ISOLATION AND CHARACTERIZATION OF TWO ACIDIC PROTEINS FROM THE 50S SUBUNIT REQUIRED FOR GTPase ACTIVITIES OF BOTH EF G AND EF T

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Received April 17, 1972

Summary: L7 and L12, two acidic proteins, were extracted from E. coli 70S ribosomes or 50S subunits by treatment with ethanol and NH_4Cl and further purified. Ability of the extracted 50S subunits to support the GTPase of EF G as well as the Phe-tRNA-dependent GTPase of EF T can be restored by the addition of L7 or L12. Moreover, CsCl-prepared 50S "cores", which lack about 7 proteins, can also be reactivated by these two proteins. In both systems, L12 is more effective than L7.

In the past few months a number of independent reports have indicated the close relationship of the 50S ribosomal sites which interact with the elongation factors G and T (T_u + T_s). These observations have been based largely on inhibition studies with the antibiotics thiostrepton, thiopeptin and siomycin (1,2) and on the mutual exclusion of EF G and EF T in binding to ribosomes (3,4). Recently, the ribosomal proteins L7 and L12 have been shown to be required for EF G-dependent GTPase (5), and Hamel and Nakamoto (6) have reported the extraction by ethanol-NH_4Cl of two ribosomal proteins (P I and P II) affecting various reactions associated with EF G and EF T (see also 7). We now report in this communication our observation that the acidic proteins L7 and L12 from the 50S subunit play a very important and common role in the ribosomal GTPase of both EF G and EF T.

MATERIALS AND METHODS

EF T, EF G and NH_4Cl-washed ribosomes from E. coli B $T2^r$ were purified as previously described (8). Ribosomal subunits were isolated by sucrose gradient centrifugation using a Beckman zonal rotor and checked for purity by

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analytical sucrose gradient centrifugation. The 30S subunits used were pure by this criterion; of the 50S subunits, preparations containing less than 5% contaminating particles were used. γ-^{32}P-GTP was prepared by the method of Glynn and Chappell (9,10) and purified by elution from DEAE cellulose in bicarbonate form with a gradient of triethylammonium bicarbonate buffer.

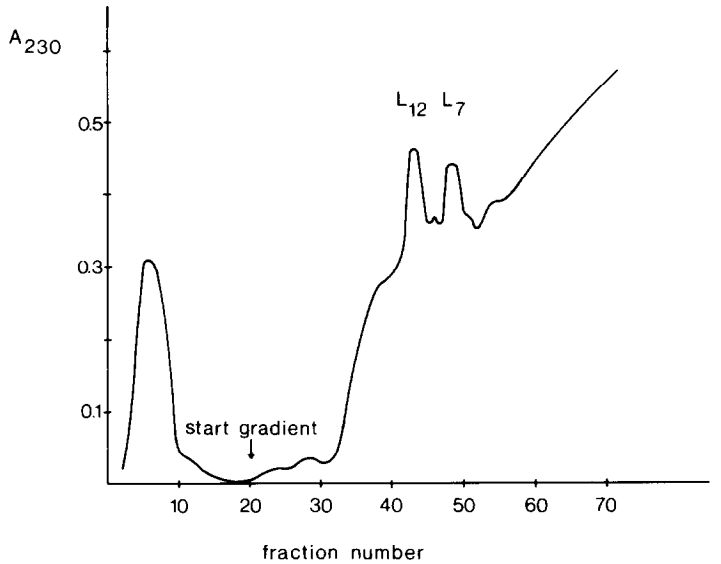

Fig. 1. DEAE Cellulose chromatography of proteins extracted from 70S ribosomes by 1 M NH_4Cl - 50% ethanol. Similar results are obtained when 50S subunits are used for extraction. A gradient of 10-150 mM NH_4-acetate (pH 5.7 - 5.0) was employed to elute L7 and L12 (11). Background absorption is due to the buffer used.

To extract the acidic proteins from 70S ribosomes or 50S subunits, a solution of 100-200 mg of ribosomes in 5-10 ml ribosome buffer (10 mM $MgCl_2$ - 60 mM KCl - 20 mM Tris-Cl, pH 7.8) was brought to a concentration of 2 M NH_4Cl, followed by addition of an equal volume of ethanol. The mixture was left on ice for 2 - 4 hrs, spun at 20,000 g for 15 min and the pellet washed once with 5-10 ml of a 1:1 mixture of ribosome buffer and ethanol. The combined supernatants were chromatographed over DEAE cellulose (Whatman DE 52) according to Möller et al.(11). The pellets were dissolved in ribosome buffer, dialyzed against the same buffer containing 50% glycerol and stored at -35°C. 50S "cores" were prepared by CsCl density-gradient centrifugation in the pres-

ence of 40 mM $MgCl_2$ as described by Meselson et al. (12). One A_{260} unit was taken to represent 25 pmoles 70S, 39 pmoles 50S or 67 pmoles 30S particles (13,14). Protein determinations were done according to Lowry et al.(15) using crystalline bovine serum albumin as standard. The molecular weight of L7 and L12 was assumed to be 12,500 (11). $tRNA^{Phe}$ was purified from tRNA purchased from Schwarz by a modification of the procedures of Gillam et al. (16) and Nishimura et al. (17).

RESULTS

EF T is known to exhibit a ribosome-dependent GTPase activity which is also dependent on aa-tRNA and is particularly observable at low GTP concentrations (10^{-6}M). As Table 1 shows, this reaction requires both ribosomal subunits and is stimulated 2-3-fold by poly U. Another ribosome-dependent GTPase is known to be exhibited by EF G and is thought to be involved in translocation.

Table 1. EF T dependent ribosomal GTPase: effect of various omissions

Experimental conditions	pmoles P_i liberated
Complete system	57.1
- poly U	22.9
- $Phe\text{-}tRNA^{Phe}$	0.51
- EF T	0.00
- 30S subunits	3.80
- 50S subunits	0.81

The complete system contained: 28 pmoles 50S subunits, a stoichiometric amount of 30S subunits and 4.2 μg EF T; for other conditions see Fig. 2. Stimulation by poly U is dependent on the 30S preparation. In several experiments an even lower activity than that shown was observed in the absence of poly U.

Extraction of 50S subunits by NH_4Cl-ethanol treatment was found to reduce their ability to stimulate the GTPase activity of both EF G and EF T to 15-25% of that shown by the native 50S (Figs.2 and 3). Addition of the NH_4Cl-ethanol extract to the reaction mixture restored the original activities in

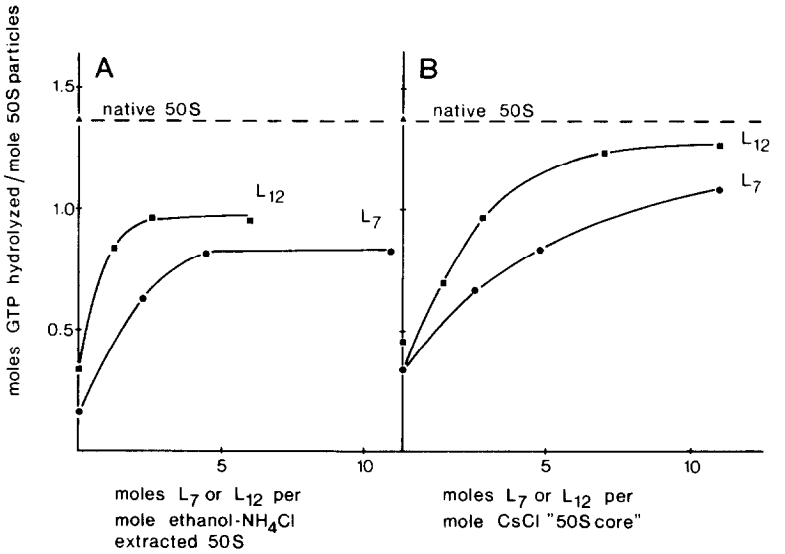

Fig. 2. EF T-dependent ribosomal GTPase: restoration by the 50S proteins L7 and L12 of the ability of (A) NH_4Cl-ethanol and (B) CsCl extracted 50S particles to stimulate the reaction. The assay system for L12 contained in a 75 µl volume: 20 mM Tris-HCl, pH 7.8 - 25 mM $MgCl_2$ - 50 mM NH_4Cl and 10% glycerol (from the ribosomes); 28 pmoles 50S subunits, 37 pmoles NH_4Cl-ethanol treated 50S particles or 33 pmoles 50S CsCl "cores" and stoichiometric amounts of untreated 30S subunits; approx. 5 µg (75 pmoles) of EF T, 0.2 A_{260} units (280 pmoles) of ^{14}C-Phe-$tRNA^{Phe}$ (purity 65%), 100-120 pmoles of γ-^{32}P-GTP (spec.act. 1000-2000 Ci/mole) and 5 µg of poly U. L12 was added to the reaction mixture immediately before incubation, preincubation in this case being unnecessary. After incubation for 2 minutes at 30°C, activity was measured as liberation of $^{32}P_i$ (22). To get better stimulation, L7 was preincubated with 30S and 50S particles for 10 min at 30° in a 40 µl reaction mixture containing 20 mM Tris-HCl, pH 7.8 - 25 mM $MgCl_2$ - 50 mM NH_4Cl; then 40 µl of a mixture containing poly U, GTP, Phe-$tRNA^2$ and EF T in 20 mM Tris-HCl, pH 7.8 - 25 mM $MgCl_2$ - 100 mM $(NH_4)_2SO_4$ was added to the reaction mixture which was then incubated for 2 min at 30°C.

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both cases. Polyacrylamide gel electrophoresis at pH 4.5 in 6 M urea (18) resolved 4 to 6 protein bands in the extract. To purify the active proteins, the extract was chromatographed on DEAE cellulose. As Fig. 1 shows, the elution pattern of 50S proteins L7 and L12 (A_1 and A_2) corresponds to that seen by Möller et al.(11); no contamination with S1 could be seen. The two proteins identified as L7 and L12 migrated as single bands in one-dimensional polyacrylamide gels. Further proof of the identity of these proteins came from bidimensional acrylamide gel electrophoresis (results not illustrated) using the method of Kaltschmidt and Wittmann (19).

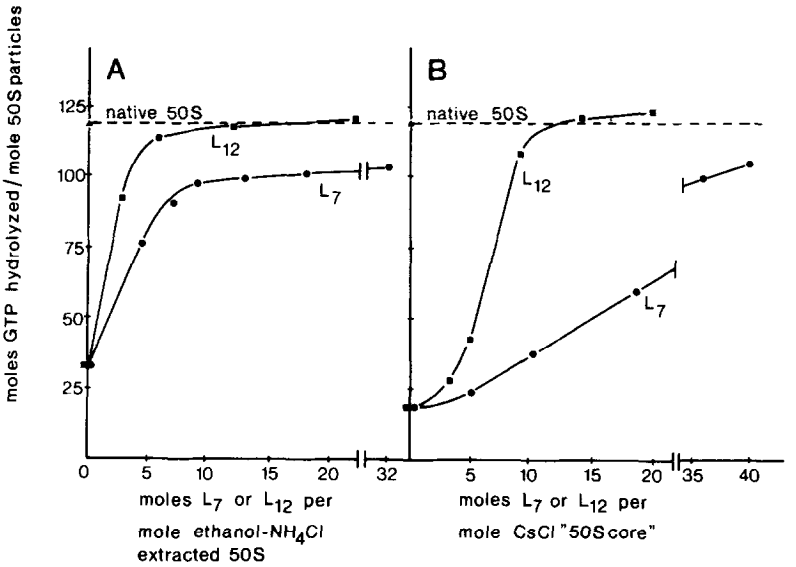

Fig. 3. EF G-dependent ribosomal GTPase: restoration by the 50S proteins L7 and L12 of the ability of (A) ethanol-NH_4Cl extracted 50S and (B) CsCl 50S "cores" to participate in the reaction. The 75 µl reaction mixture was [100 mM Tris-HCl, pH 7.8 - 14 mM $MgCl_2$ - 80 mM NH_4Cl - 14 mM 2-mercapto-ethanol (ME) - 7% glycerol (from ribosomes) - 153 µM γ-^{32}P-GTP (spec.act. 27 Ci/mole)] containing 0.5 A_{260} units 50S particles (20 pmoles), 1.5 A_{260} units 30S subunits (100 pmoles), approx. 5.0 µg EF G (60 pmoles) and up to 10 µg L7 or 5.5 µg L12. After incubation for 15 min at 30°C, the GTPase activity was measured as the amount of $^{32}P_i$ liberated (22).

Activities of L7 and L12 were assayed using either NH_4Cl-ethanol treated 50S subunits or CsCl 50S "cores", both supplemented with untreated 30S subunits. Fig. 2A shows that, in the EF T-catalyzed GTPase reaction, NH_4Cl-ethanol treated 50S subunits could be 70% reactivated by the addition of L12 alone and slightly less by L7. Fig. 2B shows an increase from about 20% to nearly full activity with CsCl 50S "cores" and saturating amounts of L7 or L12, L12 again being more effective. The GTPase of EF G was similarly affected by the absence or presence of L7 or L12 as shown in Fig. 3. It should be noted that with CsCl 50S "cores" the higher specific activity of L12 compared to that of L7 was particularly apparent. Thiostrepton and Siomycin, which are known to inhibit both the EF T- and the EF G-dependent ribosomal GTPase, were found at 10^{-5}M to inhibit both reactions in the pres-

ence of CsCl 50S "cores" and L7 or L12. Neither EF G nor EF T were complemented in their GTPase activities by L7 and L12 alone, and repeated NH_4Cl-ethanol extraction of 50S did not affect either their residual activity or their ability to be stimulated by L7 and L12.

As with the GTPase of EF G, L7 and L12 could restore the ability of NH_4Cl-ethanol extracted 50S to participate in forming the complexes [5'-guanylylmethylenediphosphonate (GMPPCP)-EF G-50S] and [fusidic acid-GTP-EF G-50S] (Table 2). L12 showed the same effect on CsCl 50S "cores"; in this case, the effect of L7 was not tested.

Table 2. Effect of L7 and L12 on the EF G-dependent binding of GTP and GMPPCP to NH_4Cl-ethanol extracted 50S and CsCl 50S "cores"

Experimental conditions	moles nucleotide bound per mole 50S particles	
	GTP (+fusidic acid)	GMPPCP
native 50S	0.45	0.48
NH_4Cl-ethanol ex.50S	0.09	0.07
NH_4Cl-ethanol ex.50S + L7		0.29
NH_4Cl-ethanol ex.50S + L12	0.43	0.41
CsCl 50S "core"	0.06	0.06
CsCl 50S "core" + L12	0.34	0.35
L12	0.00	0.00

The 65 µl reaction mixture was [60 mM Tris-HCl, pH 7.8 - 16 mM $MgCl_2$ - 80 mM NH_4Cl - 7 mM ME - 5% glycerol] containing some of the following components: 1.0 A_{260} unit 50S particles (39 pmoles), 9 µg L7 or L12 (720 pmoles) and approx. 10 µg EF G (119 pmoles). After incubation at 30°C for 10 min, either 100 nmoles fusidic acid and 150 pmoles ^{3}H-GTP (196 cpm/pmole, from NEN) or 200 pmoles ^{3}H-GMPPCP (154 cpm/pmole) in 10 µl aqueous solution was added. After further incubation at 23°C for 10 min, the reaction mixture was diluted with 2 ml of 48 mM Tris-HCl, pH 7.8 - 13 mM $MgCl_2$ - 44 mM NH_4Cl - 7 mM ME - 0.1 mM fusidic acid, if it were present during the reaction, and passed through a Millipore filter. The filter was washed one time with 2 ml of the same buffer, dried and assayed for radioactivity.

DISCUSSION

Our results show that the 50S proteins L7 and L12 play a common role in the ribosome-dependent GTPase of both EF G and EF T as well as in the binding of EF G to 50S subunits in the presence of GMPPCP or GTP and fusidic

acid. This would indicate that EF G and EF T share a common or partially overlapping 50S site which includes L7 and/or L12. Such a common site would agree with the results obtained with thiostrepton, thiopeptin and siomycin (1,2) and the competition between EF G and EF T for binding to ribosomes (3, 4). Since L7 has been found to be N-acetylated L12 (20), it would seem likely that both L7 and L12 bind to the same portion of the 50S subunit, the acetylation possibly playing an active role in the regulation of translation (11). Whether L12 and L7 are themselves a part of the active center responsible for cleavage of GTP or are only indirectly related remains to be investigated, in addition to the characterization of their optimum reassociation conditions. The possibility that extraction of L7 and L12 merely prevents 30S-50S association and thereby causes loss of activity was eliminated by our observation, in sucrose density-gradients, of 30S reassociated with either NH_4Cl-ethanol or CsCl extracted 50S. Because CsCl density-gradient centrifugation at 40 mM $MgCl_2$ is known to remove about 7 proteins from the 50S subunit (21) and L12 and L7 alone are able to restore the GTPase activities of both EF G and EF T, we conclude that, among the split proteins, L12 and L7 are the essential ones for these reactions.

Acknowledgement: We thank Dr. G. Chinali for helpful criticism and for providing, together with Mr. J. Voigt, purified ^{3}H-$tRNA^{Phe}$. We express our gratitude to Dr. F. Eckstein for synthesis of the ^{3}H-GMPPCP, to Dr. J. Modolell for providing thiostrepton and siomycin, and to Drs. D. Vazquez and J. Modolell, as well as to Dr. D. Richter, for sending us their manuscripts prior to publication. Fusidic acid was a gift from the Leo Pharmaceutical Co., Ballerup, Denmark. Excellent technical assistance was provided by Mrs. B. Klingbeil, Mrs. B. Foellmer and Miss R. Bonewald.
This work was supported by SFB 75 of the Deutsche Forschungsgemeinschaft.

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