COMPONENTS OF THE 50S RIBOSOMAL SUBUNIT INVOLVED IN GTP CLEAVAGE

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Summary: The "β core" (i.e., the 50S subunit of E. coli from which about eight "split β proteins" had been removed) can substitute for the whole 50S subunit in promoting the cleavage of GTP from the aminoacyl-tRNA-S_3-GTP complex (S_3-dependent GTP cleavage). As was reported, the S_2-dependent GTP cleavage requires, in addition to the "β core", at least one of the"split β proteins". Thiostrepton, known to inhibit both the S_2-dependent and the S_3-dependent GTP cleavage, is bound to the "β core".

Studies with cell-free systems from bacteria revealed that two peptide chain elongation factors, i.e., S_3, (the aminoacyl-tRNA binding factor) and S_2, (the translocase) are involved in GTP cleavage, each in a different phase of the elongation cycle (1,2).

This cycle begins by S_3 binding GTP and aminoacyl-tRNA. The resulting complex is bound to the A site of the ribosome-messenger complex (which has peptidyl-tRNA bound at the P site). Subsequently the GTP is cleaved, Pi and S_3-GDP complex are released from the ribosome and the α-amino group of the aminoacyl-tRNA forms a peptide linkage with the carboxyl group of the peptidyl residue which has been released from linkage to the tRNA. The newly extended peptidyl-tRNA is bound at the A site, the discharged tRNA at the P site. Finally, S_2 and GTP are bound to the ribosome, GTP is cleaved to GDP and Pi, the discharged tRNA is released from the P site, the peptidyl tRNA is shifted from the A site to the P site, the ribosome moves three nucleotides along the messenger RNA, and Pi, GDP and S_2 are released from the ribosome. This terminates the cycle (1,2).

Neither S_3 nor S_2 can promote GTP cleavage, unless bound to the ribosome. The S_3-promoted GTP cleavage also depends on aminoacyl-tRNA as well

as on the appropriate messenger RNA, and one GTP is cleaved for each aminoacyl-tRNA bound to the ribosome (3-5). S_2, however, does promote GTP cleavage in the presence of the ribosome even in the absence of messenger RNA and peptidyl-tRNA (6). It is conceivable that this so called "uncoupled GTPase activity" is an artifact of the cell-free system. As confirmed by us, (data not shown) the GTPase activities of both S_3 and S_2 are blocked by Thiostrepton (7-9). Indirect evidence was presented for the binding of this inhibitor to the 50S subunit (10-13). This may reflect that there are components of this subunit which are involved in both GTP cleaving processes.

In this study we present data on the components of the ribosome involved in GTP cleavage as promoted by S_2 and S_3, and on the site of binding of Thiostrepton on the 50S subunit.

In our experiments we made use of a procedure developed for removal of some of the proteins from the 50S subunit by centrifugation in a CsCl gradient (14,15). This treatment produces (i) a so-called β core, i.e. the 50S subunit devoid of about 8 proteins and (ii) split β proteins, i.e. a mixture of the about 8 proteins which had been removed from the 50S subunit (15).

The results in Table I: (i) verify that both 30S and 50S subunits and poly U are involved in GTP cleavage promoted by S_3 (ref. 16); (ii) reveal that β cores can substitute for 50S subunits in promoting the cleavage and (iii) split β proteins are without effect on it.

The data in Table II: (i) verify that both 30S and 50S subunits are involved in S_2-dependent "uncoupled GTPase activity" (17) and (ii) are consistent with the results of Kischa et al. (18) in showing that β cores alone do not substitute for 50S subunits, whereas β cores and split β proteins or β cores and P_1 protein do. It is probable that the P_1 protein used in our experiment is identical with the A protein used by Kischa et al. (18). The method followed for the selective removal of P_1 protein from

Table I. Ribosomal components required for the cleavage of GTP from the Phe-tRNA-S_3-GTP complex.

Components of the system	GTP cleaved (pmoles of $^{32}P_i$ released)
1. Complete system (30S+50S+poly U + Phe-tRNA-S_3-γ^{32}P-GTP complex)	3.21
2. -50S + β cores	2.50
3. -50S + β cores + split β proteins	2.33
4. -50S + split β proteins	0.42
5. -50S	0.32
6. -30S	0.32
7. -poly U	1.54

30S and 50S ribosomal subunits, β cores and split β proteins from E. coli Q13 cells were prepared essentially according to the procedure of Staehelin et al. (15). 50S subunits were dialyzed against buffer A (20 mM Tris HCl, pH 7.4, 6 mM 2-mercaptoethanol) containing 50 mM magnesium acetate. The resulting solution was made 4.8 M in CsCl and centrifuged at 41,000 rpm in the SW 50.1 rotor of the Spinco ultracentrifuge at 2° for 25 hours. The top 1 ml sample in the centrifuge tube was removed, dialyzed against buffer B (50 mM Tris HCl, pH 7.4, 50 mM NH_4Cl, 10 mM magnesium acetate, 1 mM dithiothreitol) and concentrated (split β proteins). Acrylamide gel electrophoresis (20) of this preparation in 6 M urea at pH 4.5 resulted in about 8 bands. The rest of the gradient was collected in 25 eight drop fractions. The only A_{260} absorbing peak banded at a buoyant density of 1.65 g/cm^3. The fractions containing this peak were pooled and dialyzed against buffer B (β cores). Peptide chain elongation factors were purified from Bacillus stearothermophilus (21). These factors are analogous to factors from E. coli and Pseudomonas fluorescens. S_1 corresponds to T_s and TI_s, S_2 (the translocase) to G and TII, and S_3 (the aminoacyl-tRNA binding factor) to Tu and TI_u (1). Phe-tRNA-S_3-γ-^{32}P-GTP complex was prepared according to the procedure of Skoultchi et al. (22) by incubating S_1, S_3, Phe-tRNA and γ-^{32}P-GTP and passing the reaction mixture over a Sephadex G25 column to remove GTP not bound in the complex.

Assay of the cleavage of GTP from the complex. 0.05 ml of the reaction mixture contained in buffer B (but containing 5 mM dithiothreitol instead of 1 mM,) some of the following components, as indicated in the Table: 0.5 A_{260} unit of 30S subunits, 1 A_{260} unit of 50S subunits, 1 A_{260} unit of β cores, split β proteins derived from 2 A_{260} units of 50S subunits, 5 μg of poly U. The reaction mixture was incubated at 30° for 5 min, mixed with 0.05 ml of Phe-tRNA-S_3-γ-^{32}P-GTP complex solution (containing 15 pmoles of γ-^{32}P-GTP, specific activity 120 cpm/pmole) and further incubated at 30° for 5 min. The Pi released was determined according to the procedure of Conway and Lipmann (6).

50S subunits was that of Hamel and Nakamoto (19), who discovered that this protein is involved in the S_2-dependent GTP cleavage.

Table II. Ribosomal components required for GTP cleavage as promoted by S_2 factor.

Components of the system	GTP cleaved (pmoles of $^{32}P_i$ released)
1. Complete system (30S+50S+S_2+γ^{32}P-GTP)	36.0
2. -50S + β cores	10.5
3. -50S + β cores + split β proteins	46.8
4. -50S + β cores + P_1 protein	37.7
5. -50S + split β protein	10.1
6. -50S + P_1 protein	12.2
7. -50S	8.3
8. -30S	7.6
9. -S_2	4.8

P_1 protein was released from the 50S subunits essentially according to the methods of Hamel and Nakamoto (19): 50S subunits were suspended in 20 mM Tris-HCl (pH 7.4), 10^3 mM NH_4Cl, 200 mM magnesium acetate and 1 volume of 95% ethanol was added at 0°. The reaction mixture was centrifuged to sediment the precipitate at 5,000 g for 20 min. The supernatant fraction dialyzed against buffer B was taken as P_1 protein.

Assay of GTP cleavage as promoted by S_2: 0.1 ml of the reaction mixture contained in buffer C (40 mM Tris-HCl, pH 7.4, 160 mM NH_4Cl, 10 mM magnesium acetate, 10 mM dithiothreitol) some of the following components as indicated in the Table: 0.5 A_{260} unit of 30S subunits, 1 A_{260} unit of 50S subunits, 1 A_{260} unit of β cores, split β proteins derived from 2 A_{260} units of 50S subunits, 2 μg of S_2. The reaction mixture was incubated at 30° for 10 min, subsequently it was made 20 mM in γ^{32}P-GTP and was further incubated for 10 min. The P_i released was determined as indicated in the Legend to Table I.

The results in Table III reveal that β cores and P_1 protein do not substitute for 50S subunits in polyPhe-tRNA synthesis, whereas β cores and split β proteins do.

To narrow the localization of the binding site of Thiostrepton within the 50S subunit we prepared ^{35}S labelled Thiostrepton. The results of experiments with this compound in Table IV: (i) reveal that Thiostrepton binds to the β core of the 50S subunit and (ii) are consistent with the possibility that each β core may bind a single Thiostrepton molecule.

Table III. Ribosomal components required for polyPhe-tRNA synthesis from Phe-tRNA as directed by poly U.

Components of the system	^{14}C phenylalanine incorporated into polyPhe-tRNA (pmoles)
1. Complete system (30S+50S+poly U + ^{14}C Phe-tRNA+S_1+S_2+S_3+GTP)	11.1
2. -50S + β cores	0.7
3. -50S + β cores + split β proteins	10.0
4. -50S + β cores + P_1 protein	1.2
5. -50S + split β proteins	1.2

0.125 ml of the reaction mixture contained in buffer C some of the following components: 5 μg of poly U, 2 A_{260} units of ^{14}C-Phe-tRNA carrying 41 pmoles of Phe residues, 0.1 μg of S_1, 0.1 μg of S_2, 0.2 μg of S_3. The amount of 30S and 50S subunits, β cores, split β proteins and P_1 protein was the same as indicated in the Legend to Table II. The reaction was started by adding 250 nmoles of GTP. After incubation at 37° for 10 min the amount of Phe residues incorporated into hot acid insoluble material was determined (22).

(To convert this possibility into a certainty, it had to be shown that each β core or 50S subunit does bind Thiostrepton.) (See also 7,11,13.) The fact that Thiostrepton binds to a component of the ribosome which is involved in both S_2- and S_3-dependent GTP cleavage processes is in line with the fact that it blocks both processes.

Labelled Thiostrepton may serve as a tool in the future for identifying the component (or components) in the β core to which the inhibitor is bound. This component is either (i) a binding site for S_2 and for aminoacyl-tRNA-S_3-GTP complex and (ii) a part of both GTP cleavage systems, or interacts with these in an allosteric fashion. (See also 7,10.) It will be interesting to learn if the Thiostrepton-binding component undergoes conformational changes during either or both of the two GTP cleavages.

Table IV. Binding of thiostrepton to various components of the ribosome.

Components	Thiostrepton bound (cpm/pmole of component)
30S	9
50S	55
β cores	61
β cores + split β proteins	59

Preparation of ^{35}S-labelled thiostrepton according to the procedure of Dr. E. Meyers: 50 mCi of $H_2{}^{35}SO_4$ (specific activity 100 mCi/mg) was added to 250 ml of a medium in which Streptomyces azureus (ATCC 14921) had been growing at 25° for 56 hrs in flasks in a rotary shaker. The fermentation was continued for 72 hrs. The antibiotic was extracted from the mycelial mat with chloroform and the thiostrepton was crystallized by adding 95% ethanol to the chloroform extract, and was recrystallized twice by the same procedure. The yield was 2 mg, the specific activity 1.06×10^6 cpm/mg. 1.6 mg of the crystalline material was dissolved in 1 ml of dimethylsulfoxide.

Assay for thiostrepton binding: 1.6 μg of the ^{35}S thiostrepton preparation was incubated in 0.5 ml of buffer B (but containing 2 mM dithiothreitol instead of 1 mM) at 30° for 5 min with some of the following components: 300 pmoles of 30S subunits, 200 pmoles of 50S subunits, 300 pmoles of β cores, split β proteins derived from 600 pmoles of 50S subunits. The reaction mixture was cooled to 0° and was passed over a Sephadex G-50 column to separate the thiostrepton bound to the ribosomal components from unbound thiostrepton. The fractions were assayed for absorbancy at 260 mμ and counted in a Dioxan based scintillator. The thiostrepton content of our crystalline preparation was determined in the following way: binding of labelled thiostrepton to limiting amounts of β cores was tested in a series of samples under identical conditions except that sample I contained labelled thiostrepton and all the other samples contained in addition to the same amount of labelled thiostrepton as in sample I, varying amounts of unlabelled, presumably pure, thiostrepton. It was calculated from the results that the thiostrepton content of our preparation was 30% and that all radioactivity was in thiostrepton. The molecular weight of this antibiotic was reported to be approximately 1,600 (23,24). Taking this into consideration it was calculated that the specific activity of the labelled thiostrepton was 56 cpm/pmole.

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