The Activity of the Acidic Phosphoproteins from the 80 S Rat Liver Ribosome

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The selective removal of acidic phosphoproteins from the 80 S rat liver ribosome was accomplished by successive alcohol extractions at low salt concentration. The resulting core ribosomes lost over 90% of their translation activity and were unable to support the elongation factor 2 GTPase reaction. Both activities were partially restored when the dialyzed extracts were added back to the core ribosome. The binding of labeled adenosine diphosphoribosyl-elongation factor 2 to ribosomes was also affected by extraction and could be reconstituted, although not to the same extent as the GTPase activity associated with elongation factor 2 in the presence of the ribosome. The alcohol extracts of the 80 S ribosome contained mostly phosphoproteins P1 and P2 which could be dephosphorylated and rephosphorylated in solution by alkaline phosphatase and protein kinase, respectively. Dephosphorylation of the P1/P2 mixture in the extracts caused a decrease in the ability of these proteins to reactivate the polyphenylalanine synthesis activity of the core ribosome. However, treatment of the dephosphorylated proteins with the catalytic subunit of 3':5'-cAMP-dependent protein kinase in the presence of ATP reactivated the proteins when compared to the activity of the native extracts. Rabbit antisera raised against the alcohol-extracted proteins were capable of impairing both the polyphenylalanine synthesis reaction and the elongation factor 2-dependent GTPase reaction in the intact ribosomes.

Since the detection of acidic protein L7/L12 in the large subunit of Escherichia coli ribosomes and its associated activities (1-5), several studies have been made on eukaryotic analogues of bacterial L7/L12. Analogous proteins have been found in ribosomes from species such as: Saccharomyces cerevisiae (6-8), Artemia salina (9, 10), rat liver (11-15), HeLa and Krebs ascites cells (16, 18), and others (19, 20). In general, alcohol extraction of large ribosomal subunits isolated from both prokaryotes and eukaryotes has yielded proteins with similar physical properties, which include: electrophoretic mobility, molecular weight, the tendency to aggregate in solution, solubility in 50% alcohol, multiplicity of copies/ribosome, and, in most eukaryotic species, phosphorylation. It has been shown that immunological cross-reactivity exists between alcohol-extractable proteins of ribosomes isolated from species with distant evolutionary relationships (13, 14, 20). There are accounts of bacterial L7/L12 interacting with yeast ethanol-extracted ribosomes to give active hybrid ribosomes (15). These results suggest that ribosomes have evolved in a way that has conserved the function of their large subunit acidic proteins (14, 21).

This report concerns the activities of acidic phosphoproteins from the 80 S rat liver ribosome and their involvement in protein elongation reactions. We have used procedures which we previously described (21) for extracting and reconstituting whole ribosomes in order to measure the activity of the ethanol-soluble proteins. We have identified the active proteins in the extracts as phosphoproteins P1 and P2 previously characterized by Tsurugi and co-workers (22), and have now been able to show functional significance of these proteins.

Materials and Methods

Materials—[^H]Phenylalanine, [^3H]NAD*, [^32P]NAD*, [^32P]GTP, and[^32P]ATP were purchased from New England Nuclear. Puromycin dihydrochloride, sodium deoxycholate, dithiothreitol, 2-mercaptoethanol, agarose-immobilized alkaline phosphatase, and ADP-Sepharose were purchased from Sigma. E. coli strain B transfer RNA and E. coli paste were obtained from Grand Island Biological Company. 3':5'-cAMP-dependent protein kinase catalytic subunit (porcine heart) was a gift from Dr. Susan Taylor and Norman Nelson.

80 S Ribosomes—Puromycin-treated 80 S rat liver ribosomes were prepared according to the method of Siler and Moldave (23). pH 5.2 supernatant containing elongation factors 1 and 2 was prepared as described by Moldave and Skogerson (24). Elongation factor 2 from rat liver was purified according to the procedure of Moldave et al. (25). E. coli[^H]phosphorylation transfer RNA was prepared by the method of Siler and Moldave (23).

EF 2^*GTPase Assays—Ribosome-dependent EF-2 GTPase assays were carried out according to the procedure previously described (28). Controls for background counts due to hydrolysis of GTP by purified EF-2, 80 S ribosomes or core ribosomes, and for unabsorbed[^32P]GTP were summed and subtracted from the total counts observed to determine the ribosome-dependent GTP hydrolysis. Diphtheria toxin A-chain was purified from whole diphtheria by the following procedure of Everse et al. (27). Whole diphtheria toxin was purchased from Connaught Laboratories Inc. and further purified by the procedure of Everse et al. (27).

Labeling of EF 2—The labeling of purified EF-2 was accomplished by the procedure of Bermeck and Matthaei (29), where diphtheria toxin A-chain was used to catalyze the attachment of either[^H]ADPR or[^32P]ADPR to EF-2 using substrates[^32P]NAD* or[^32P]NAD* respectively. The labeled EF-2 was then repurified over DEAE-Sephadex A-50 to remove unreacted substrate and diphtheria toxin A-chain. Labeled EF-2 was dialyzed before use against a buffer containing 0.35 M sucrose, 0.05 M Tris-HCl, 0.05 mM KCl, 0.004 mM MgCl2, 0.006 M 2-mercaptoethanol, pH 7.5 (buffer A).

Binding of EF 2—Binding experiments were carried out similar to the procedure described by Bermeck and Matthaei (29). 80 S ribosomes or 80 S core ribosomes were mixed with an excess of labeled EF-2 in 1.0 ml of buffer A containing a 1 mg/ml concentration of bovine serum albumin and 1 mM GTP, then incubated at 37°C for 10
min. The ribosomal bound EF-2 was then separated from the free EF-2 by either 1) centrifugation at 100,000 × g for 30 min, or 2) filtration through small Sepharose 6B-CL columns which had been pre-equilibrated with buffer containing 1 mM GTP.

**Extraction of Ribosomes**—The procedure for extracting 80 S ribosomes was carried out exactly as we have previously described (21). The final concentration of salts after addition of cold ethanol was 0.175 M sucrose, 0.025 M Tris-HCl, 0.075 M KCl, 0.002 M MgCl$_2$, 0.003 M 2-mercaptoethanol, pH 7.5. A 0.1 g/ml solution of KCl was used to increase the KCl concentration from 0.025 to 0.075 M. In all cases where 80 S ribosomes were used in this report, the extraction procedure described above was carried out twice at 0 °C. All manipulations of the ribosomal extract proteins were done in glass containers due to the absorption of the protein to plastic surfaces.

**Phosphatase and Kinase Treatment**—Treatment of protein extracts from the ribosome with agarose-immobilized alkaline phosphatase was carried out by suspending prewashed agarose–enzyme beads in the dialyzed protein extract at pH 7.8 for 20–40 min at 37 °C. The protein concentration of the extract was 1–3 μg/ml. The agarose–enzyme beads were then separated from the protein solution by filtration through glass wool.

Treatment of the dephosphorylated protein extracts with protein kinase was carried out as follows: 3 ml of protein extract (1–3 μg/ml), previously treated with immobilized alkaline phosphatase, was incubated with 2 μg of the catalytic subunit of 3′:5′-cAMP-dependent protein kinase (porcine heart) and 33 nmol of ATP in buffer A. The mixture was dialyzed against 2 liters of buffer A for several hours, then applied over a ADP-Sepharose column (0.5 × 1.0 cm) to remove the kinase enzyme before reconstitution. Extracts to be compared in activity to the kinase- or alkaline phosphatase-treated extracts were treated in the same way except the kinase or ATP was omitted.

Two-dimensional gel electrophoresis of ribosomal extracts was performed exactly as described by Tsurgui et al. (22) except that the pH of the second dimension was run at pH 3.9 rather than 4.05.

Silver staining of one-dimensional SDS gels was carried out according to the procedure of Switzer et al. (30).

**Preparation of Antiserum**—The preparation of rabbit antiserum against ribosomal extracts was accomplished by injecting a solution of the proteins in phosphate-buffered saline (2.0 mg/ml) with an equal volume of Freund’s adjuvant into rabbits every 7 days. Injection was done subcutaneously at multiple sites on the neck and back of the animal. Ouchterlony double diffusion was used to determine if the rabbit’s antisera, 50% ammonium sulfate fraction, contained antibodies to the ribosomal extracts. 6 weeks of injection of the proteins was necessary to produce an immune fraction.

**RESULTS**

**Extraction and Reconstitution of Ribosomal Activities**—We have determined that extraction of the 80 S ribosome with 50% ethanol at 0.075 M KCl, 0.002 M MgCl$_2$ affects the removal of a select few proteins which can be dialyzed and reconstituted with the 80 S core ribosome to produce a partially reactivated ribosome. Attempts to extract the 60 S rat liver ribosome by this method resulted in 60 S core ribosomes which could not be reconstituted for activity.

Fig. 1 shows the polyphenylalanine synthesis activities of: 80 S ribosomes, 80 S core ribosomes, and 80 S core ribosomes reconstituted with an excess of the dialyzed ethanol extract. EF-2 GTPase activities of the same three types of ribosomes are shown in Fig. 2.

The extraction of 80 S ribosomes produced 80 S cores which were devoid of EF-2 GTPase activity. This total loss of EF-2 GTPase activity was different from the polyphenylalanine synthesis activity, which showed 5% residual elongation activity remaining in the same preparation of 80 S core ribosomes. The residual activity in the polyphenylalanine synthesis reaction could be explained by an incomplete removal of the ethanol-soluble proteins, but this conclusion is inconsistent with both the EF-2 GTPase activity data and the fact that further extractions failed to remove the residual translation activity. The total loss of EF-2 GTPase activity in the 80 S core is indicative that the ethanol-extracted proteins are essential for this reaction.

The extent of reconstitution in the assays was 33% in the EF-2 GTPase reaction and 70% in the polyphenylalanine synthesis reaction.

In addition to the GTPase activity of EF-2, the binding of the labeled elongation factor to the extracted and reconstituted ribosome was measured. Quantitation of the binding of EF-2 to the ribosome was complicated by the lack of an effective method of labeling the EF-2 molecule. Labeled GDP and GTP have been used to monitor the formation of the ternary complex between EF-2, the ribosome, and the guanosine nucleotide; however, this method gives an indirect
measurement of the amount of EF-2 that binds to the ribosome and is complicated by other GTP binding sites on the ribosome (39). Bermeck and Matthaei have shown that ADPR-EF-2, produced from the reaction of EF-2, diphtheria toxin A-chain, and NAD⁺, binds to the ribosome in the presence of GTP, GDP, and GMP-PCP with the same affinity as that of the native elongation factor (31, 32). The attachment of ADPR to EF-2 then affects functions of the elongation factor other than its binding to the ribosome.

The binding of [α-32P]ADPR-EF-2 to 80 S ribosomes, 80 S core ribosomes, and the reconstituted ribosome is shown in Fig. 3. The data demonstrate a 76% decrease in ADPR-EF-2 binding to the ribosome after extraction, while reconstitution increased the binding of the ADPR-EF-2 to the ribosome by a factor of 2, recovering nearly half of the binding capacity of the unextracted ribosome.

**Inhibition of Ribosomal Activities with Antibodies**—An antiserum against the ethanol-extracted proteins was prepared by immunizing rabbits with milligram quantities of protein removed from the ribosome by extraction with 50% ethanol, 0.075 M KCl. The IgG fraction from this serum was obtained by ammonium sulfate fraction followed by dialysis. Control serum was also prepared in an analogous manner and compared in activity to the immune serum by measuring the capacity of these two fractions to inhibit the polyphenylalanine synthesis reaction of either whole ribosomes or reconstituted ribosomes. Fig. 4A shows the inhibition of polyphenylalanine synthesis activity of intact 80 S ribosomes preincubated with control and immune IgG fractions. The reconstitution of 80 S core ribosomes with the extract proteins preincubated with the same immunoglobulin fractions is shown in Fig. 4B. Fig. 4C shows the EF-2 GTPase activity of intact ribosomes which had been preincubated with control and immune IgG fractions. In C the background GTPase activity associated with the antibody solution was separately measured in the absence of the ribosome and subtracted from the total hydrolysis activity to determine only the ribosome-dependent activity.

The inhibition of the polyphenylalanine synthesis reaction by high concentrations of immune IgG fraction was not complete either when the unextracted 80 S ribosome was preincubated with antibody solution or when the dialyzed proteins from extracted ribosomes were incubated with immune antibody solution prior to reconstitution with the 80 S core ribosome. In contrast, the EF-2 GTPase reaction was completely inhibited by the immune IgG fraction while only slightly inhibited with the same concentration of control IgG fraction. The results from the antibody inhibition experiments are consistent with the extraction/reconstitution data in that the EF-2 GTPase activity was affected to a greater extent by the removal of the ethanol-soluble proteins or their complexation with antibodies. On the other hand, the poly(U) reaction, which has an absolute requirement for elongation factor 2, appears to have retained a portion of its activity with the extraction of the ethanol-soluble proteins or in the presence of antibodies prepared against them.

**Resolution of Ethanol-extracted Proteins**—Experiments were carried out in an effort to separate the various proteins in the ethanol extracts of the ribosome so that reconstitution activities of each protein could be measured individually. The concentrated extracts from 405 mg of 80 S ribosomes were electrofocused from pH 3.5-10.0. The dialyzed fractions obtained from this column were assayed for protein content and activity as measured by the ability to reconstitute 80 S core...
The peak fractions of the column restored 35-40% of the solved extract are shown in the upper portion of the figure. Ribosomes. The protein and activity profile of the electrofocusing column and the unre- cusing column is shown in Fig. 5. 18% SDS-polyacrylamide gels run on the peak fractions of the column and the unre- cusing away from the other proteins and did not show activity the peaks with corresponding reconstitution activities allowed several conclusions to be drawn.

Electrofocusing did not separate the four SDS gel protein bands of the extract (these bands will be referred to as bands 1-4 from smallest to largest); however, the proteins content of the peaks with corresponding reconstitution activities allowed for pH 5-60% isoelectricfocusing column and electrofocused from pH 3.5-10.0 in a 5-60% glycerol gradient for 72 h. The fractions obtained from draining the column were individually dialyzed and analyzed for pH 3.4-5.0 in 5.0 M, 0.075 M KC1 extraction ethanol, 0.075 M KC1 extraction procedure of Switzer et al. (30).

To prove this hypothesis, samples from the two peaks c produced in a very small amount in peak c while it was not in peak b. Peak b, which contained only bands 2 and 3, had activity when reconstituted with the 80 S core ribosome; therefore, bands 2 and 3 were the only proteins necessary to observe reactivation of 80 S core activity.

Two-dimensional electrophoresis gels performed according to the procedure of Lastick and McConkey (31) revealed that the proteins in peak c were acidic phosphoproteins P1 and P2 previously characterized by Tsurugi et al. (22). In addition, the two-dimensional gel pattern of the unresolved extract was the same as that of the proteins in peak c (Fig. 6, A and B). By visual inspection during electrophoresis, ferritin did not migrate into the second dimension gel because the red protein band remained in the first dimension tube gel after the second dimension would either precipitate the proteins or cause them to electrophorese out of the gel and into the upper running buffer reservoir. On the other hand, the proteins in peak b showed the same mobility as the two upper bands in peak c when the samples were analyzed by one-dimensional SDS gels. Therefore, the proteins in peak b were likely to be forms of P1 and P2 which were more phosphorylated than the ones seen in the two-dimensional gels of the protein in peak c. To prove this hypothesis, samples from the two peaks present in a very small amount in peak c while it was not in peak b. Peak b, which contained only bands 2 and 3, had activity when reconstituted with the 80 S core ribosome; therefore, bands 2 and 3 were the only proteins necessary to observe reactivation of 80 S core activity.

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were treated with alkaline phosphatase according to the procedure of Arpin et al. (11), then analyzed on both two-dimensional gels and SDS gels. Fig. 6, C and D, shows the two-dimensional gels of the proteins from peaks b and c treated with alkaline phosphatase before electrophoresis. The two-dimensional gel patterns of the two sets of alkaline phosphatase-treated proteins are the same. The one-dimensional SDS gels of the alkaline phosphatase-treated proteins show that the mobility of the proteins in SDS was not affected by the phosphatase treatment (Fig. 6E). Therefore, the two bands in peak b and the two upper bands in peak c were the same proteins differing only in the extent of phosphorylation.

The conclusion drawn from the above data was that SDS gel bands 2 and 3 were acidic phosphoproteins P2 and P1, respectively. P2 was assigned to band 2 and P1 to band 3 because of the molecular weights of these proteins which had been previously reported (22). Band 1 (Mr = 12,700), seen in the SDS gels of the proteins in peak c and in the unresolved extract, was not present on the anodic portion of the two-dimensional gel of the proteins in peak c since all of the protein spots found on the two-dimensional gels were accounted for as forms of P1 and P2. The band was likely to have electrophoresed toward the cathode in the first dimension of the two-dimensional gel run on the proteins in peak c; however, examination of the two-dimensional gel run with opposite polarity in the first dimension did not reveal any protein spots. The fact that band one did not appear on the two-dimensional gels may have been due to the difference in staining procedures used for one-dimensional and two-dimensional gels. One-dimensional SDS gels were stained by the silver staining method of Switzer et al. (30) while two-dimensional gels could only be stained with protein dyes (Coomassie brilliant blue was used) since the silver-staining procedure produced strong backgrounds with gels which had been run in urea. Band 1 did not appear on one-dimensional SDS gels that had been stained with Coomassie blue but did appear when the same gels were silver-stained. Therefore, band 1 was present in the unresolved extract and in fractions from the isoelectricfocusing column in too small amounts to have been seen on the two-dimensional gels.

Treatment of Ribosomal Extracts with Alkaline Phosphatase—Since an assay has become available for measuring the

Fig. 6. Two-dimensional urea electrophoresis gels of key fractions from the electrofocusing column profile shown in Fig. 5. Two-dimensional electrophoresis gels run according to the procedure of Lastick and McConkey (31) on the unresolved 50% ethanol, 0.075 M KCl extract of 80 S ribosomes (A), the protein in the peak at pH 4.4 (fraction 24) from the protein profile shown in Fig. 5 (B), the protein in the peak at pH 4.4 previously treated with 2.0 μg of alkaline phosphatase (bovine intestinal mucosa) (C), the protein in the peak at pH 2.0 previously treated with 2.0 μg of alkaline phosphatase (D). The spots marked AP correspond to alkaline phosphatase. E shows one-dimensional acrylamide gels run in the presence of sodium dodecyl sulfate on the unresolved extract treated with alkaline phosphatase (I), fraction 24 (the peak at pH 4.4) (2), fraction 12 (the peak at pH 2.0) (3), fraction 24 previously treated with alkaline phosphatase (4), and fraction 12 previously treated with free alkaline phosphatase (5). Bands marked AP correspond to alkaline phosphatase.
activity of the ethanol-extracted proteins of the ribosome, this assay was used to compare the relative abilities of control and alkaline phosphatase-treated extract proteins to reactivate 80 S core ribosomes. To avoid carryover of alkaline phosphatase into the ribosome assay mixture, agarose-immobilized alkaline phosphatase was used in place of the free enzyme so that the reconstitution could be carried out in the absence of phosphatase activity, which has been shown to inhibit the polyphenylalanine synthesis activity of 80 S ribosomes (32). The polyphenylalanine synthesis activity of 80 S core ribosomes reconstituted with control and alkaline phosphatase-treated extracted protein is shown in Table I. The activities of unextracted 80 S ribosomes and 80 S core ribosomes alone are also shown in the table for comparison.

The activity of the 80 S core ribosome reconstituted with untreated extract protein was 5.9 times higher than the activity of the same 80 S core ribosome reconstituted with alkaline phosphatase-treated extract was similar to that of the unreconstituted core ribosome. Therefore, the alkaline phosphatase-treated extract apparently had lost the ability to stimulate the polyphenylalanine activity of 80 S core ribosomes.

The loss of reconstitution activity in the treated extract was not due to its absorption to agarose-enzyme resin since the eluate showed the same protein bands as the untreated material when analyzed on SDS-polyacrylamide gels. After removal of the agarose-enzyme beads, the alkaline phosphatase-treated extract was devoid of phosphatase activity as measured by p-nitrophenylphosphate hydrolysis at pH 9.5. An additional control in which unextracted 80 S ribosomes were preincubated with an excess of the alkaline phosphatase-treated extract showed that the alkaline phosphatase-treated extract did not inhibit intact ribosomes. Therefore, the failure of the alkaline phosphatase-treated extract to reactivate 80 S core ribosomes could not be attributed to the inhibitory effect of contaminating alkaline phosphatase (Table I).

The removal of covalently bound phosphate by the agarose-enzyme beads was demonstrated by two-dimensional gels. Fig. 7 shows the two-dimensional electrophoresis gels of the control extract (Fig. 7A) and the alkaline phosphatase-treated extract (Fig. 7B). The pattern of the control extract is identical to the pattern of the unresolved extract (Fig. 6A), while the pattern of the alkaline phosphatase-treated extract is the same as that of the free alkaline phosphatase-treated extract in Fig. 6C. Therefore, the immobilized enzyme was capable of dephosphorylating proteins P1 and P2 to the same extent as the free enzyme.

Treatment of the Ribosomal Extracts with Protein Kinase—Labeling experiments with $[^{32}P]ATP$ and protein kinase showed that the catalytic subunit of 3':5'-cAMP-dependent protein kinase (porcine heart) could covalently attach $[^{32}P]phosphate$ to the proteins in the ethanol extract, while cGMP-dependent protein kinase could not. The catalytic subunit of protein kinase was used in these experiments because it phosphorylates substrate proteins in the absence of cAMP (Fig. 8).

The realization that protein kinase was capable of phosphorylating proteins in the 50% ethanol extract of the ribosome prompted an experiment in which the phosphorylated extracts were assayed for the ability to reconstitute 80 S core ribosome relative to the untreated extracts. In order to observe the largest activity difference between extracted proteins treated and not treated with protein kinase, the alkaline phosphatase-treated extract was used for the phosphorylation experiments. This approach had a second significance in that a reactivation of the phosphatase-treated extract by protein kinase would fortify the hypothesis that the removal of covalent phosphate was the means by which the phosphatase enzyme impaired the activity of the extracted protein.

The polyphenylalanine synthesis activity of 80 S ribosomes
reconstituted with: untreated, immobilized alkaline phosphatase-treated extract, and protein kinase-treated extract which had been previously treated with immobilized alkaline phosphatase is shown in Table I. The protein kinase and alkaline phosphatase-treated extract did reactivate 80 S core ribosomes to a measurable extent. Control activities shown in Table I, where native 80 S ribosomes were preincubated with the same three types of extracts described above, demonstrated that the phosphatase- and kinase-treated extracts did not affect the activity of unextracted ribosomes. In addition, a reconstitution was carried out with alkaline phosphatase-treated extract treated with protein kinase without ATP. The activity of the extract in that condition was nearly the same as that of the extract treated with alkaline phosphatase (Table I). The results indicate that the action of the phosphatase and protein kinase enzymes was inhibitory in the case of the former and stimulatory in the case of the latter.

It should be noted that treatment of the ethanol extract with protein kinase and ATP without prior phosphatase treatment did not increase the activity of the resulting extract over that of the native extract (Table I). Therefore, the stimulatory effect of protein kinase on the extract protein was observed only with extracts pretreated with alkaline phosphatase. This finding might be explained if the native extract contained phosphorylated forms of P1 and P2 which were already as active as possible when measured by ribosomal assays that are available at present.

Analysis of the protein kinase-treated extract by two-dimensional gel electrophoresis is shown in Fig. 7C. Since [γ-32P]ATP was used as a substrate for the protein kinase reaction, autoradiographs could be used to detect the labeled protein in the two-dimensional gel (Fig. 7D). The autoradiographic pattern of protein kinase-treated extracted protein is complicated by the presence of 32P-labeled protein impurities which do not appear on the stained gel but do appear in the autoradiograph. The spots on the autoradiograph which are due to the autophosphorylation of protein kinase catalytic subunit itself and its trace impurities are indicated on the figure as pk. The spots in the autoradiograph which are due to phosphorylation of trace impurities (proteins which cannot be seen in the stained gel) predominate over the actual labeled forms of P1 and P1; however, the labeling of P1 and P2 is evident from the autoradiograph.

The effect of protein kinase on whole ribosomes were measured by incubating varying amounts of the kinase catalytic subunit with ATP (100 pmol/assay) and 80 S ribosomes, then assaying for polyphenylalanine synthesis activity. Consistent with several previous reports (33–38), protein kinase phosphorylation of ribosomes did not enhance their translation activity.

The results from a similar experiment in which 80 S ribosomes were treated with free alkaline phosphatase showed that much more free phosphatase enzyme was required to cause 50% inhibition of ribosomal activity than the amount used in the dephosphorylation of the extract solutions. Therefore, alkaline phosphatase did not have a strong inhibitory effect on the ribosomes and was likely to have acted in a less specific manner, that of hydrolyzing GTP, the substrate for the assay.

**DISCUSSION**

In our previous report (21) we presented a method for reconstituting polyphenylalanine synthesis activity with 80 S rat liver ribosomes which had been extracted with 50% ethanol solution. We followed the procedure of Arpin et al. (11) who extracted 60 S rat liver ribosomal subunits with 50% ethanol at low salt concentration to obtain phosphoproteins P1 and P2. P1 and P2 from the 60 S rat liver ribosome had been previously characterized by Tsurgi et al. (22) and by Leader and Coia (18). None of these reports, however, contain information of the activities of the ethanol-extractable proteins. Sanchez-Madrid et al. (8) described experiments in which 80 S rat liver ribosomes were extracted and assayed for polyphenylalanine synthesis and EF-2 GTPase activities. This report described only the effect of extraction on the rat liver ribosome and did not include reconstitution data. In addition, these investigators used extraction procedures which involved far higher salt concentrations than the ones described by Arpin et al. for selectively removing phosphoproteins P1 and P2 (11).

The overall findings presented in this report demonstrate the necessity of proteins P1 and P2 for the activity of the rat liver ribosome. Ethanol extraction and reconstitution methods have been used to probe the activity of these proteins in an attempt to assign to them a functional role in the protein elongation process. P1 and P2 have been shown to be essential for the GTPase reaction of elongation factor 2. Our ability to observe the reconstitution of the EF-2 GTPase reaction in this report and not in our previous report was likely due to an improved method of handling the ribosomal extracts, since the observation was made that the dilute extract protein absorbed to plastic surfaces but not to glass.

The binding of ADPR-EF-2 to extracted and reconstituted ribosomes differed from the elongation factor’s GTPase activity in that residual binding was observed with 80 S core ribosomes which were devoid of EF-2 GTPase activity. This incomplete removal of EF-2 binding in comparison to the EF-2 GTPase data indicates that the ethanol-extracted proteins

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**Fig. 8.** Autoradiographs of 18% polyacrylamide-SDS gels of ribosomal ethanol extract proteins treated with protein kinase and [γ-32P]ATP. A is the catalytic subunit of protein kinase (pk) incubated with [γ-32P]ATP alone. Solutions of dialyzed ethanol extract protein (1–2 μg of protein/ml) were treated with B, 1.0 μg of protein kinase catalytic subunit and [γ-32P]ATP and C the solution in A with treated with 200 μl of agarose-immobilized alkaline phosphatase.
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play a less significant role in the binding of the elongation factor than they do with its GTPase activity. The separation of the proteins in the ethanol extracts of the ribosome by isoelectric focussing demonstrated that acidic phosphoproteins P1 and P2, in aggregated forms, make up the active constituents of the ethanol extract. The forms of P1 and P2 which electrophoresed at pH 4.4 had been previously observed by Arpin et al. (11) and Tsurugi et al. (22), but the active forms which electrophoresed below pH 2.0 had not been described by these investigators.

The existence of covalent phosphate on P1 and P2 has been reaffirmed and further implicated as being essential for the activity of these proteins on the ribosome. The combined data indicate that the two enzymes used to phosphorylate and dephosphorylate P1 and P2 in the ribosomal extract were capable of effecting the activity of the proteins while in solution but not while on the ribosome.

A number of investigations have been made on the effect of kinases on mammalian ribosomes (14, 33-38). The results from the last studies, however, have failed to demonstrate a direct effect of phosphorylation on the activity of the isolated ribosome or its subunits. The curious existence of multiple phosphorylated forms of P1 and P2, evidenced by the two-dimensional gels, is a phenomena which exists with only one other ribosomal protein (32). If phosphorylation is a means by which the activity of these proteins is regulated, then the question arises as to why so many different phosphorylated forms of these proteins are found in the liver ribosome. A possible explanation of the occurrence of the multiple phosphorylated forms of P1 and P2 might be that the degree of phosphorylation is related to the extent of activation of their activity on the ribosome. Alternatively, a simpler hypothesis could be made that the physical structure of P1 and P2 requires covalent phosphate in order for the proteins to retain solubility before assembling onto a ribosome. The latter hypothesis is reasonable on the basis that the two proteins are rich in hydrophobic amino acids (22) which, in the absence of phosphate groups, would tend to lessen the solubility of the molecules.

Genet et al. (34) have observed phosphorylation of acidic ribosomal proteins by kinases associated with polysomes but not found in purified ribosomes. This finding goes against the hypothesis that phosphorylation of P1 and P2 is a regulatory mechanism since the polysomal kinases that Genet et al. observed are in close proximity intercellularly to any protein being synthesized on the ribosome including P1 and P2, which would become phosphorylated before they assembled onto a newly forming ribosome. If this were the case then covalently bound phosphate may be essential for the viability of P1 and P2 without being a regulatory handle on their activity. This conclusion is consistent with the fact that no kinase has yet been found to stimulate the activity of fully assembled ribosomes while kinases have been found to phosphorylate P1 and P2 (14). The further investigation of the structure and function of proteins P1 and P2 will undoubtedly lead to a better understanding of the translational events in mammalian ribosomes.

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