RECONSTITUTION OF THE 50S RIBOSOME SUBUNIT. ROLE OF PROTEINS L 7 AND L 12 IN THE GTPase ACTIVITIES. SITE OF ACTION OF THIOSTREPTON

JUAN P.G. BALLESTA and D. VAZQUEZ
Instituto de Biologia Celular, Velazquez 144, Madrid 6, Spain

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1. Introduction

Upon treatment of *Escherichia coli* ribosomes with 1 M ammonium chloride in the presence of 50% ethanol, ribosome-derived protein deficient particles are obtained. These particles are inactive for the GTPase activities dependent on ribosomes and either elongation factor G (EF G) or elongation factor T (EF T) [1]. The deficient particles recover these GTPase activities by addition of the released proteins [1–4] which have a strong acidic character and have been identified as proteins L 7 and L 12 [3, 4] following the nomenclature of Wittmann [5]. The same two proteins have been independently isolated and studied by Möller and coworkers [6, 7]; proteins L 7 and L 12 were also found in their systems to be involved in the recovery of the G-dependent GTP hydrolytic activity in the reconstitution of 50 S-derived cores deprived of a number of proteins [6].

We have studied the effect of alcohols in EF G- and EF T- dependent functions of the 50 S ribosome subunit. Ethanol and methanol strongly stimulate the EF G-dependent hydrolysis of GTP in the presence of 50 S-derived cores obtained by dissociation of some proteins in CsCl gradients [8]. The alcohol effect is more relevant in studies on EF T-dependent activities. EF T-dependent binding of Phe-tRNA$_{Phe}$ to ribosomes is hardly affected by moderate concentrations of methanol whereas the GTP hydrolysis is considerably enhanced under the same conditions. This effect results in an uncoupling of the EF T-dependent aminoacyl-tRNA binding and the GTP hydrolysis [9] which are believed to take place normally in the proportion 1:1. Furthermore this methanol- and EF T-dependent uncoupled GTP hydrolysis, which takes place in the presence of ribosomes or their 50 S subunits, is resistant to antibiotics of the siomycin and thiostrepton group [9] which are known to block EF T-dependent aminoacyl-tRNA binding [10, 11].

We have recently studied the effect of methanol on the activities of 50 S cores deprived of proteins L 7 and L 12 (P1-50 S cores) and the possible role of these two proteins on EF T-dependent activities of the 50 S ribosome subunit. The results obtained are presented in this contribution.

2. Methods

Ribosomes were prepared from *E. coli* D-10 cells by grinding with alumina [12] and washed 5–6 times with 20 mM Tris-HCl buffer, pH 7.4, containing 1 M ammonium chloride and 40 mM Mg acetate. Sucrose gradient centrifugation in a zonal rotor was carried out for the preparation of ribosomal subunits [13].

Ethanol precipitation of 50 S ribosomal subunits was carried out in the presence of 1 M ammonium chloride for the preparation of L 7 and L 12 deficient ribosomes and the fraction containing these two proteins; this fraction was used without further separation of the L 7 and L 12 proteins [1]. Two dimensional electrophoresis in polyacrylamide [5] confirmed that our P1-50 S cores were deprived of proteins L 7 and L 12. A similar analysis of the released fraction was shown to contain only L 7 and L 12 proteins and minor traces in some preparations of L 1 and L 3.

Preparation of β- and γ-cores from 50 S ribosomal

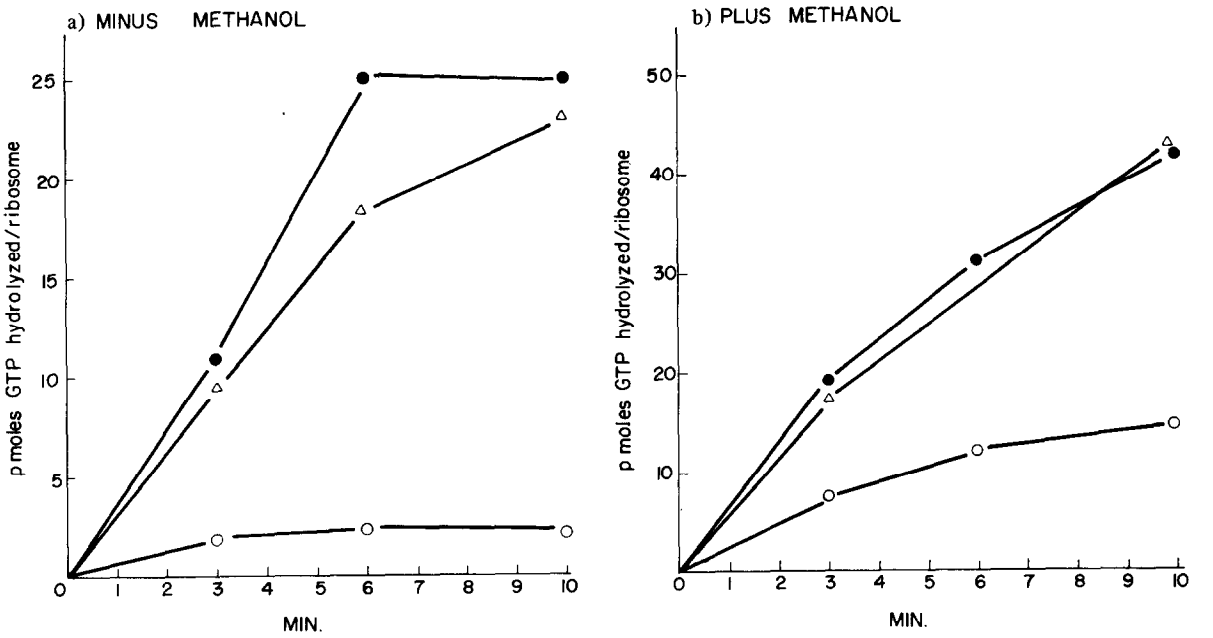

Fig. 1. Effect of methanol on EF G-dependent GTPase of Pl-50 S cores. Reactions were carried out in 0.1 ml of 20 mM Tris-HCl pH 7.8, containing 125 mM NH_4Cl, 5 mM Mg acetate, 50 μM [γ-^{32}P] GTP, 0.38 μM 30 S subunits and 3.8 μg of EF G. Methanol when present was 20% (v/v). 50 S subunits and Pl-50 S cores were present when indicated at a concentration 0.35 μM. Incubation was at 30°. 0.03 ml aliquots were taken at the indicated times and extracted as described [16]. (●—●—●) 50 S subunits; (○—○—○) P1-50 S cores; (△—△—△) P1-50 S cores plus L 7 and L 12 proteins.

subunits was carried out according to Maglott and Staehelin [14]. Proteins separated in the isolation of γ-cores ($SP_{50\text{-}\gamma}$) were used without a further separation. Two dimensional electrophoresis in polyacrylamide has shown that β-cores lack seven of the proteins of the 50 S subunits but still have L 7 and L 12 proteins. The γ-cores are derived from the β-cores by dissociation of protein L 7 and six more proteins but, at least some of the γ-cores, still have protein L 12.

Electrophoretically pure EF T and EF G were prepared following the method of Parmeggiani et al. [15]. [γ-^{32}P] GTP was prepared according to Glynn and Chappel [17]. The assays for EF G- and EF T-dependent activities have been described previously [8, 9].

3. Results

3.1. *Effect of methanol on P1-50 S cores*

The EF G-dependent GTPase activity of the P1-50 S cores was studied either in the presence or in the absence of methanol (fig. 1). In the absence of methanol the protein deficient particles were inactive for GTP hydrolysis as previously observed by other workers [1–3] but the addition of the released proteins restored their activity to a value similar to that of the control 50 S subunits (fig. 1a). However, in the presence of methanol there is a significant GTPase activity of the P1-50 S cores which, depending on the preparation, usually ranges from 30–50% of the control 50 S subunits (fig. 1b).

P1-50 S cores have also been found to be inactive, in the absence of methanol, in complementing EF T for GTP hydrolysis [1–3] but recover this activity in the presence of proteins L 7 and L 12 (fig. 2a). In the presence of methanol the EF T-dependent activity is considerably enhanced in all cases and the P1-50 S cores activity is 30–50% of the control 50 S subunits (fig. 2b). In the presence of methanol the EF T-dependent GTPase activity measured is mainly uncoupled from aminoacyl-tRNA binding as we have previously reported [9].

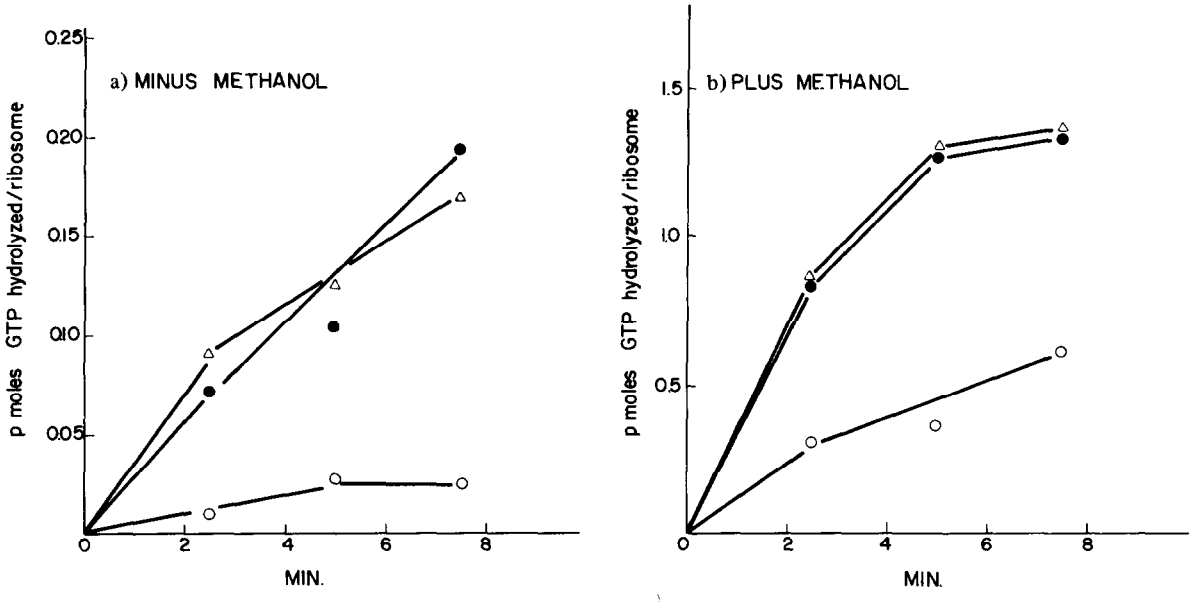

Fig. 2. Effect of methanol on EF T-dependent GTP hydrolysis carried out by Pl-50 S cores. Conditions were similar to those described in fig. 1, except that in addition all reaction mixtures contained 40 μg/ml of poly (U) and 0.16 μM Phe-tRNA. (●—●—●) 50 S subunits; (○—○—○) Pl-50 S cores; (△—△—△) Pl-50 S cores plus L 7 and L 12 proteins.

3.2. *Reconstitution of activity with γ-cores and L 7 and L 12 proteins*

We have previously studied EF G-dependent activity of β- and γ-cores and found that β-cores have a good activity but a slower rate than 50 S subunits. GTP hydrolysis in the presence of γ-cores was very poor but the activity was recovered by addition of the seven proteins separated in the preparation of γ-cores from the β-cores [8]. However, as shown in fig. 3, addition of proteins L 7 and L 12 to the γ-cores causes a significant recovery of the EF G-dependent GTPase activity (fig. 3A) and a somewhat poorer recovery of the EF G-dependent GTPase activity (fig. 3B). Since the same γ-core particles recover their EF T- and preferentially EF G-dependent GTPase activities by addition of the proteins separated in the preparation of γ-cores it is obvious that lack of recovery of activities from γ-cores is not due to inactivation of these particles.

3.3. *Effect of thiostrepton on Pl-50 S cores and 50 S reconstituted subunits*

Since the thiostrepton group of antibiotics have been found to inhibit EF G- and EF T-dependent activities [10, 11] and proteins L 7 and L 12 have been shown to be required for both EF G- and EF T-dependent GTPase activities [2–4], proteins L 7 and L 12 might be the target for thiostrepton action [8]. In order to test this possibility we have studied in the methanol system, the recovery of EF T- and EF G-dependent GTPase activities of Pl-50 S cores when L 7 and L 12 proteins were added either before or after thiostrepton. The results showed that thiostrepton inhibited in all cases EF G-dependent GTPase and the extent of inhibition was similar whether the antibiotic was added before or after addition of proteins L 7 and L 12. There is a significant GTPase activity in Pl-50 S cores in the absence of proteins L 7 and L 12 which is also inhibited by thiostrepton (table 1). However the uncoupled EF T-dependent GTPase, which is also observed using Pl-50 S cores, not only is not inhibited but is enhanced by thiostrepton; this enhancement is equally observed when the antibiotic is added before or after proteins L 7 and L 12.

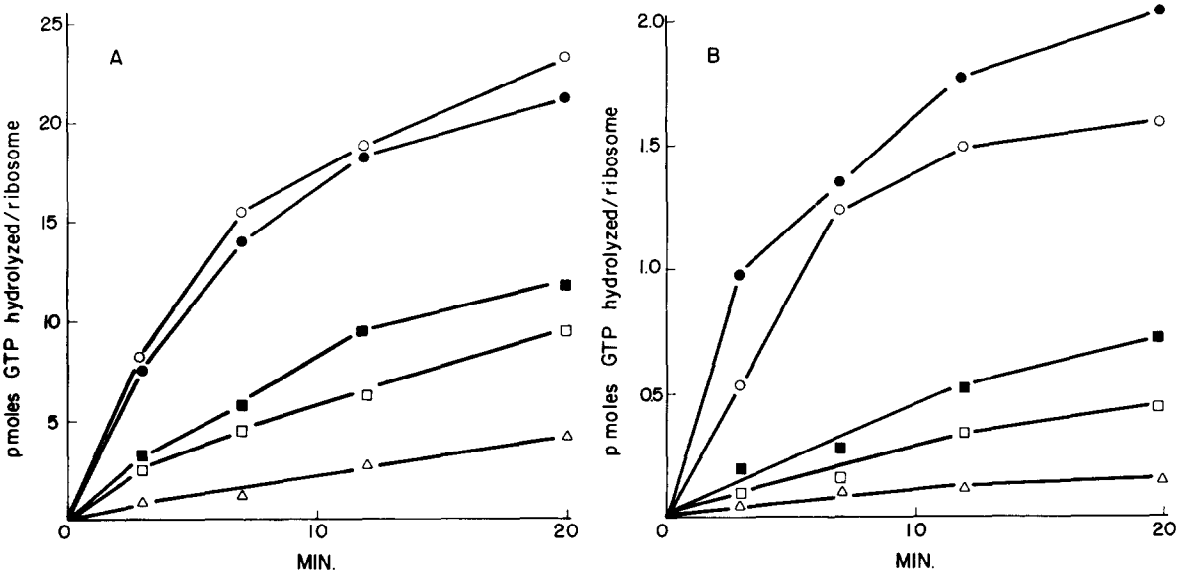

Fig. 3. Effect of proteins L7 and L 12 in GTP hydrolysis by CsCl-derived-cores. Conditions were as described in fig. 1 and fig. 2 for EF G- and EF T-dependent assays, respectively, except that in this case particles were 0.40 μM and both reactions were carried out in 20% (v/v) methanol. A) EF G-dependent GTPase. B) EF T-dependent GTPase. (●—●—●) 50 S subunits; (△—△—△) γ-cores; (●—●—●) γ-cores plus $SP_{50\text{-}\gamma}$; (□, ■) γ-cores plus different preparations of L 7 and L 12.

4. Discussion

We have confirmed the requirement of L 7 and L 12 proteins for the ribosome to hydrolyze GTP in the presence of EF G and EF T [1–3]. However the role of these two proteins in the mechanism of hydrolysis is not clear. They might be a part of a GTPase active center of the ribosomal structure where the GTP molecule is hydrolyzed when either EF G or complex II (aminoacyl–tRNA–EF T_u-GTP) interacts. Alternatively they might form the EF G and EF T binding sites or even, without being a part of either of these sites, might be responsible for an allosteric effect which is necessary if the ribosome is to have the right conformation. Our results, showing the Pl-50 S cores, lacking L 7 and L 12 proteins, are able to carry out substantial GTP hydrolysis in the presence of methanol and either of the elongation factors argue against the first possibility but are compatible with the other two hypotheses. Our finding that ribosomes in the presence of methanol can be active in both EF G- and EF T-dependent GTPase (table 1) certainly favours the third hypothesis and suggests that under physiological conditions proteins L 7 and L 12 induce a ribosome conformation required for activity. The presence of methanol appears to overcome, at least partially, the requirement of proteins L 7 and L 12 by inducing the required ribosomal conformation.

The results obtained using CsCl derived cores indicate as expected, that many other proteins are needed in addition to L 7 and L 12 for the ribosomes to be active in EF G-dependent GTPase, although it is interesting that these two proteins alone are sufficient to restore up to 50% of the activity of γ-cores which lack half of their structural proteins. This fact confirms the key role that L 7 and L 12 play in this activity and in addition indicates that the γ-cores GTPase center is partially active as previously reported [8]. The reconstruction of the γ-cores EF T-dependent GTPase activity is poorer than that of EF G-dependent activity.

Our results using CsCl cores seem to conflict with those of Sander et al. [3] who have reported an almost total reconstruction of "CsCl cores" GTPase activity by L 7 and L 12 proteins and with those of Sopori and Lengyel [16] who found that their β-cores

Table 1
Effect of thiostrepton on activity and reconstruction of 50 S and P1-50 S cores.

Additions		GTP hydrolyzed (pmoles/ribosome)			
First incubation	Second incubation	EF T	Stimulation (%)	EF G	Inhibition (%)
P1-50 S cores	--	0.92	--	6.2	--
P1-50 S cores + TS	--	1.91	107	0.4	96
P1-50 S cores + L 7 + L 12	--	2.30		19.1	--
P1-50 S cores + L 7 + L 12	TS	3.95	71.7	2.3	88
P1-50 S cores + TS	L 7 + L 12	3.89	69.1	1.3	93
50 S	--	2.77		30.2	--
50 S + TS	--	4.11	48.3	7.7	75

Reactions were carried out in 50 μl containing 125 mM ammonium chloride, 20 mM Tris-HCl pH 7.8, 5 mM Mg acetate, 45 pmoles of either 50 S or P1-50 S cores, 45 pmoles of 30 S and 20% (v/v) methanol. In addition 5 μM [γ-^{32}P] GTP and 2.5 μg of EF T were added to study EF T-dependent GTP hydrolysis and 50 μM [γ-^{32}P] GTP and 4.1 μg of EF G were present when EF G-dependent GTPase was studied. Thiostrepton (TS) was added, when required, at 20 μM final conc. The first incubation was carried out for 10 min at 30° without radioactive GTP. Then the mixtures were cooled at 0° and the products listed under "second incubation" were added when required. The samples were then taken to 30° for 10 min and the reaction started by the addition of radioactive GTP. Final incubation was carried out for 10 min at 30°. Extent of inhibition or stimulation by thiostrepton is calculated taking as controls the same experimental systems without addition of the antibiotic.

were strongly stimulated by L 7 and L 12 proteins. These apparently conflicting results might be explained on the basis of the different core preparations used in each case. Sander et al. [3] prepared their cores in the presence of 40 mM Mg^{2+} and under these conditions the resulting particles must be closer to our β-cores (prepared in 50 mM Mg^{2+}) than to γ-cores (prepared in 10 mM Mg^{2+}). In addition different strains of *E. coli* have been used in the various published works and this can affect the resulting cores. In fact the particles used by Sander et al. [3] derived from *E. coli* B ribosomes lack proteins L 7 and L 12 while the cores obtained from *E. coli* A 19 using similar conditions [15] have these two proteins present.

The data expressed in table 1 show that the L 7 and L 12 proteins are not necessary for the inhibitory or stimulatory action of thiostrepton and in addition demonstrate that the antibiotic does not interfere with the reconstitution of the Pl-50 S cores. These results are in agreement with previous reports indicating that thiostrepton binds to cores deficient in L 7 and L 12 proteins [4, 18]. Although L 7 and L 12 proteins and thiostrepton binding sites on the ribosomal structure appear to be closely related they do not bind on the same site(s).

Acknowledgements

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