ATPase and GTPase Activities Associated with a Specific 5S RNA-Protein Complex*

(*B. stearothermophilus*/50S ribosomal proteins/fusidic acid/thiostrepton/reconstitution)

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ABSTRACT An RNA-protein complex consisting of 5S RNA and two ribosomal proteins, B-L5 and B-L22, was isolated from *Bacillus stearothermophilus* ribosomes and found to be active in GTP hydrolysis. This activity was not influenced by elongation factor G. Further analysis of this complex showed that it was also able to hydrolyze ATP. Inhibition studies revealed that ATP was a non-competitive inhibitor of GTP and that GTP was also a noncompetitive inhibitor of ATP, indicating that two different enzymatic sites were involved. Differences in pH optimum and optimal temperature also point to a two-site enzyme complex. Both enzymatic hydrolyses were inhibited by thiostrepton and fusidic acid, which are known inhibitors of protein synthesis.

In order to accomplish protein synthesis, ribosomes perform an intricate pattern of enzymatic activities (for recent reviews on the structure and function of bacterial ribosomes see refs. 1-3). One promising way to separate the many different ribosomal functions from each other is to reduce the complex ribosomal particle to smaller subparticles, which in one way or the other still exhibit one of the ribosomal activities. This subparticle would be more suited for analysis of the particular activity in terms of its structural and functional characteristics. The experiments described here, deal with such a subparticle from *Bacillus stearothermophilus* 50S ribosomes, namely its specific 5S RNA-protein complex (4).

This complex contains mainly the two 50S ribosomal proteins, B-L5 and B-L22. Four other proteins, B-L6, B-L10, B-L26, and B-L27, were also present in the complex, but in smaller amounts [as shown by two-dimensional acrylamide-gel electrophoresis (4)]. Since the *B. stearothermophilus* 5S RNA-protein complex is generally more stable than the corresponding *Escherichia coli* complex, it was used to initiate the functional studies reported below.

MATERIALS AND METHODS

Preparation of Materials. *B. stearothermophilus* ribosomes and ribosomal subunits were prepared as described (5). 5S RNA, prepared by phenol extraction of 70S or 50S ribosomes, was purified by Sephadex G-100 chromatography (6). Specific 5S RNA-protein complexes were reconstituted and isolated

as described (4). These 5S RNA-protein complexes did not show any loss of activity when stored for 1 year at -80° .

Assay for ATPase and GTPase Activity. The method used was essentially that described by Parmeggiani *et al.* (7) except that elongation factor G (EF-G) was omitted from the assay. Usually 0.07 A_{260} unit of 5S RNA-protein complex (corresponding to 0.082 nmol of 5S RNA) was incubated in 100- μ l volumes in the presence of 0.12 mM $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ or ATP, 0.1 mM MgCl_2 , 3 mM NH_4Cl , 6 mM 2-mercaptoethanol, and 0.1 M Tris·HCl. ATPase assay was at pH 7.8, at 60° for 30 min. GTP hydrolysis was measured at pH 7.4 and 45° for 30 min. After incubation, the samples were placed on ice. 5 μ l of 50 mM KH_2PO_4 and 100 μ l of 1 M HClO_4 were added, followed by mixing and centrifugation at 2000 rpm ($500 \times g$) for 2 min. An aliquot of the supernatant (100 μ l) was tested for liberated phosphate, with 300 μ l of 20 mM ammonium molybdate and 200 μ l of isopropanol acetate. After the samples were mixed thoroughly for 1 min, they were centrifuged at 2000 rpm for 2 min. 200 μ l of the organic phase were withdrawn and counted with 5 ml of standard counting solution in a Packard liquid scintillation counter.

Unless otherwise stated, the specific activities of GTP and ATP used in the assay were 1-5 Ci/mol. For all points in the figures the appropriate blank, obtained under identical conditions in the absence of 5S RNA-protein complex, was subtracted.

RESULTS

GTPase and ATPase Activity Associated with Specific 5S RNA-Protein Complex. There is evidence for a common binding site of EF-G and EF-Tu on the ribosome (8-11). It has been suggested that the GTP hydrolysis associated with these two factors is accomplished at the same center on the ribosome (12-14). Therefore we analyzed our specific 5S RNA-protein complex for GTPase activity. Indeed such enzymatic activity was observed. Incubation of constant amounts of $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ with increasing concentrations of *B. stearothermophilus* 5S RNA-protein complex exhibited a linear increase in GTP hydrolysis (Fig. 1). This hydrolysis was not influenced by addition of EF-G (unpublished results).

Checking GTPase activity in the presence of all common mono-, di-, and trinucleotides revealed that the adenine nucleotides, especially ATP, exhibited a strong inhibitory effect (Horne, J. & Erdmann, V. A., manuscript in preparation). Therefore *B. stearothermophilus* 5S RNA-protein complex was incubated with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. The 5S RNA-

Abbreviations: pppGpp, guanosine-pentaphosphate; ppGpp, guanosine-tetraphosphate; EF, elongation factor.

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FIG. 1. ATP (●—●) and GTP (○—○) hydrolysis dependent upon *B. stearothermophilus* 5S RNA-protein complex concentration. 10 μ l of 5S RNA-protein complex corresponds to 0.015 A_{260} unit. Assay conditions were as described in *Methods* except that the specific activity of the triphosphates was 0.2 Ci/mol.

protein complex showed also significant ATPase activity (Fig. 1). We then made an attempt to find the optimal conditions for both hydrolytic activities.

Ionic Conditions. To determine which cations are required for optimal GTP and ATP hydrolysis we tested both enzymatic activities in the presence of 3 mM Mg^{++} and the following monovalent cations: NH_4^+ , K^+ , Na^+ , and Li^+ . Maximal GTPase and ATPase activities were obtained with 3 mM NH_4Cl . Next, the NH_4Cl concentration was held constant at 3 mM and different concentrations of the following divalent cations were tested: Mg^{++} , Mn^{++} , Fe^{++} , Ca^{++} , Zn^{++} , Co^{++} , Cu^{++} , and spermidine. At 0.1 mM, $MgCl_2$ was the best divalent cation, yielding optimal activities for both the ATP and GTP hydrolysis. There was no activity of either enzymatic hydrolysis in the presence of 1 mM EDTA.

The Effect of pH on GTPase and ATPase activities is shown in Fig. 2. Both hydrolytic activities had a broad pH optimum.

FIG. 2. Effect of pH on GTPase (○—○) and ATPase (●—●) activity.

FIG. 3. Temperature dependence of GTPase (●—●) and ATPase (○—○) activity.

GTP was best hydrolyzed at pH 7.4, whilst maximal ATP hydrolysis took place at pH 7.8 (Fig. 2). pH curves in which Mg^{++} was replaced by Ca^{++} showed similar characteristics, which is in contrast to findings for membrane ATPase of *E. coli* (15).

Optimal Temperature and Time of Incubation. We found that GTP was hydrolyzed best by the 5S RNA-protein complex at 45° (Fig. 3). In contrast, ATP was hydrolyzed best at much higher temperatures. The temperature dependence of ATP hydrolysis repeatedly showed two optima, one at 60° and the other at 75° (Fig. 3). Since 60° is the optimal growth tempera-

FIG. 4. Time dependence of GTPase (○—○) and ATPase (●—●) activity.

Fig. 5. Determination of K_m and V_{max} for GTP (○—○) and ATP (●—●) hydrolysis. S corresponds to substrate concentration.

ture for *B. stearotherophilus*, we decided to do all subsequent ATP hydrolysis assays at this temperature. Fig. 4 shows the time curve for hydrolysis of ATP and GTP by the 5S RNA-protein complex. Both types of hydrolysis were linear between 0 and 100 min of incubation.

K_m and V_{max} for ATP and GTP. Kinetic studies were done and plotted according to Lineweaver-Burk (Fig. 5). A K_m of 70 μ M and a V_{max} of 140 pmol/min was obtained for ATP hydrolysis; a K_m of 20 μ M and a V_{max} of 40 pmol/min was obtained for GTP hydrolysis. In comparison, Kaziro *et al.* (16) have reported a K_m for GTP of the EF-G-dependent *E. coli* GTPase of 90 μ M.

Inhibition Studies. Experiments in which the hydrolysis of three different concentrations of ATP was measured in the presence of increasing amounts of GTP revealed that GTP is a noncompetitive inhibitor of ATP (Fig. 6). The K_i was 340 μ M. Similarly, noncompetitive inhibition of the GTPase activity by ATP was found; the K_i was 2 μ M (Fig. 7).

Thiostrepton (17–20) and fusidic acid (14, 21, 22) inhibit protein synthesis by interfering with the known functions of EF-G and EF-Tu (23–25). Therefore, both antibiotics were tested for their effects on GTPase and ATPase activity. Fig. 8

Fig. 6. Inhibition of ATPase activity by GTP. K_i for the noncompetitive inhibitor GTP was 0.34 mM. ATP concentration: (●—●) 0.02 mM; (○—○) 0.04 mM; (●—●), 0.12 mM.

Fig. 7. Inhibition of GTPase activity by ATP. K_i for the noncompetitive inhibitor ATP was 2 μ M. GTP concentration: (●—●) 0.013 mM; (○—○) 0.03 mM; (●—●) 0.1 mM.

clearly shows that these antibiotics inhibit both enzymatic activities. In another experiment (not shown here) ouabain, a known inhibitor of *E. coli* ATP phosphohydrolase (26), did not influence hydrolysis of ATP or GTP.

DISCUSSION

The specific *B. stearotherophilus* 5S RNA-protein complex analyzed here had been shown by two-dimensional polyacrylamide electrophoresis to consist only of 5S RNA and the 50S ribosomal proteins, B-15 and B-L22, plus, in smaller concentrations, B-L6, B-L10, B-L26, and B-L27 (4). The results presented in this communication establish that, in association with this complex, ATP as well as GTP can be hydrolyzed. It is the first time that such hydrolytic activity has been shown to be independent of a factor and that it can be directly attributed to ribosomal proteins. The necessity of GTP hydrolysis in protein synthesis has been well established (24), and increasing evidence has accumulated that EF-G (23, 24, 27) and EF-Tu (28–32) are involved when GTP is hydrolyzed. Recently it was found that EF-G and EF-Tu bind to the same site on the ribosome (8–11) and, therefore, a single GTPase site has been proposed for the ribosome (12–14). Since the complex described here exhibits GTPase activity and since it is

Fig. 8. Inhibitory effects of thiostrepton and fusidic acid upon ATP (●—●) and GTP (○—○) hydrolysis. Assay conditions as described in the text except that thiostrepton was tested in the presence of 0.8% dimethylsulfoxide and fusidic acid in the presence of 20% methanol.

also inhibited by fusidic acid and thiostrepton, which are known inhibitors of GTPase activity associated with translocation, it is possible that one or more of the proteins in the complex represents the ribosomal GTPase enzyme.

The other enzymatic activity associated with the 5S RNA-protein complex is the ability to split ATP. That GTP and ATP are hydrolyzed at two different sites is suggested by the observation that ATP and GTP are noncompetitive inhibitors of each other. This finding is further supported by the fact that the pH and temperature optima for both types of hydrolysis are different. The only ATPase so far known to be associated with bacterial ribosomes is the one involved in the formation of "magic spot," i.e., pppGpp and ppGpp (33-38), in which ATP serves as the donor for the pyrophosphate in the 3' position of the guanosine nucleotide (37). Since GTP or GDP or both accept the two phosphates from ATP, it would seem necessary that during "magic spot" formation GTP and ATP would have to be in close proximity on the ribosome. The ATPase discovered in association with the 5S RNA-protein complex yields, under the conditions described here, inorganic phosphate. However, under conditions not described here pyrophosphate formation has also been observed.

Activities similar to those described here for *B. stearothermophilus* have also been found with specific *E. coli* (Gaunt-Klöpfer, M. & Erdmann, V. A., manuscript in preparation) and rat-liver (Grummt, F., Grummt, I. & Erdmann, V. A., manuscript in preparation) 5S RNA-protein complexes, which would point to a universal functional requirement of these activities in pro- and eukaryotic ribosomes.

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