn, 1969), and run at 10 mA/gel. The gels were stained in Coomassie Brilliant Blue, destained, and sliced into 2-mm sections. The slices were dried and counted in a liquid scintillation counter.

Preparation of Ribosomal Subunits by Zonal Centrifugation. Ribosomes were obtained from the reticulocyte lysate according to the method of Traugh et al. (1973), and suspended in reticulocyte buffer. The ribosomes were brought to 500 mM KCl–2 mM $MgCl_2$, incubated for 10 min at 30 °C, applied to a hyperbolic sucrose density gradient (10–38% sucrose w/v in buffer B) according to Eikenberry et al. (1970) in the Beckman zonal rotor Type Ti XV, and centrifuged for 6.5 h at 31 000 rpm. The purified subunit regions were pooled, concentrated, and stored at −70 °C as previously described (Traugh et al., 1973).

Phosphorylation of Ribosomal Subunits in Vitro. Protein kinases were prepared from the ribosome-free supernatant of rabbit reticulocytes by chromatography on DEAE-cellulose and phosphocellulose (Traugh and Traut, 1974). The phosphorylation mixture contained 20 mM Tris-HCl (pH 7.5), 3 mM $MgCl_2$, 1.4 μM cAMP (where indicated), 0.14 mM [γ-^{32}P]ATP, 1 mM dithiothreitol, purified ribosomal subunits, and protein kinase. The reaction was incubated for 60 min at 30 °C and terminated by addition of an equal volume of 8 M urea–6 M LiCl. A unit of protein kinase activity has been defined previously (Traugh and Traut, 1974). Enzyme units of cAMP-regulated protein kinase were measured using histone. Cyclic nucleotide-independent protein kinase activity was measured with casein as substrate.

Two-Dimensional Polyacrylamide Gel Electrophoresis. Ribosomal proteins were separated by two-dimensional polyacrylamide gel electrophoresis as described by Howard and Traut (1973; Howard et al., 1975) with the following modifications. Urea (Mallinckrodt) was purified by passing through a mixed bed resin (Bio-Rad), and stored frozen in 50-ml aliquots with 1 mM methylamide (Sigma). *N,N'*-Methylenebisacrylamide (Eastman) was recrystallized from acetone before use. Ribosomal proteins were extracted from the RNA by addition of an equal volume of 8 M urea–6 M LiCl and precipitated in 10% trichloroacetic acid. The protein precipitate was washed twice with ten volumes of ethanol–diethyl ether (3:1) and once with diethyl ether. The proteins were solubilized by suspension overnight in 0.1 ml of 8 M urea adjusted to pH 5.0 with HCl, and 1 mM β-mercaptoethanol. Just before applying each sample to the first-dimensional gel, the pH was adjusted to 8.4 with 1 M Tris. Electrophoresis in the first dimension was at 4 °C. Samples migrating toward the cathode were run for 7 h at 5 mA/gel, and samples migrating toward the anode were run for 2 h. The gels were dialyzed against 8 M urea–0.3 N HCl for 10 min and twice against 8 M urea (pH 4.5) for 10 min, rinsed in distilled water, and applied to the second dimension (Avital and Elson, 1974). Electrophoresis in the second dimension was at 150 V for 7 h at room temperature. The gels were stained overnight with Coomassie Brilliant Blue, destained, covered with Saran Wrap, and analyzed by autoradiography by placing the wrapped gel directly against Kodak No-Screen Medical x-ray film.

Determination of the Specific Activity of S13. Protein S13 was excised from a two-dimensional gel slab as a strip (4.4 × 0.6 cm), and the Coomassie Brilliant Blue stain was monitored at 600 nm using a scanning attachment with the Gilford 240 spectrophotometer. The gel was cut into 0.4-cm segments, dried, and counted in a liquid scintillation counter. The area beneath the scan was traced, divided into fractions corresponding to the 0.4-cm gel segments, cut out, and weighed. Specific activity was calculated from the amount of ^{32}P and Coomassie Brilliant Blue in each fraction.

Results

Bound Phosphate Associated with Crude Ribosomes. Radioactive phosphate was incorporated into rabbit reticulocytes intracellularly, by incubation of washed cells for 2 h in a nutritional medium containing ^{32}P (Bitte and Kabat, 1974). The cells were lysed and protein-synthesizing complexes were isolated by centrifugation through a cushion of sucrose. Bound radioactive phosphate in the ribosome-free supernatant fraction and the isolated crude ribosome fraction was measured by precipitation in trichloroacetic acid heated at 90 °C for 15 min. The amount of radioactive phosphate bound to proteins associated with the protein-synthesizing complex fraction was 70 times greater than the bound phosphate in the supernatant fraction. Thus in the cytoplasm of the enucleated reticulocyte, 20–30% of the total exchangeable phosphoryl groups are bound to protein associated with the crude ribosome complex.

In order to identify the phosphorylated components of the protein-synthesizing complex, the crude ribosome fraction was dissociated into individual components by incubation in 0.5 M KCl followed by centrifugation in a sucrose density gradient containing 0.5 M KCl. The radioactivity of each fraction was monitored by measurement of precipitable counts after incubation for 10 min at 90 °C in 10% trichloroacetic acid. Greater than 60% of the protein-bound radioactivity was released by dissociation of the ribosomes into subunits; these proteins remained localized near the top of the gradient. Approximately 20% of the total radioactive phosphate remained with the 40S subunit. Less than 15% was associated with the 60S subunit. The distribution of radioactive phosphoprotein throughout the sucrose density gradient was examined further by electrophoresis of portions of the gradient fractions in polyacrylamide gels containing sodium dodecyl sulfate (Figure 1). The stained protein pattern of each fraction is shown in the upper right-

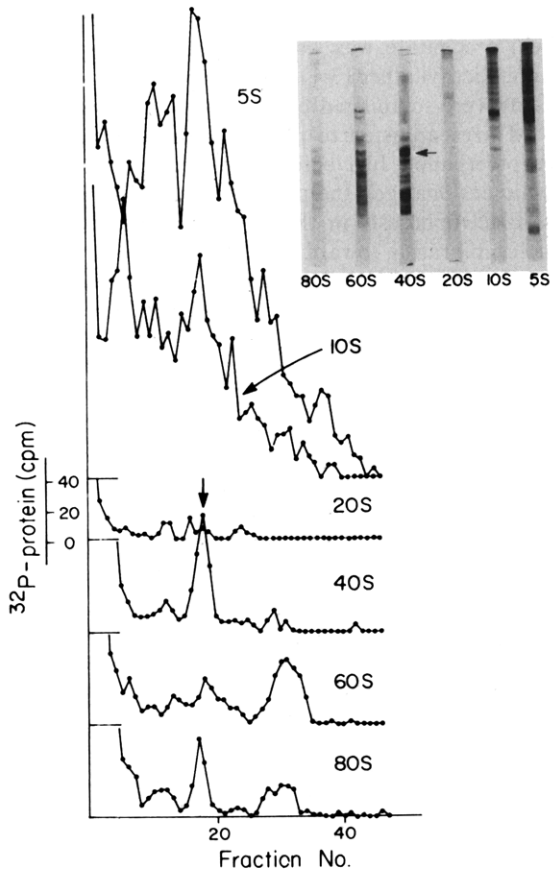

FIGURE 1: Fractionation of phosphoproteins associated with the protein-synthesizing complex. Protein-synthesizing complexes phosphorylated in situ were dissociated into individual components by centrifugation and analyzed by gel electrophoresis in sodium dodecyl sulfate as described in Methods.

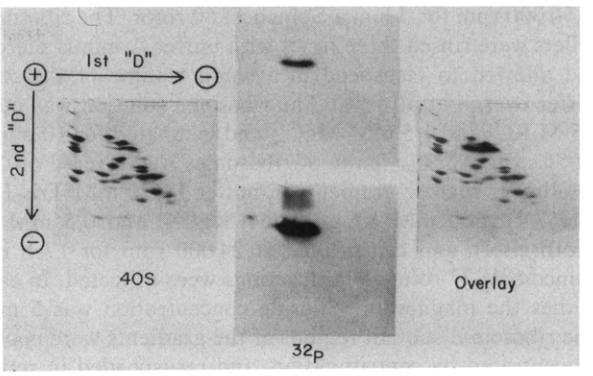

FIGURE 2: Analysis of 40S ribosomal proteins phosphorylated in situ by two-dimensional polyacrylamide gel electrophoresis. Total protein extracted from 0.750 mg of 40S ribosomal subunits phosphorylated in situ was analyzed by two-dimensional polyacrylamide gel electrophoresis followed by autoradiography as described in Methods. 40S, protein pattern stained with Coomassie Brilliant Blue; ^{32}P, upper autoradiogram exposed for 12 h, lower autoradiogram exposed for 4 days; overlay, stained protein pattern with superimposed autoradiogram.

hand portion of the figure. Radioactive phosphate was quantified by cutting each gel in 2-mm slices, and counting the dried gel slices in a liquid scintillation counter.

In the 40S subunit a single major protein band is phosphorylated with a molecular weight of 35000 (Traugh et al., 1973). This protein band is indicated by an arrow (Figure 1). In the 60S subunit, phosphate is associated with several radioactive bands. The small fraction of 80S ribosomes that did not dissociate in the high salt, contain the phosphorylated bands from both subunits. The majority of radioactivity released from the ribosomes in the 0.5 M KCl wash fraction is associated with proteins of molecular weight greater than 35000. The radioactivity present at the top of several of the gels does not correspond to stained protein bands, and is removed by heating the samples in 10% trichloroacetic acid prior to electrophoresis.

Identification of 40S Ribosomal Proteins Phosphorylated in Situ by Two-dimensional Electrophoresis. After dissociation of the ribosomes into subunits by sucrose density gradient centrifugation in high salt buffer, the individual subunit regions were pooled and analyzed by two-dimensional polyacrylamide gel electrophoresis. The small subunits were treated with LiCl–urea to extract the total protein, and the proteins were analyzed and identified as described by Howard et al. (1975). Figure 2 shows the stained protein pattern of the 40S subunit and autoradiograms of the phosphorylated proteins. In the upper autoradiogram, a single phosphorylated spot having an elongated shape is present. This protein is identified as S13. When the same gel is exposed for a longer period of time, as shown in the lower autoradiogram, two radioactive spots are visualized. These proteins appear as a symmetrical doublet trailing, in the first dimension, the protein spot previously shown to contain proteins S1, S2, and S4 (Howard et al., 1975). We have been unable to identify these proteins further due to the small amount of modified material. Together, these spots contain less than 1% of the total radioactivity observed in the small subunit by two-dimensional electrophoresis after a 2-h incubation with radioactive phosphate. The absence of other phosphorylated proteins shown to be associated with the 40S subunit by one-dimensional electrophoresis procedures (Figure 1) indicates these proteins are not an integral part of the 40S subunit since all of the identified ribosomal proteins isolated from the small subunit of rabbit reticulocytes run toward the cathode in the second dimension at pH 8.6 (Howard et al., 1975).

Several Phosphorylated Forms of Protein S13 are Present in Reticulocytes. The major phosphorylated protein in the 40S subunit is identified as S13 using two-dimensional polyacrylamide electrophoresis (Howard et al., 1975). The leading half of the elongated protein pattern is densely stained; the trailing portion, while equal in length, contains approximately 25% of the total protein. The radioactive profile is about the same intensity along the entire length. When the autoradiogram is positioned on the stained gel pattern (Figure 2, Overlay), it can be seen that the leading edge of S13 is not radioactive, not phosphorylated, and the trailing portion contains a large fraction of the total phosphate. In order to identify the entire elongated protein as S13, the stained area was divided into 4-mm portions and each fraction was defined in a third electrophoretic step in polyacrylamide gels containing sodium dodecyl sulfate. The radioactivity and the protein migrate to the same position in each gel. Using appropriate standards the molecular weight of this protein was determined to be 35000 (data not shown).

In order to calculate the specific activity of the radioactive phosphoryl groups along the length of the modified protein, the amount of Coomassie stain was quantified spectrophotometrically, and the radioactivity was monitored. Bickle and Traut (1971) have demonstrated that the amount of Coomassie Brilliant Blue bound to ribosomal proteins is

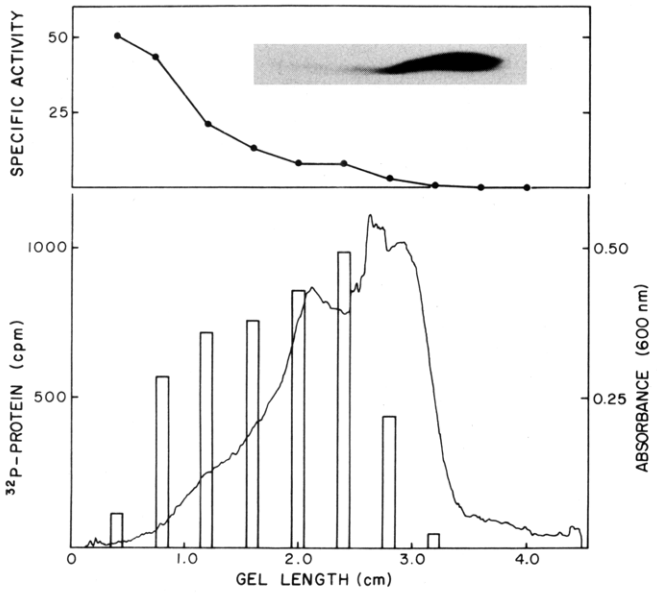

FIGURE 3: Specific activity of protein S13 phosphorylated in situ. The ribosomal proteins from 40S subunits were separated by two-dimensional polyacrylamide gel electrophoresis as described in Figure 2, and the specific activity of S13 was analyzed as described in Methods. Specific activity (●–●); ^{32}P-protein, cpm (bar graph); absorbance, 600 nm (—). A photograph of the stained protein is shown in the insert.

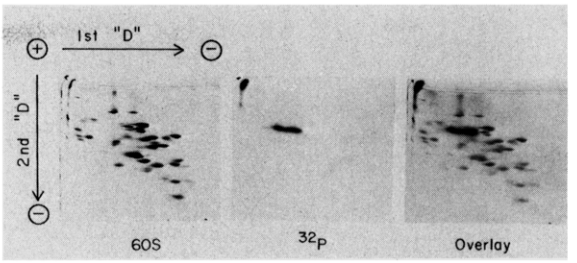

FIGURE 4: Two-dimensional polyacrylamide gel electrophoresis of 60S ribosomal proteins phosphorylated in intact reticulocytes. Total protein extracted from 1.1 mg of 60S ribosomal subunits phosphorylated in situ was analyzed by two-dimensional electrophoresis followed by autoradiography as described in Methods. 60S, ribosomal proteins from the 60S subunit visualized by Coomassie Brilliant Blue; ^{32}P, autoradiogram exposed for 12 days; overlay, stained protein pattern with superimposed autoradiogram.

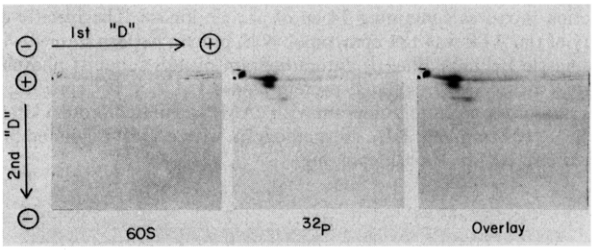

FIGURE 5: Two-dimensional polyacrylamide gel electrophoresis of 60S ribosomal proteins phosphorylated in situ which migrate toward the anode in the first dimension. Procedures were identical with those utilized in Figure 4 except that the anode and cathode were reversed in the first dimension and electrophoresis was for 2 h.

proportional to the amount of protein. The gel containing the stained protein was removed from the two-dimensional slab and spectrophotometrically scanned at 600 nm. This gel was then cut in 4-mm slices, dried, and counted in a liquid scintillation counter. From the ratio of radioactive phosphate to protein (Figure 3) it is apparent that the elongated pattern is due to an increased number of phosphoryl groups retarding migration of the protein at pH 8.6. The specific activity decreased from 50 to 0 along the length of the protein.

Phosphorylated Proteins Associated with the 60S Subunit are not Ribosomal Structural Proteins. Phosphate associated with the large subunit after modification in situ is present in at least five protein bands after disc gel electrophoresis in sodium dodecyl sulfate (Figure 1). When the large subunits are treated with LiCl–urea, and the total protein is subsequently analyzed by two-dimensional polyacrylamide gel electrophoresis, it can be observed that a single phosphorylated protein migrates with the majority of the 60S proteins toward the cathode (Figure 4). The remainder of the labeled proteins either stayed at the origin or ran in the opposite direction. The single phosphorylated protein migrating toward the cathode does not correspond to any of the 60S proteins, but has the properties characteristic of S13, the phosphorylated protein in the 40S subunit. We have observed that at 5 mM $MgCl_2$, some of 40S subunits dimerize, and are associated with the 60S subunit fraction. When the $MgCl_2$ concentration in the sucrose density gradient is lowered from 5 to 2 mM, 40S contamination of the 60S subunits is negligible. Since three 60S subunit proteins migrate toward the anode at pH 8.6 (Howard et al., 1975; Sherton and Wool, 1974), a portion of the phosphorylated 60S proteins was run in the reverse direction in the first dimension. Figure 5 shows the stained protein pattern and the autoradiogram of the two-dimensional gel. Three radioactive spots were observed. None of these spots coincide with the three identified ribosomal proteins. These proteins are probably highly phosphorylated protein contaminants which are part of the protein-synthesizing complex.

In order to examine whether the structural proteins of the large subunit were in fact devoid of radioactive phosphate, and not simply better substrate for endogeneous phosphoprotein phosphatase, the ribosomes were isolated in buffer containing 50 mM phosphate instead of Tris-HCl. This concentration of phosphate was shown to completely inhibit phosphoprotein phosphatase activity from rabbit reticulocytes with ribosomal substrates (H. N. Lightfoot and J. A. Traugh, unpublished observations). When the 80S ribosomes were examined by two-dimensional gel electrophoresis, radioactive phosphate was found with the 40S protein, S13 (data not shown). No other radioactive spots were observed by autoradiography. The pattern of radioactive phosphate bound to S13 was similar if not identical with that obtained by isolation of ribosomal subunits in the absence of phosphate. This indicates that the absence of phosphate bound to proteins in the 60S subunit is not due to phosphoprotein phosphatase activity during the isolation procedure.

In Vitro Phosphorylation of 40S Subunits by cAMP-Regulated Protein Kinases. Criteria have been established previously for the identification of protein kinase activities regulated by cAMP and those independent of control by cyclic nucleotides (Traugh et al., 1974). Four protein kinase activities are present in rabbit reticulocytes (Traugh and Traut, 1974); three are regulated by cAMP and have identical substrate specificity. Two of these protein kinases are dependent on cAMP for activity (II and III_H). The third activity (I) is free catalytic subunit derived from the cAMP-regulated holoenzymes. The activity of a fourth pro-

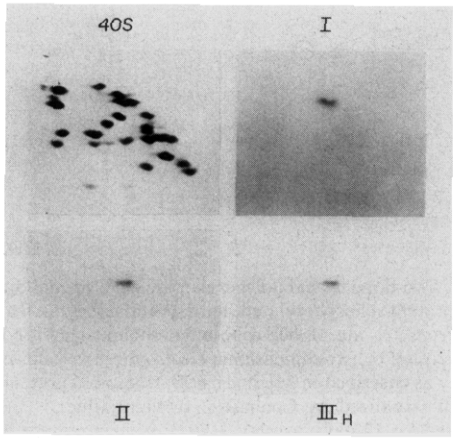

FIGURE 6: Phosphorylation of 40S ribosomal subunits with cAMP-regulated protein kinases and [γ-^{32}P]ATP. Phosphorylation of 40S (0.735 mg) subunits was carried out as described in Methods in 0.4-ml reaction mixtures containing 74 eu of protein kinase. The specific activity of the ATP was 181 cpm/pmol. 40S, protein pattern stained with Coomassie Brilliant Blue; I, autoradiogram of 40S subunits phosphorylated with cAMP-regulated protein kinase I, 1.7×10^5 cpm/mg of 40S ribosomes; II, autoradiogram with cAMP-regulated protein kinase II, 1.3×10^5 cpm/mg; III_H, autoradiogram with cAMP-regulated protein kinase III_H, 1.3×10^5 cpm/mg.

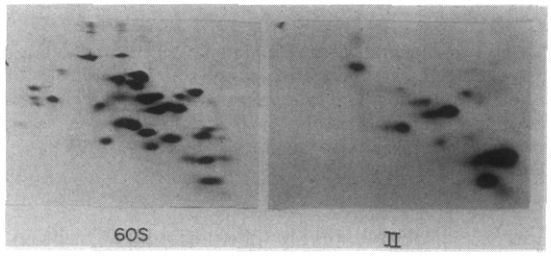

FIGURE 7: Phosphorylation of 60S ribosomal subunits with cAMP-regulated protein kinase II and [γ-^{32}P]ATP. Phosphorylation reaction mixtures, described in Methods, contain 110 eu of cAMP-regulated protein kinase II and 1.12 mg of 60S subunits in 0.2 ml. The specific activity of the ATP was 181 cpm/pmol. 60S, protein pattern stained with Coomassie Brilliant Blue; II, autoradiogram, 1.4×10^5 cpm/mg of 60S ribosomes.

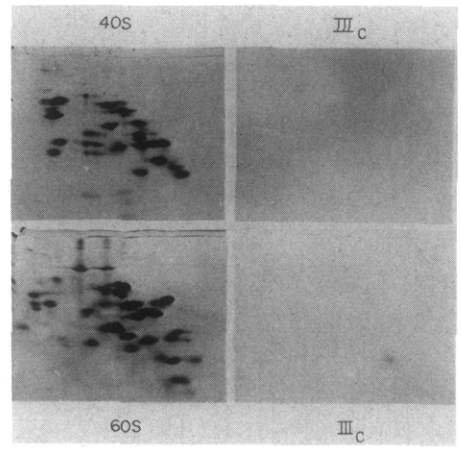

FIGURE 8: Modification of ribosomal subunits with cyclic nucleotide-independent protein kinase III_C. The procedure for phosphorylation in vitro is described in Methods. 40S subunits (0.735 mg) were incubated in 0.4-ml reaction mixtures containing 74 eu of III_C. 60S subunits (1.12 mg) were incubated in 0.1 ml containing 110 eu of III_C. 40S, protein pattern of 40S subunits stained with Coomassie Brilliant Blue. III_C, upper right; autoradiogram; 0.2×10^5 cpm/mg of 40S ribosomes. 60S, protein pattern of 60S subunits stained with Coomassie Brilliant Blue. III_C, lower right; autoradiogram; 0.4×10^5 cpm/mg of 60S ribosomes.

tein kinase (III_C) is independent of cyclic nucleotides and has been shown to phosphorylate different substrates (Traugh and Traut, 1974).

Purified 40S subunits were phosphorylated in vitro with the three cAMP-regulated protein kinases. Figure 6 shows the standard stained pattern of 40S ribosomal proteins after separation by two-dimensional electrophoresis, and autoradiograms of the radioactive phosphate incorporated from [γ-^{32}P]ATP. All three of these enzymes phosphorylate a single identical protein in the 40S subunit. The migration characteristics of this protein in the two-dimensional gel electrophoresis system are those of S13. When this modified protein is excised and further defined by electrophoresis in polyacrylamide gels containing sodium dodecyl sulfate, the molecular weight is 35 000. Thus the protein which is phosphorylated intracellularly by incubation of intact reticulocytes with radioactive inorganic phosphate can be modified in vitro by cAMP-regulated protein kinases. The number of phosphoryl groups incorporated into this protein is dependent on the $MgCl_2$ concentration, the monovalent cation concentration, and the ratio of protein kinase to substrate. Under optimal conditions, an average approaching four phosphate groups can be added to the 40S ribosomal subunits in vitro. All of this phosphate is incorporated into protein S13 as observed by gel electrophoresis.

Phosphorylation of 60S Ribosomal Subunits by cAMP-Regulated Protein Kinases. Upon addition of the cAMP-regulated protein kinases to isolated 60S subunits from rabbit reticulocytes, a number of ribosomal proteins are specifically phosphorylated. Figure 7 shows a typical stained pattern of 60S subunit proteins, and an autoradiogram of the radioactive phosphate incorporated by cAMP-regulated protein kinase II. An identical phosphorylation pattern is obtained with each of the cAMP-regulated protein kinases. However, the phosphate incorporated into 60S subunit proteins by these kinases is not bound to proteins which have been shown to be receptor proteins in intact cells. It is important to note that under optimal conditions, the moles of phosphate incorporated per 60S subunits in vitro is approximately one. Thus the 6–12 proteins modified accept minimal amounts of phosphate.

Phosphorylation of Ribosomal Subunits by Cyclic Nucleotide-Independent Protein Kinase. Protein kinase III_C was shown previously to phosphorylate proteins associated with the ribosome using either ATP or GTP as the phosphate donor (Traugh et al., 1973). When 40S and 60S ribosomal subunits were incubated with kinase III_C and [γ-^{32}P]ATP, and the phosphorylated proteins were analyzed by two-dimensional electrophoresis, the majority of the covalently bound phosphate did not migrate with ribosomal structural proteins (Figure 8). One 60S ribosomal subunit structural protein was slightly phosphorylated by this kinase. It was also modified by the cAMP-regulated protein kinases. However, phosphorylation of this protein was not observed in situ. Thus the phosphorylation of ribosomal subunits previously observed by kinase III_C was due to small amounts of nonribosomal proteins which remain associated with the subunits under our isolation conditions. Ventimiglia and Wool (1974) observed that the protein kinase preparation transfering the γ-phosphoryl group of GTP to 40S proteins contains several phosphorylated proteins which adhere to the large subunit. These proteins were

not observed in any of our gel electrophoresis systems at the concentrations of protein kinase used, nor is the enzyme identical with III_C.

Discussion

The ribosomal proteins from a variety of tissues have been separated by two-dimensional electrophoresis and the reproducible protein patterns have been identified (for review, see Traugh and Traut, 1973). These results show mammalian ribosomes contain approximately 70 different proteins and the majority, if not all, of these proteins are homologous between various tissues of the same animal (Sherton and Wool, 1974) and between different species of mammal (Delaunay et al., 1972, 1973b).

We have shown by two-dimensional gel electrophoresis followed by electrophoresis of the excised proteins in sodium dodecyl sulfate disc gels, that one 40S ribosomal structural protein is phosphorylated in rabbit reticulocytes in situ. Greater than 99% of the phosphate is incorporated into a single protein previously identified as S13 (Howard et al., 1975). Protein S13 is present in several different states within the cell; these are determined by the number of phosphate groups bound to the protein. This is evidenced by specific activity measurements along the protein after gel electrophoresis at pH 8.6. Gressner and Wool (1974a) have shown that S6, the only protein phosphorylated in regenerating rat liver, contains more than one phosphoryl group per protein molecule, and may have as many as six phosphoryl moieties. This protein is also modified in normal liver after inhibition of protein synthesis by cycloheximide and puromycin (Gressner and Wool, 1974b). Protein S6 corresponds to the protein modified in the 40S subunit of Krebs II ascites cells (Rankine and Leader, 1975), and S13 in rabbit reticulocytes as judged by the nearly identical patterns after two-dimensional polyacrylamide gel electrophoresis using minor modifications of the technique of Kaltschmidt and Wittmann (1970), and by molecular weight estimations. The molecular weight for the phosphorylated protein in rat liver is 36000 (Gressner and Wool, 1974), and 36000 in Krebs II ascites cells. With rabbit reticulocytes we routinely obtain a value of 35000 (Traugh et al., 1973; unpublished data) although Howard et al. (1975) estimated 32000. The phosphorylated protein in 40S subunits prepared by Kabat with cited molecular weights of 33000 (1970) and 27500 (Cawthon et al., 1974) has been identified in our laboratory as S13.

Kabat and coworkers had shown the stimulation of phosphorylation of the 40S protein in rabbit reticulocytes by addition of cAMP to the nutrient medium during incubation with ^{32}P (Cawthon et al., 1974). Barden and Labrie (1973) have demonstrated an increase in the phosphorylation of a single ribosomal protein when pituitary gland slices were incubated with dibutyryl cAMP. Glucagon injection into whole animals increased phosphorylation of a single ribosomal protein in rat liver (Blat and Loeb, 1971). Thus regulation of phosphorylation of a single ribosomal protein by cAMP may be a universal phenomenon in mammalian tissues.

Less than 1% of the total phosphate observed with the 40S subunit proteins after two-dimensional electrophoresis is observed as a symmetrical doublet. The protein(s) may be ribosomal in origin, and displaced in the first dimension at pH 8.6 due to the addition of the phosphoryl group. Anderson et al. (1975) have observed a complex migration pattern of a similar if not identical protein in regenerating rat liver, and suggest that this protein may be dually modified. Alternatively, the symmetrical doublet may be proteins associated with the crude ribosome complex which were not completely removed by the high salt wash procedure. At present, we have been unable to determine the origin of these proteins due to a lack of material.

Protein S13 is modified in vitro by the three cAMP-regulated protein kinases from rabbit reticulocytes. Up to four phosphoryl groups can be added to the protein under optimal conditions. In much of the early work with partially purified protein kinases, more than one 40S protein was phosphorylated. We have observed that the structure of the subunits is important in conferring specificity of phosphorylation in vitro. Additional proteins in the 40S subunit are phosphate acceptors under conditions which lead to subunit inactivation.

Although the phosphorylation of specific 60S ribosomal proteins is catalyzed by cAMP-regulated protein kinases in vitro, these modifications have not been observed in situ. The phosphorylation of the 60S subunit appears to be an artifact of extensive purification of the ribosomes possibly due to unfolding of the ribosomal subunit, removal of protective nonribosomal proteins, or removal of inhibitors of protein kinase activity associated with the large subunit. The possibility remains that a very small fraction of the total 60S subunit population may be phosphorylated in situ.

Other phosphorylated proteins are associated with the ribosomal subunits; however, these proteins do not migrate with previously identified ribosomal structural proteins upon electrophoresis, and are present in less than stoichiometric concentrations in relation to the subunits. Thus, it is assumed that these proteins are highly phosphorylated contaminating nonribosomal proteins which were not completely removed by the high salt. Indeed, many of the enzymatic activities involved in protein synthesis are tightly bound to the ribosome and only removed by several cycles of high salt washes.

Although phosphorylation of ribosomal proteins was originally observed in 1970 (Kabat, and Loeb and Blat) the relationship between phosphorylation of ribosomal proteins and protein synthesis has not been established. This has been due in part to artifacts produced by the protein kinases in vitro. In addition, isolated 40S ribosomal subunits contain covalently bound phosphate which was added intracellulary. We have recently isolated several phosphoprotein phosphatase activities from rabbit reticulocyte lysates which remove the phosphoryl groups added to S13 either in situ or in vitro (Lightfoot et al., 1975). We are currently in the process of examining the function of cAMP-regulated phosphorylation of the 40S ribosomal subunit by examining the partial reactions of protein synthesis using phosphorylated and dephosphorylated ribosomal subunits.

Acknowledgments

We thank Dr. David Kabat for supplying 40S and 60S rabbit reticulocyte ribosomal subunits phosphorylated in situ for the initial stages of the study.

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Two-Dimensional Gel Electrophoresis of Membrane Proteins†

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ABSTRACT: A high-resolution method for two-dimensional separation of membrane proteins is described. It involves a nondiscriminating solubilization of a membrane preparation with sodium dodecyl sulfate, followed by electrophoresis in the first dimension according to charge (by isoelectric focusing). The electrophoresis in the second dimension is in the presence of sodium dodecyl sulfate, thus separating proteins on the basis of molecular weight. Electrophoresis in the first dimension is either on a thin slab gel, or on a small-diameter tube; electrophoresis in the second dimension is on a thin slab gel. Up to 100 μg of protein can be analyzed. The two-dimensional system is a modification of the one recently described by O'Farrell (1975). About 150 different proteins can be visualized in *Escherichia coli* or *Salmonella typhimurium* cell envelopes; examples of differences between mutant and wild-type strains are presented. The method is applicable also to membrane preparations from other sources: a two-dimensional separation of plasma membrane proteins from HeLa cells is presented.

One of the main problems encountered in the study of membrane proteins is the lack of an adequate assay for their presence and of good methods for their separation. Recently several one-dimensional electrophoretic methods, mainly on acrylamide gels in the presence of sodium dodecyl sulfate (Ames, 1974), have been applied successfully to the analysis of membrane proteins (for example, Ames, 1974; Fairbanks et al., 1971; Neville, 1971; Schnaitman, 1973). By use of this powerful method about 30–60 protein bands can be identified and separated in *Escherichia coli* and *Salmonella typhimurium* membranes (Ames, 1974).

† From the Department of Biochemistry, University of California, Berkeley, California 94720. *Received August 25, 1975.* This study was supported by National Institutes of Health Grant AM 12121 from the National Institute of Arthritis and Metabolic Diseases to G.F.-L.A.