THE INVOLVEMENT OF 50S RIBOSOMAL PROTEIN L11 IN THE EF-G DEPENDENT GTP HYDROLYSIS OF *E. COLI* RIBOSOMES

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Received 17 April 1975

1. Introduction

A number of 50S ribosomal proteins are known to be directly or indirectly involved in the EF-G dependent GTPase reaction of *E. coli* 70S ribosomes [1]. In addition to the requirement for the proteins L7 and L12, L10 is necessary for the binding of L7 and L12 to the ribosome [2,3]. Furthermore, L6 is essential for the reconstitution of the GTPase activity of CsCl cores prepared at low magnesium concentration [2].

Howard and Gordon [4] adapted a procedure [5] to remove, in addition to L7/L12, the proteins L10 and L11 selectively from the 50S ribosome. The resulting core particles were reported to be fully active in peptidyl transferase activity [6,7], but did not bind thiostrepton [8]. In the present communication we show that these cores missing L11, when supplemented with L10 and L7, are inactive in EF-G dependent GTPase, but retain the capacity to bind GMPPCP in the presence of EF-G.

The effect of thiostrepton on the GMPPCP binding of cores lacking L11 is also investigated.

2. Materials and methods

2.1. *Ribosomal particles*

Ribosomes from *E. coli* MRE 600 were isolated as described elsewhere [9] and washed two times through a 1.1 M sucrose cushion with Standard Buffer (20 mM Tris–HCl (pH 7.4), 10 mm Mg $(OAc)_2$, 60 mM NH_4Cl and 6 mM 2-mercaptoethanol) made up to 1 M NH_4Cl. Then, the ribosomes were dialysed against Standard Buffer containing 1 mM Mg $(OAc)_2$ instead of 10 mM. Next, the subunits were separated as described earlier [9], except that the buffer used in the zonal centrifugation was Standard Buffer containing 1 mM $Mg(OAc)_2$.

Ribosomal cores were prepared as described previously [4,5]. The several 50S core preparations used were defined as follows. P_0 core: 50S ribosomes extracted twice with high salt and ethanol at 0°C according to [5], P_{0-37} core: P_0 core extracted twice at 37°C; P_{37} core: 50S ribosomes extracted twice at 37°C. The proteins split off during the preparation of P_{37} cores were defined as SP_{37}.

All ribosomal subunits and cores were stored in Standard Buffer at −30°C.

2.2. *Proteins*

L7 was isolated as previously described [10]. L10 was isolated according to [11]. L11 was isolated by LiCl-urea extraction according to Hindennach et al. [11] followed by chromatographic purification according to Mora et al. [12]. All proteins gave one single spot on two dimensional polyacrylamide gels [13]. The amino acid compositions were in agreement with published values [10,12,14].

L7 was labeled via reductive methylation with 0.05% [^{3}H] formaldehyde (spec. act. 100 Ci/mol) as described by Amons and Möller [15]. The specific activity of the methylated protein was 1300 cts/min/μg, which corresponds to one methyl group per 5 molecules of L7.

EF-G was prepared essentially according to Kaziro et al. [16]. Protein was determined according to Lowry et al. [17] using crystallin bovine insulin as a standard.

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2.3. *Assay conditions*

The GTPase assay was performed as described earlier [2] with the following modifications. Preincubation was carried out for 5 min at 37°C in a 25 μl reaction mixture containing the ribosomal cores, proteins and 100 mM Tris–HCl (pH 7.4), 22 mM $Mg(OAc)_2$, 170 mM NH_4Cl and 2 mM 1,4-dithioerythritol (DTE). After the addition of 30S ribosomes and EF-G, the reaction was started with 50 nmol [γ-^{32}P]GTP (spec. act. 1 Ci/mol). The end volume was 50 μl, the salt concentrations being 55 mM Tris–HCl, 12 mM $Mg(OAc)_2$, 90 mM NH_4Cl and 1 mM DTE. The incubation was carried out for 10 min at 37°C.

The conditions for GMPPCP binding (including the preincubation step) were the same as described for the GTPase assay. After incubation in the presence of 150 pmol of [^{3}H]GMPPCP (spec. act. of 1.2 Ci/mmol) instead of the GTP, the reaction mixture was cooled to 0°C. The ribosome bound radioactivity was determined by Millipore filtration using the following washing buffer: 10 mM Tris–HCl (pH 7.4), 10 mM $Mg(OAc)_2$ and 80 mM NH_4Cl.

The reaction mixtures contained 2% glycerol from the EF-G preparation and in experiments with thiostrepton 2% DMSO. All data were corrected for the activities of 30S ribosomes alone.

2.4. *Materials*

[^{3}H]GDP, [^{3}H]GMPPCP and [γ-^{32}P]GTP were obtained from the Radiochemical Centre, Amersham, England. [^{3}H]formaldehyde was purchased from New England Nuclear and thiostrepton from Squibb.

3. Results

Two dimensional electrophoresis [13] of the core proteins showed that our P_0 cores were lacking the proteins L7, L8, L12 and L33. In our hands P_{0-37} cores and P_{37} cores were indistinguishable and lacked both the proteins L10 and L11 compared with P_0 cores. Moreover, both the P_{0-37} cores and P_{37} cores were deficient in L1 and L5 when compared with P_0 cores.

P_0 cores and P_{0-37} cores had only a slight activity in the EF-G dependent GTPase reaction (table 1). In contrast to P_0 cores, P_{0-37} cores cannot be reactivated solely by the addition of L7. Although L10 is necessary for the binding of L7 to the P_{0-37} core (see below) the addition of L10 to a mixture of P_{0-37} cores and L7 results only in a small stimulation of the GTPase reaction (table 1, lines 6 and 7). However, a striking increase in GTPase activity could be obtained by including L11 in the reconstitution mixture (table 1, line 9). Supplementation of L7, L10 and L11 with a partially purified protein fraction containing L1 and L5 had no further effect on the rate of GTP hydrolysis. The same results were obtained with P_{37} cores instead of P_{0-37} cores.

Table 1
The effect of various proteins on the EF-G dependent GTP hydrolysis of 50S ribosomal cores

Experimental conditions	% of control
1. 50S (control)	100
2. P_0	7
3. P_0 + L7	97
4. P_{0-37}	3
5. P_{0-37} + SP_{37}	97
6. P_{0-37} + L7	3
7. P_{0-37} + L7 + L10	19
8. P_{0-37} + L7 + L11	20
9. P_{0-37} + L7 + L10 + L11	60
10. P_{0-37} + L10 + L11	1

Where indicated, the following amounts of protein were incubated with 30 pmol (0.75 A_{260} units) of 50S particles: 15 μg of SP_{37}, 150 pmol (1.8 μg) of L7 and 30 pmol (0.6 μg) of L10 or L11. Further, 60 pmol (0.85 A_{260} units) of 30S ribosomes and 125 pmol (10 μg) of EF-G were present. The GTPase activity of the control was 915 mol GTP hydrolyzed/mole 50S particle.

Since, under our conditions of ionic strength, the presence of 70S couples is essential for the EF-G dependent GTPase activity [18], we investigated the role of L11 in the subunit association. From fig.1 it is clear that L11 has no influence on the degree of association of 30S subunits and 50S P_{0-37} cores supplemented with L7 and L10.

Previous studies have indicated a cluster of proteins, composed of a least L7, L12 and L10, on the 50S ribosome ([1], review). In order to find out whether L11 is required for the association of this cluster to the ribosome, we investigated the binding of [^{3}H] methylated L7 to the P_{0-37} core (table 2). Methylation of L7 has no effect on its activity [15]. As seen

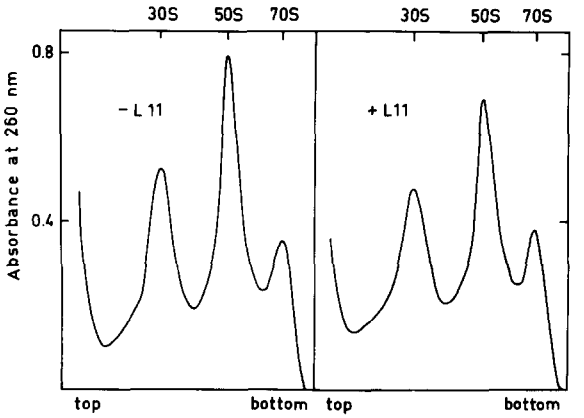

Fig.1. The influence of L11 on the association of P_{0-37} cores with 30S ribosomes. 0.12 nmol (3 A_{260} units) of P_{0-37} cores, 0.6 nmol (7.2 μg) of L7, 0.12 nmol (2.4 μg) of L10 and, where indicated, 0.12 nmol (2.4 μg) of L11 were preincubated 5 min at 37°C in a 100 μl volume. After the addition of 0.24 nmol (3.4 A_{260} units) of 30S ribosomes, the end volume was adjusted to 200 μl. The buffer compositions were the same as described for the EF-G dependent GTPase assay with the exception that 0.05 M Tris–HCl was replaced by 0.05 M Triethanolamine–HCl (pH 7.5). After incubation for 10 min at 37°C, the reaction mixtures were cooled to 0°C and 20 μl of a 2.5% aqueous solution of glutaric aldehyde was added. After incubation for 10 min at 0°C, the reaction mixtures were layered on a 15–30% linear sucrose gradient containing 0.05 M Triethanolamine–HCl (pH 7.5), 0.01 M $Mg(OAc)_2$, 0.08 M NH_4Cl and 3 mM 2-mercaptoethanol. The gradients were centrifuged at 25 000 rev/min for 18 hours at 5°C in a Spinco SW 27.1 rotor.

Table 2
The binding of L7 to 50S ribosomal cores

Experimental conditions	Moles of L7 bound per mole of core
1. P_0	2.1
2. P_{0-37}	0.1
3. P_{0-37} + L10	1.5
4. P_{0-37} + L11	0.1
5. P_{0-37} + L10 + L11	1.8

0.36 nmol (9 A_{260} units) of 50S cores, 1.8 nmol (22 μg) of [^{3}H] L7 and, where indicated, 0.36 nmol (7.2 μg) of L10 or L11 were preincubated 5 min at 37°C in a 0.3 ml volume. After the addition of 0.72 nmol (10.2 A_{260} units) of 30S subunits, the end volume was adjusted to 0.6 ml. The buffer compositions were the same as described for the EF-G dependent GTPase assay. After incubation for 10 min at 37°C, the reaction mixtures were centrifuged 4 hr at 40 000 rev/min at 10°C in a Spinco SW 50.1 rotor equipped with 0.6 ml adapters. The pellet was dissolved in a solution containing 0.02 M Tris–HCl (pH 7.4) and 0.1% SDS and counted in 10 ml of Bray's solution.

from table 2, only the addition of L10 enables L7 to bind to the P_{0-37} core. Aside from a small increase in the binding of L7 upon addition of L11, the latter protein does not seem to be necessary for the binding of L7 to the ribosome.

The activity of the EF-G dependent GMPPCP binding depends on the presence of L7 and L10 on the ribosomal core (table 3). However, there is no requirement for L11 to get maximal GMPPCP binding activity compared to the control with the SP_{37} split proteins (table 3, lines 5, 7 and 9). Despite the need for L11 in the GTP *hydrolysis*, this protein has no effect on the GMPPCP *binding* reaction. Essentially the same conclusion was drawn with respect to the binding of GDP in the presence of EF-G and sodium fusidate (results not shown).

The effect of thiostrepton on the EF-G dependent binding to GMPPCP for different combinations of cores and proteins is given in table 3. Although L11 has been reported [8] to be the thiostrepton binding protein, we found that the amount of inhibition of P_{0-37} cores supplemented with L7 and L10 is hardly influenced by L11 (table 4, lines 7 and 9).

4. Discussion

The availability of core particles lacking a few well-defined ribosomal proteins offered the possibility to examine their role in the EF-G mediated reactions concerning guanosine nucleotides. Our experiments prove that the stimulation of the GTPase activity of P_{0-37} cores by L11 is not due to a stringent requirement of this protein for the association of the subunits. Furthermore we found that L11 is not required for the L10 dependent binding of L7 to the P_{0-37} cores. However, Howard and Gordon [4] found that L11 is necessary for the physical reconstitution of L10 and L7/L12 to the P_{0-37} core. To explain the situation, the reconstitution conditions may be decisive. Howard and Gordon reconstituted

Table 3
The effect of various proteins on the binding of GMPPCP to cores in the presence of EF-G

Experimental conditions	% of control	
	– Thiostrepton	+ Thiostrepton
1. 50S (control)	100	1
2. P_0	31	1
3. P_0 + L7	86	9
4. P_{0-37}	24	10
5. P_{0-37} + SP_{37}	62	1
6. P_{0-37} + L7	29	
7. P_{0-37} + L7 + L10	61	18
8. P_{0-37} + L7 + L11	38	
9. P_{0-37} + L7 + L10 + L11	62	9
10. P_{0-37} + L10 + L11	37	

Refer to the legend of table 1 for the amounts of ribosomal particles and proteins. The control represents 8.5 pmol of [^{3}H]GMPPCP bound to the filter. Where indicated, 20 μM of thiostrepton was present. In this case a second preincubation was preformed for 5 min at 37°C before the final addition of [^{3}H]GMPPCP.

their cores with proteins fractions at 0°C in a low salt buffer, whereas our reconstitution experiments were carried out at 37°C at a much higher salt concentration. On the basis of our findings, it seems highly unlikely that the cluster of proteins L7, L12 and L10 is bound to the ribosome through L11. On the other hand our experiments cannot exclude that the stimulation of the GTP hydrolysis by L11 is expressed through the interaction of this protein with L7/L12 and L10, the latter proteins forming probably the factor binding site on the ribosome [1,19]. However, in contrast to the GTPase reaction, the mutual dependent binding of nucleotides and EF-G to the ribosome does not require L11. In analogy with other enzyme-catalyzed reactions, it would be advantageous to distinguish a site of hydrolysis and a separate site of nucleotide binding on the ribosome. We suggest that these sites may extend to more than one protein, L11 being the actual site of GTP hydrolysis but not necessarily the physical binding site of the nucleotide.

Earlier experiments performed in this laboratory also point to an important role of L11 in the GTPase reaction. Photo-affinity labeling studies of Maassen and Möller [20] using a GDP analog bound to 70S ribosomes in the presence of EF-G and fusidate showed that L11, L18 and to minor extend L5 are labeled. On irradiation of the same analog in the presence of the *B. stearothermophilus* 5S RNA–protein complex, BL10 and BL22 are labeled [21]. This 5S RNA protein complex is found to be active in GTP hydrolysis [22]. As judged from the electrophoretic behaviour, BL10 and BL22 seem similar to *E. coli* L11 and L18 [21,23]. These experiments were the first direct evidence that L11 and L18 are at or near the region where the phosphate moiety of the nucleotide is situated on the ribosome.

The simplest view to explain the situation would be that L11 catalyzes the hydrolysis of the γ-phosphate ester. This would leave L18 in conjunction with the elongation factor as a possible candidate for the nucleotide binding site on the 50S ribosome.

Interestingly, photo-affintiy labeling studies of Hsiung et al. [24,25] with a peptidyl tRNA analog bound to the ribosomal P or the A site, showed that the main reaction products are L11 and L18. These results suggest that the P and the A site are located in a region near the GTPase centre.

Our experiments with thiostrepton show that this antibiotic can still function in the absence of L11. This suggests that protein(s) other than L1, L5, L8,

L11 and L33 are the main target of thiostrepton binding. The finding that L11 is the thiostrepton binding protein [8] and the observed inhibition of this antibiotic in the absence of L11 could be reconciled on the assumption that the antibiotic has a complex functional binding site, involving several ribosomal proteins.

Acknowledgements

We thank Drs J. Maassen and L. Slobin for the critial reading of the manuscript and Mrs A. Rutten and Miss T. Dijksman for technical assistence. This investigation was supported in part by a grant from the Netherlands Organization for the Advancement of Pure Research (Z.W.O.) under auspices of the Netherlands Foundation for Chemical Research (S.O.N.).

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