DETERMINATION OF AN UPPER LIMIT TO THE PHOSPHORUS CONTENT OF POLYPEPTIDE CHAIN ELONGATION FACTOR AND RIBOSOMAL PROTEINS IN ESCHERICHIA COLI

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SUMMARY: Following reports of phosphoprotein in mammalian ribosomal proteins, a similar study was undertaken in E. coli. The ribosomal proteins contained phosphate which was in the order of 1 mole/mole of subunit, but this phosphorus did not co-electrophorese with any ribosomal protein. The soluble proteins were also analyzed, and from the background level of phosphorus radioactivity in the region of the elongation factors in the gel, it was concluded that there was no greater than 1 mole/mole for all three factors together.

Mammalian ribosomes have been found to contain phosphoproteins, and these proteins accept phosphate in vitro (1-3). This study was aimed at probing into the possibility of regulation at the translational level by protein phosphorylation in bacterial systems. There has only been one report of protein phosphokinase of bacterial origin (4). Therefore, in order to avoid the vagaries of in vitro systems, the incorporation of ^{32}P into various fractions was investigated using growing cells. The ^{32}P incorporation into the elongation factors was determined by making use of the known mobility of the factors T_u, T_s, and G on polyacrylamide gels. The apparent protein-bound radioactivity was estimated in the proteins extracted from the separated ribosomal subunits. This was then compared with ribosomal proteins on sodium dodecyl sulfate (SDS) electrophoresis. It is concluded that protein phosphorylation is probably not important in regulating the function of bacterial ribosomal proteins and elongation factors.

MATERIALS AND METHODS

Growth conditions. E. coli S/6 (a derivative of B) was grown on a modification of the Tris-buffered low phosphate minimal medium of Kjeldgaard (6).

This medium was supplemented with P_i, $^{32}P_i$, or casamino acids and carbon source as indicated in the legends. All cultures were grown at 37° on a rotary shaker, with 5-ml volumes. Log-phase cells were inoculated into the radioactive medium, and grown through three generations in log phase. Growth was stopped by chilling, and the cells were centrifuged and washed with 10 ml of the above medium also containing 1 mM Na_2HPO_4.

The labeled cells were then fractionated after mixing with 1 g wet weight of carrier cells grown in a rich broth-yeast extract-glucose medium (as described in ref. 5). Cell breakage and fractionation was based on previously described methods.

The tRNA was quantitatively precipitated from the S-100 fraction by titration with protamine sulfate. The supernatant material contained all the T_u, T_s, and G factors from the original S-100 (assayed by previously described methods 5, 7). This material was analyzed by electrophoresis with no further treatment. The same system as before (5) was used. The staining profile was recorded with the linear transport attachment of the Gilford spectrophotometer, and the gel was then cut into 0.9 mm slices for radioactivity determinations. The slices were placed in vials, dried, and counted in a scintillation counter.

The crude ribosomal pellet was then purified by a simplification of the method of Kurland (8), designed to permit the use of this small scale. One cycle of ammonium sulfate precipitation and one sedimentation through 0.5 M NH_4Cl-10% sucrose were used. The pellet was taken up in 50 mM KCl, 10 mM Tris-HCl, pH 7.8, 0.1 mM dithiothreitol (DTT), and layered over a 28-ml 10-30% linear sucrose gradient and a 1-ml cushion of 30% sucrose. The same dissociating buffer was used in the gradient but with 1 mM $MgCl_2$. This was centrifuged in the Spinco SW25.1 rotor at 25,000 rpm for 14.5 hr. The pooled subunit peaks were then concentrated by ethanol precipitation, following the procedure devised by Falvey and Staehelin (9) for reticulocyte ribosomal subunits. The subunit pellets were taken up in 1 ml of 10 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 0.1 mM DTT, and dialyzed against the same buffer.

The protein was extracted from the dialyzed subunits, following the acetic acid extraction procedure of Hardy et al. (10). The protein was dialyzed against 6 M urea, 0.012 M methylamine, pH adjusted to 6.5 with H_3PO_4. This material was then applied directly to SDS gels. The procedure of Weber and Osborn (11) was followed exactly. Densitometry and counting were as above for the soluble protein fraction.

RESULTS

In order to obtain an estimate of what percentage of total cellular phosphoprotein appears in the ribosomal and elongation factor protein fractions, aliquots were taken from different stages in the procedure described under Materials and Methods. The samples were mixed with carrier protein, heated in 5% trichloroacetic acid (TCA) for 10 min to hydrolyze the RNA, collected on a filter, and the radioactivity was determined. The results are given in Table 1. The unhydrolyzed radioactivity is also included in order to indicate the contribution of RNA phosphorus. Of the putative phosphoprotein in the S-100 fraction, 10% remained in solution after protamine sulfate precipitation and therefore cannot be attributed to tRNA. In addition, the hot TCA-stability of this fraction indicates that it is free of nucleic acids. This material was analyzed further as described below.

Table 1 also shows that of the hot acid-stable radioactivity which pelleted with the crude ribosomes, only 0.5% survived the ribosome-washing procedure. Most of the material removed was precipitated at lower ammonium sulfate concentration than the ribosomes, or was released in the high salt wash. It can be seen from Table 1 that the high salt wash fluid contained a sizeable quantity of hot acid-insoluble radioactivity. Of the material that was associated with the purified ribosomes, approximately 30% was recovered in the extracted proteins. This was free of RNA-^{32}P, as can be seen from its hot acid stability. This material was analyzed further (see below).

The protamine sulfate supernatant fraction as shown in Table 1 was analyzed further by polyacrylamide disc electrophoresis. The polypeptide chain elongation factors comigrated in a peak which was readily identifiable from the

TABLE 1. Distribution of Hot TCA-Insoluble ^{32}P in Various Cellular Fractions

Fraction	Hot TCA-stable ^{32}P (cpm)	Hot TCA-stable ^{32}P / Cold TCA-insoluble ^{32}P
S-100	1.50×10^6	21%
Protamine supernatant	7.50×10^4	86%
Crude ribosomes	2.50×10^6	8%
Purified ribosomes	8.47×10^3	1%
High salt wash	6.33×10^4	6%
30S subunit	1.89×10^3	0.8%
30S protein	3.08×10^2	97%
50S subunit	6.58×10^3	1.3%
50S protein	2.02×10^3	70%

The cells were grown in the basal medium described in Materials and Methods, supplemented with 0.2% glucose, 1 mg/ml Difco Bacto casamino acids, and 1 mCi of $^{32}P_i$, carrier free, from 0.1 to 0.4 A_{450}. They were fractionated as described in Materials and Methods, and the radioactivity was determined in aliquots after precipitation in 5% TCA, together with an additional 50 μl of unlabeled carrier S-100, heating for 10 min at 90°, where indicated, filtration on GFC glass fiber filters, and counted.

staining pattern. This corresponded with the band previously recognized (5) in electrophoresis of the S-100. The staining pattern of the protamine sulfate supernatant fraction was indistinguishable from that obtained with the S-100. Fig. 1 shows the stain and radioactivity profiles of this fraction. No peak of radioactivity was coincident with the elongation factor band - the major peak in the stained gel - and significant radioactivity smeared through the length of the gel. It has been calculated from the protein content of this band and the background radioactivity in it, and from the molecular weights of the factors (12), that there cannot be more than 1 mole of phosphate per mole of all three fractions together.

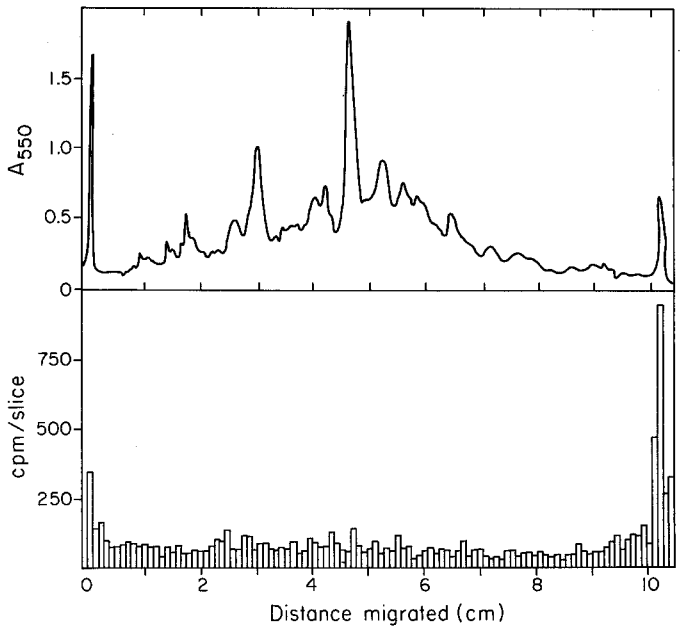

Fig. 1. Disc electrophoresis of the ^{32}P-labeled soluble proteins. The protamine sulfate supernatant fraction from Table 1 (100 μl) was applied to the polyacrylamide gel and run and analyzed as described in Materials and Methods.

In an attempt to determine whether the phosphate associated with the ribosomal proteins (see Table 1) was of any metabolic significance, the determination was carried out with cells grown in different media. The results, given in Table 2, are based on the ratio of the ^{32}P in the protein and in the RNA moieties of the subunits, using the known molecular weight and number of phosphate residues in the RNA chains (13). The phosphorus content decreased, either under phosphate starvation or in the absence of a supply of amino acids The nature of the carbon source made little difference.

The ribosomal protein radioactivity was analyzed further by electrophoresis in SDS. The resulting patterns are shown in Fig. 2 for the 30S and 50S subunits. In neither case did the radioactivity coincide with any of the stained ribosomal proteins.

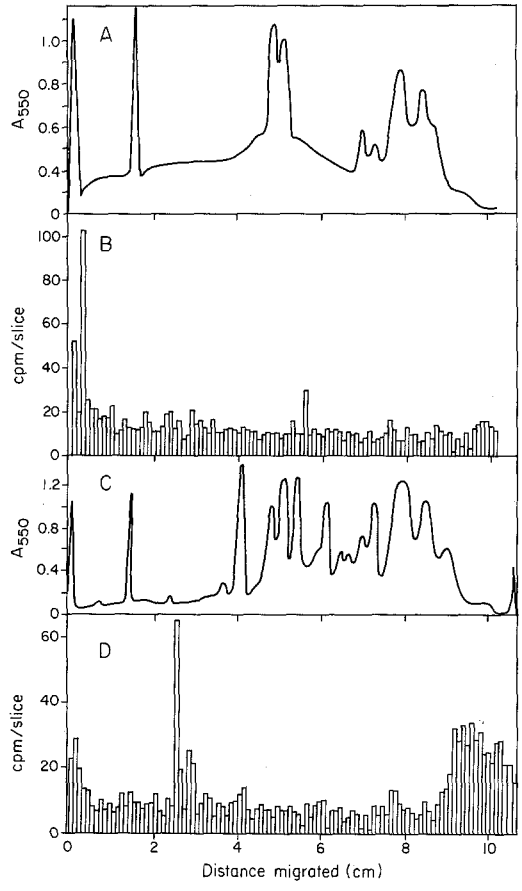

Fig. 2. SDS electrophoresis of the ^{32}P-labeled proteins from the ribosomal subunit. The proteins derived from the cells grown in log phase in casamino acids in Table 2 were applied to the SDS gels after dialysis, as described in Materials and Methods, and run and analyzed. A. 30S stain. B. 30S radioactivity. C. 50S stain. D. 50S radioactivity.

DISCUSSION

These experiments place an upper limit on the phosphate content of the elongation factor and ribosomal proteins. No determinations have been made on the initiation factor proteins. In fact, there were significant amounts of hot TCA-insoluble radioactivity in the high salt wash fluid of the ribosomes. If a control of translation were exercized by means of protein phosphorylation, it could be at the level of chain initiation.

TABLE 2. Distribution of Protein Phosphate in Ribosomes from Different Growth Conditions

Medium	Moles P/Mole of 30S	50S
Casamino acids - glucose, log phase	0.7	3.7
Casamino acids - glucose, starved	0.3	1.9
P_i - glucose, log phase	0.4	0.8
P_i - glycerol, log phase	0.2	1.0
P_i - glutamate, log phase	0.1	1.5

Cells were grown as in Table 1, but with 2.5 mCi of $^{32}P_i$ in each culture, and either 0.4 mg/ml casamino acids for the log phase cells or 0.2 mg/ml for the starved cells, or 0.05 mM K_2HPO_4 for the P_i-grown cells. The starved cells were incubated for 2 hr after growth had stopped in that medium. Glycerol or glutamate were 20 mM. The ribosomal subunits were prepared from purified ribosomes as described in Materials and Methods, and the proteins extracted.

It seems more likely that such controls should take place at the level of transcription. A stimulation of the activity of RNA polymerase by phosphorylation of sigma factor has been reported (14). It is suggested that the phosphoprotein found associated with the ribosomes here is normally associated with the DNA, is released when the extracts are DNase-treated, and associates artifactually with the ribosomes.

There have been reports of phosphoprotein associated with mammalian ribosomes (1-3); whether this represents a control system unique for eukaryote systems remains to be seen.

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