SELECTIVE PHOSPHORYLATION FROM GTP OF PROTEINS L7 AND L12 OF E. COLI 50S RIBOSOMES BY A PROTEIN KINASE FROM RABBIT RETICULOCYTES

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SUMMARY

A protein kinase, purified in this laboratory from the supernatant fraction of rabbit reticulocytes and found to be independent of cyclic nucleotides and to preferentially utilize GTP as a phosphoryl donor, was tested for its substrate specificity with respect to proteins of the 50S ribosomal subunit of Escherichia coli. Two dimensional gel electrophoresis of proteins from subunits labeled with $\gamma[^{32}P]$-GTP showed that only proteins L7 and L12 were phosphorylated by the kinase.

A protein kinase from rabbit skeletal muscle (1) specific for histone as opposed to casein and dependent on cyclic AMP has previously been shown in this laboratory to phosphorylate specific proteins in the 50S subunit of E. coli (2) (Issinger, Kiefer and Traut, unpublished results). The phosphorylated proteins were all relatively basic judged from their position on two dimensional gel electropherograms. More recently a protein kinase which differs from the rabbit skeletal muscle enzyme has been isolated from rabbit reticulocytes by Traugh, Mumby and Traut (3). It has several novel properties (4): chief among these are 1) it is relatively specific for casein as opposed to histone, thus placing it in a group of kinases with specificity for acidic proteins (5,6); 2) that it is not stimulated by cyclic nucleotides; 3) that it has a high specificity for GTP as compared to ATP as a phosphoryl donor [Ventemiglia and Wool described an enzyme with similar substrate specificity (7)]; 4) that it has a different substrate specificity for ribosomal proteins of rabbit reticulocytes than either the rabbit skeletal muscle kinase or an enzyme with similar properties isolated from reticulocytes or erythrocytes (3,4).

As a first step in the investigation of the properties of the GTP specific protein kinase, we examined its specificity with respect to phosphorylation of proteins in the intact 50S ribosomal subunit from E. coli. Although the enzyme fraction was slightly contaminated with the ATP requiring histone specific kinase mentioned above, the use of GTP-[^{32}P] as phosphoryl donor permitted investigation of the specificity of the GTP requiring enzyme even though it was not purified to homogeneity. With GTP and E. coli 50S subunits as substrates the only proteins phosphorylated by the reticulocyte enzyme, previously shown to prefer casein and acidic proteins as substrates, were proteins L7 and L12. These proteins are unique among 50S ribosomal proteins in several respects: 1) They are more acidic; 2) they have been highly conserved in evolution and have been shown by Sherton and Wool (8) to have homologies with certain eucaryotic ribosomal proteins; 3) they have been implicated in all of the ribosomal functions related to GTP hydrolysis (9, and references therein).

MATERIALS AND METHODS

Purified ribosomal subunits from E. coli were prepared as described by Traugh and Traut (2). Ribosomal proteins were prepared by acetic acid extraction according to Hardy *et al.* (10). Protein kinase was prepared as described by Traugh and Traut (4).

The enzyme fraction used throughout the experiments reported here corresponds to that designated Peak III in the purification on DEAE cellulose (3) and will be referred to as DEAE-III enzyme in this report.

γ[^{32}P]ATP and γ[^{32}P]GTP were prepared by a modification of the methods of Glynn and Chappell (11). Further description of methods used is given in the legends to the figures.

RESULTS AND DISCUSSION

The discovery of a protein kinase with specificity for acidic proteins and the substantial information concerning the primary structure and functional

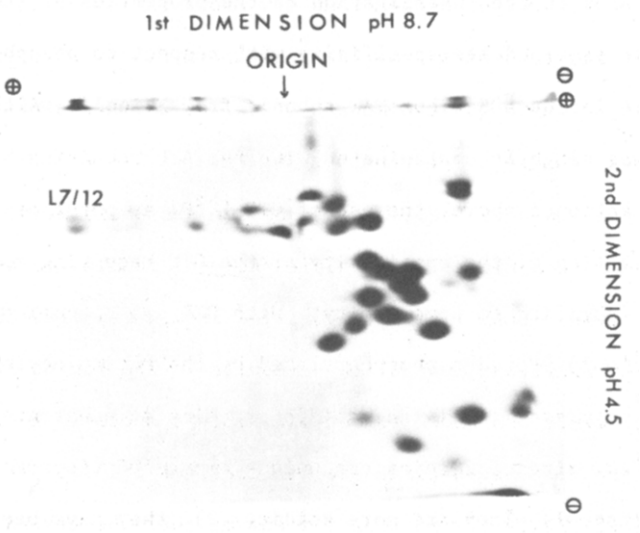

Figure 1: 50S ribosomal proteins from E. coli separated in a 2-dimensional gel electrophoresis system and stained with Coomassie blue (15).

properties of the two acidic 50S E. coli ribosomal proteins L7 and L12 prompted us to ask whether or not these proteins might be substrates for the new protein kinase. Accordingly attention was focused on the results obtained when intact E. coli 50S subunits were incubated with the DEAE III enzyme with either $\gamma[^{32}P]$- ATP or $\gamma[^{32}P]$GTP as substrate. The formation of phosphoproteins was analyzed by radioautography of two dimensional electropherograms of ribosomal protein which provide unambiguous identification of all ribosomal proteins including L7 and L12 as shown by Kaltschmidt and Wittmann (12).

A stained two dimensional gel pattern of nonradioactive 50S ribosomal proteins is shown in Fig. 1. The positions of proteins L7 and L12 are designated. Superimposition of the different autoradiograms which follow with the stained pattern corresponding to each (not shown) made possible the identification of the proteins which became phosphorylated. Fig. 2a shows the pattern of radioactive ribosomal proteins after incubation with the DEAE III enzyme and $\gamma[^{32}P]$ATP. Three major spots correspond to phosphorylated ribosomal proteins. These are L7/L12 (which are not resolved), L2 and L16. The radioactive spots above L7/L12 do not coincide with ribosomal proteins. The position is indicative of an acidic

protein with a molecular weight higher than any known ribosomal protein. Controls were performed omitting ribosomes from the reaction mixture. The same spot was apparent, indicating either that the kinase itself or another protein in the enzyme fraction is a substrate for phosphorylation.

Proteins L2 and L16 have been shown to become phosphorylated from $\gamma[^{32}P]$ATP with both purified protein kinase from rabbit skeletal muscle (2) and with the histone specific enzymes (DEAE I and II) purified from rabbit reticulocytes (3, Issinger, Kiefer and Traut, unpublished results). The kinase fraction, DEAE III, used in these experiments was known to contain histone specific activity (results not shown (3). Since the histone specific enzymes does not utilize GTP as a phosphoryl donor (3,4), the specificity of the DEAE III enzyme could be tested even in presence of the former by employing GTP instead of ATP as substrate. These results are shown in Fig. 2b. Under these conditions only the acidic proteins L7/L12 became phosphorylated. The additional spot due to a protein present in the enzyme fraction appears with GTP as well as ATP.

It was not possible by radioautography to resolve the radioactive spots corresponding to L7 and L12 and as a consequence it was impossible to establish unambiguously whether L7, or L12, or both proteins were phosphorylated.

The radioactive spot corresponding to L7, L12 was shifted slightly to the left with respect to the stained spot. It would be expected that the addition of phosphate groups would increase the mobility of acidic proteins toward the anode. The fact that the radioactive spot does not coincide precisely with the stained spot indicates that only a fraction of the protein became phosphorylated. This would be expected since proteins L7/L12 were in excess over GTP in these experiments.

Although GTP hydrolysis is a necessary step in protein synthesis no phosphorylated intermediates have been reported. Since proteins L7/L12 are known to be involved in GTP hydrolysis, and since the present findings implicate the same proteins as phosphate receptors it was important to establish the amino acid residues to which phosphate became bound. Protein kinases as

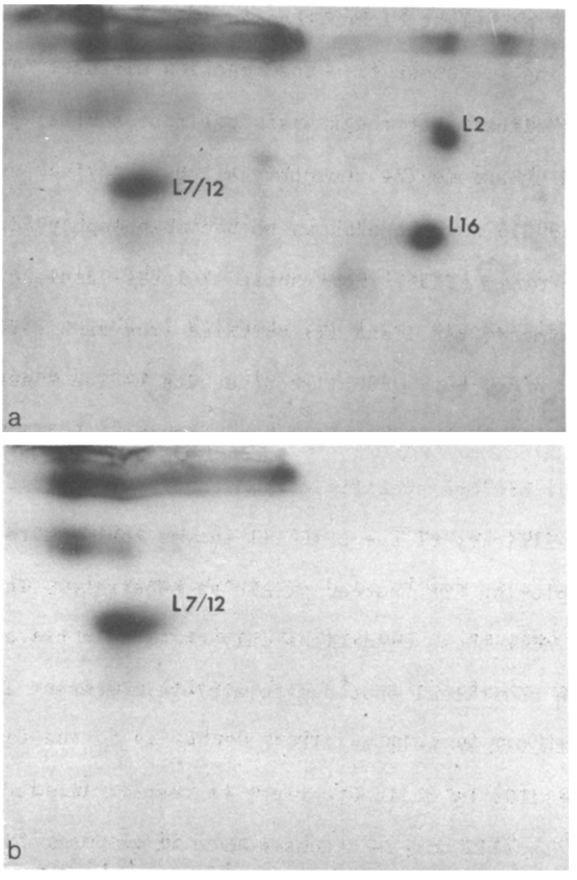

Figure 2: Autoradiogram showing the phosphorylation patterns obtained with 50S subunits incubated with DEAE III enzyme and ATP^{32} or GTP^{32}. Phosphorylation of the ribosomal subunits was performed as follows: The reaction mixture of 150 µl contained 13 mM MES-buffer (2[N-morpholino]-ethane sulfonic acid), pH 6.9; 5 mM $MgCl_2$; 3.25 nmole γ-[^{32}P]ATP or γ-[^{32}P]GTP (spec. activities: 8000 cpm and 3000 cpm/pmole, respectively); 2 mg 50S ribosomal subunits (final conc. 13.3 mg/ml); and 125 µg of the casein specific protein kinase fraction, peak III isolated from a DEAE column as described previously by Traugh, Mumby and Traut (3). The mixture was incubated for 15 min at 30°C and then chilled. The $MgCl_2$ concentration was raised to 100 mM and the mixture was diluted with 300 µl of glacial acetic acid. After 45 min at 0° with occasional shaking, the precipitated RNA was pelleted by centrifugation. The supernatant fraction containing the ribosomal proteins was dialyzed against 7.5% propionic acid at 25°C for 10 hrs at 0°. The dialyzed proteins were lyophilized and then suspended in 60 µl of a buffer which contained 9 M urea and 2 mM dithioerythritol and which was otherwise identical to the buffer used in the first dimension of the two dimensional electrophoresis method previously described by Howard and Traut (4). Polyacrylamide gel electrophoresis in two dimensions was carried out according to Howard and Traut (14). The sample was divided in two portions of 30 µl each and applied to two different gel tubes with an inner diameter of 2 mm. The basic proteins were separated in gels 7.5 cm long and the acidic proteins in gels 5.5 cm long.

generally defined are specific for serine and threonine residues; phosphorylated intermediates which might conceivably be related to turnover during the protein synthesis cycle might be predicted to consist of more labile linkages; e.g. phosphate bound to carboxyl groups or to histidine. Previous studies by Traugh and Traut on the action of rabbit skeletal muscle kinase on E. coli ribosomes demonstrated the formation of serine and threonine phosphate (2). Partial acid hydrolysis of the total 50S proteins incubated with DEAE III kinase and $\gamma[^{32}P]$GTP was carried out according to a method described by Bylund and Krebs (13). The result is shown in Fig. 3. Most of the radioactivity was found to be bound to serine.

It is advantageous to study the action of our different kinases from rabbit reticulocytes and skeletal muscle on E. coli ribosomes along with eucaryotic ribosomes. This is because of the more extensive information available on the separation, structure and function of the bacterial proteins. The apparent absence of protein kinase in E. coli raises doubts as to the functional significance of phosphorylation of ribosomes in this organism. However, on the assumption that there was an evolutionary development from the procaryotic to the eucaryotic ribosomes predicts certain homologies between the structures of procaryotic and eucaryotic ribosomal components. In fact, it has recently been shown that L7/L12 from E. coli can functionally replace the corresponding proteins in the 60S subunit from rat liver ribosomes and that they crossreact immunochemically. We postulate a similar relationship between the ribosomal

Gels were run with 5 mA/tube for 3 to 4 hours, the polarity reversed for each of the two gels. After electrophoresis the gels were directly used for separation of the proteins in the second dimension. No dialysis step as described originally by Kaltschmidt and Wittmann (12) was performed. After the electrophoresis of the proteins in the second dimension the gels were stained with Coomassie Blue R 250 as described by Casjens et al. (15), photographed and then dried under vacuum at 90°C according to the method described by Maizel (16). The dried gels were exposed for autoradiography on a Kodak No-Screen Medical X-ray film for 3-6 hours. Longer exposure up to 24 hours did not reveal additional radioactive spots.

Figure 2a: Pattern with ATP^{32}

Figure 2b: Pattern with GTP^{32}

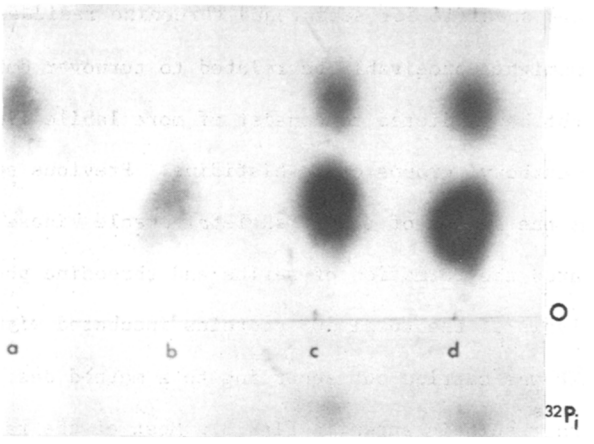

Figure 3: Electrophoresis and autoradiogram of partial acid hydrolysate of 50S ribosomal proteins after incubation of subunits with DEAE III enzyme and [^{32}P]GTP. Analysis of phosphoserine and phosphothreonine was carried out according to Bylund and Krebs (13). Approximately 200 µg of extracted protein was partially hydrolyzed in 1.9 N HCl for 6 hrs at 110°C in a vacuum sealed tube. The HCl was removed under vacuum at 40° and the mixture was suspended in 60 µ H_2O and directly applied to a sheet of Whatmann No. 3 MM paper. Electrophoresis was carried out in a formic acetic acid buffer system at pH 1.9 for 50 min at 3000 V. Nonradioactive phosphoserine and phosphothreonine were run as standards and stained with ninhydrin. The time of exposure of the autoradiogram was from 2 to 3 hours.

a. phosphothreonine; b. phosphoserine; c. ^{32}P-L7/L12-hydrolysate;

b. ^{32}P-L7/L12-hydrolysate and nonradioactive phosphoserine.

proteins susceptible to phosphorylation from eucaryotes and from their procaryotic ancestors. If phosphorylation has an influence on the regulation of the translation mechanisms in vivo then homologous peptides or amino acid sequences may already be found in procaryotic systems susceptible to phosphorylation. The coincidence that the same proteins, L7, L12, are involved in GTP hydrolysis and are the unique 50S protein substrates for a GTP dependent protein kinase is intriguing. Studies are in progress on the action of the GTP protein kinase on rabbit reticulocyte ribosomes. Regardless of the functional significance of phosphorylation of ribosomal proteins the availability of protein kinases having different specificity provides a valuable probe for exploring the topography of ribosomal proteins in the intact particle.

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REFERENCES

1. Reimann, E. M., Brostrom, C. O., Corbin, J. D., King, C. A. and Krebs, E. G. (1971) Biochem. Biophys. Res. Commun. 42, 187-194.
2. Traugh, J. A. and Traut, R. R. (1972) Biochemistry 11, 2503-2508.
3. Traugh, J. A., Mumby, M. and Traut, R. R. (1973) Proc. Nat. Acad. Sci. U.S.A. 70, 373-376,
4. Traugh, J. A. and Traut, R. R. (1974) J. Biol. Chem. 249, 1207-1212.
5. Bingham, E. W., Farrell, H. M. and Basch, J. J. (1972) J. Biol. Chem. 247, 8193-8194.
6. Traugh, J. A., Ashby, C. D. and Walsh, D. A. (1974) Methods Enzymol., in press.
7. Ventimiglia, F. A. and Wool, I. G. (1974) Proc. Nat. Acad. Sci. U.S.A. 71, 350-354.
8. Sherton, C. C. and Wool, I. G. (1974) J. Biol. Chem. 249, 2258-2267.
9. Fakunding, J. L., Traut, R. R. and Hershey, J. W. B. (1973) J. Biol. Chem. 248 8555-8559.
10. Hardy, S. J. S., Kurland, C. G., Voynow, P. and Mora, G. (1969) Biochemistry 8, 2897-2905.
11. Glynn, J. H. and Chappell, J. B. (1964) Biochem. J. 90, 147-149.
12. Kaltschmidt, E. and Wittmann, H. G. (1970) Anal. Biochem. 36, 401-412.
13. Bylund, D. and Krebs, E. G. (1974) J. Biol. Chem., in press.
14. Howard, G. and Traut, R. R. (1974) Methods Enzymol. 30, 526-539.
15. Casjens, S., Hohn, T. and Kaiser, A. D. (1970) Virol. 42, 496-507.
16. Maizel, J. V. (1971) Methods Virol. 5, 179-246.