ACTIVITY OF PROTEIN-DEFICIENT 30S RIBOSOMAL SUBUNITS IN ELONGATION FACTOR G-DEPENDENT GTPASE

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SUMMARY. E. coli 30S ribosomal subunits reconstituted from RNA and mixtures of purified ribosomal proteins lacking a single protein were examined for their activities in polyphenylalanine synthesis and GTP hydrolysis. Similar experiments were performed with a partial reconstitution system (core particles plus split proteins). Only six 30S ribosomal proteins--S4, S7, S8, S9, S11, and S15--were required for GTPase activity. Several others, including S5, which were required for polyphenylalanine synthesis, were dispensable in the GTPase reaction.

INTRODUCTION. The elongation factor G (EF-G)-dependent GTPase reaction, involved in the translocation step of protein synthesis, is catalyzed by 50S subunits alone, but the activity of 50S subunits is greatly stimulated by the addition of 30S subunits (1, 2). Using the total reconstitution system of Traub and Nomura (3), we have examined the ability of 30S subunits reconstituted in the absence of individual proteins to participate in this reaction. The results, reported here, indicate that although most of the 30S proteins are required for polypeptide synthesis, only six--S4, S7, S8, S9, S11, and S15--are required for the GTPase reaction. Recently, Marsh and Parmeggiani (2) conducted a similar study using partial reconstitution from CsCl core particles and split proteins (4, 5) and concluded that both S5 and S9 were required for GTPase activity. Because we had found that S5 was dispensable in this reaction, we also conducted experiments with the partial reconstitution system, and the results confirmed our original conclusion.

MATERIALS AND METHODS. Ribosomal subunits, 30S cores, and 30S proteins were prepared as described previously (6). γ-^{32}P-GTP was prepared by the method described by Penefsky (7) for the synthesis of γ-^{32}P-ATP. Partially purified G-factor was prepared by the method of Gordon et al. (8).

Total reconstitution of 30S subunits from 16S RNA and proteins was performed as described previously (6). The reconstitution mixtures, containing two A_{260} equivalents of proteins for each A_{260} unit of RNA, were incubated at 42° for 1 hour in 0.03 M Tris-HCl, pH 7.4 at 24°, 20 mM $MgCl_2$, 0.33 M KCl, and 6 mM mercaptoethanol. For split-core reconstitution split proteins were incubated with core particles in the same buffer at 42° for 45 min.

Assays were performed directly, without isolating the reconstituted particles. The assay for poly U-dependent polyphenylalanine synthesis has been described previously (6). The GTPase assay was a slight variation of the one used by Marsh and Parmeggiani (2). 0.25 A_{260} units of reconstituted particles were diluted to a volume of 75 µl, containing 0.52 A_{260} units of 50S subunits, 5 µg of EF-G, and 5 nmoles of γ-^{32}P-GTP in 50 mM Tris-HCl, pH 7.8 at 20°, 20 mM $MgCl_2$, 110 mM KCl, 40 mM NH_4Cl, and 6 mM mercaptoethanol. After incubation for 15 min at 30°, the reaction was stopped by the addition of 25 µl of 2 M $HClO_4$. Five µl of 20 mM KH_2PO_4, 0.3 ml of 0.02 M sodium molybdate, and 0.5 ml of isopropyl acetate were added to each tube. The tubes were shaken and centrifuged briefly, and 0.2 ml of the organic phase was withdrawn and placed in scintillation vials for counting.

Two-dimensional polyacrylamide gel electrophoresis was performed by the method of Kaltschmidt and Wittmann (9).

RESULTS.

Total Reconstitution. 30S particles reconstituted in the absence of single proteins were assayed both for poly U-dependent polyphenylalanine synthesis and EF-G-dependent GTPase, and the results are presented in Table 1. Most of the particles showed nearly full GTPase activity. Particles reconstituted in the absence of S1, S6, S13, or S20 showed normal GTPase activity as well as normal or only slightly reduced polyphenylalanine synthesis. When S2, S3, S5, S10, S12, S14, S18, or S19 was omitted, the resultant particles showed greatly reduced levels of polyphenylalanine synthesis but

Table 1. Activities of 30S particles reconstituted from 16S RNA and proteins in polyphenylalanine synthesis and EF-G-dependent GTPase.

Omitted Protein	Percent Polyphe	Percent GTPase	Omitted Protein	Percent Polyphe	Percent GTPase
S1	92±10	93± 5	S12	22± 5	106± 2
S2	34± 5	87± 5	S13	66± 6	97±14
S3	4± 4	88±12	S14	10± 7	108± 4
S4	4± 4	10± 2	S15	32±18	11± 5
S5	23± 4	98± 6	S16	82±15	75±13
S6	95± 4	120± 8	S17	20± 3	67± 4
S7	21± 4	45± 5	S18	32±13	109±13
S8	4± 4	0± 0	S19	22± 9	118±17
S9	26± 3	45± 8	S20	68± 2	80±11
S10	6± 3	94± 4	S21	31± 5	72± 4
S11	1± 1	35±11			

Legend to Table 1.

Reconstitution and assays were performed as described in Materials and Methods, except that -S16 particles were reconstituted for 2 hours instead of 1 hour. Each reconstitution mixture contained all 21 30S proteins except the one indicated. The results are expressed as percent activity relative to control reconstituted particles containing all 21 proteins. In a typical experiment, 0.5 A_{260} units of control particles polymerized 70 pmoles of phenylalanine in 30 min at 37°, as compared to 77 pmoles for 30S subunits; 50S subunits alone hydrolyzed 31 pmoles of GTP in 15 min at 30°; this blank was subtracted from each sample. With this subtraction 0.25 A_{260} units of control particles hydrolyzed 274 pmoles of GTP in 15 min at 30°, as compared to 260 pmoles for 30S subunits. The values shown are averages of two, or in some cases three, experiments with different preparations of reconstituted particles.

normal activity in GTP hydrolysis. When S16 was omitted, full restoration of both polyphenylalanine synthesis and GTP hydrolysis was obtained if the reconstitution was allowed to proceed for more than two hours, in agreement with previous results (W. Held and M. Nomura, unpublished experiments; see ref. 10). Particles reconstituted in the absence of S17 or S21 showed slight reductions in GTPase activity. Only six proteins were definitely required for the GTPase reaction--S4, S7, S8, S9, S11, and S15.

Partial Reconstitution. The protein composition of the core particles and split protein fraction was analyzed by two-dimensional polyacrylamide gel electrophoresis. We found that S1, S2, S3, S5, S9, S10, and S14 were completely removed by CsCl treatment, and no other proteins were substantially removed, in agreement with results reported previously (11, 12).

Table 2. Activities of 30S particles reconstituted from cores and split proteins in polyphenylalanine synthesis and EF-G-dependent GTPase.

Omitted Protein	Percent Polyphe	Percent GTPase
S1	76 ± 18	76 ± 13
S2	32 ± 1	102 ± 11
S3	1 ± 0	157 ± 3
S5	28 ± 5	151 ± 29
S9	27 ± 13	84 ± 10
S10	4 ± 2	164 ± 3
S14	1 ± 0	176 ± 6
Cores	0 ± 0	6 ± 5

Legend to Table 2.

Reconstitution and assays were performed as described in Materials and Methods. Each reconstitution mixture contained all seven split proteins except the one indicated. The results were calculated as in Table 1, and the levels of activity were similar to those noted there. The values shown are averages of three experiments with different preparations of reconstituted particles.

The results of single-protein omission experiments are shown in Table 2. The various particles had activities in polyphenylalanine synthesis nearly identical to the analogous particles produced by total reconstitution (Table 1), with the exception of the particle reconstituted in the absence of S1, which, here and in other experiments, showed reduced activity. The significance of this reduction is not clear, since the omission of S1 in the total reconstitution produced particles which showed normal activity in both assays.[1]

In the GTPase assay, the results were somewhat different than those obtained with total reconstitution. The omission of S1 or S9 produced slight reductions in activity, whereas a strong requirement for S9 was seen with total reconstitution. The omission of S2 had no effect on the GTPase activity of the reconstituted particles, while particles reconstituted in the

[1] With regard to S9, the original studies using the partial reconstitution system found that S9 was dispensable for polypeptide synthesis (12). Subsequent experiments using total reconstitution have produced quite variable results. The reason for this variability is not clear (W. Held, personal communication).

absence of S3, S5, S10, or S14, which displayed close to 100% activity in the total reconstitution experiment, here showed greater activity than control particles containing all seven split proteins. In a separate experiment not shown, it was found that the percent activity of the defective particles compared to the control is approximately independent of the molar ratio of split proteins to 30S cores in the reconstitution mixture, and hence the GTPase activities greater than 100% cannot readily be explained as artifacts due to the presence of excess proteins. The reason for the discrepancies between the results obtained by these two methods is unknown.

We also examined the GTPase activities of core particles to which various amounts of single split proteins were added. The results, not shown here, were nearly identical to those reported by Marsh and Parmeggiani (2). S2, S5, or S9 added singly to core particles in sufficient excess partially restored GTPase activity, and S5 and S9 added together in stoichiometric amounts produced particles having greater activity than native 30S subunits.

DISCUSSION. The total reconstitution experiments demonstrate that most 30S proteins can be omitted from reconstitution mixtures without substantially altering the GTPase activity of the resultant particles. Of particular interest are the particles reconstituted in the absence of S2, S3, S5, S10, S12, S14, S18, or S19, which showed greatly reduced levels of polyphenylalanine synthesis but normal levels of GTPase activity. These results strongly indicate that these eight proteins are required not for GTPase activity but for other functions of the 30S subunit. The partial reconstitution experiments support this conclusion with respect to S2, S3, S5, S10, and S14. The reason for the discrepancy between these results and those of Marsh and Parmeggiani (2), who found that S5 was required for the GTPase reaction, is not apparent. Our experiments showed the same type of behavior for S5 as both we and they found for S2, that is, the addition of S5 alone to cores stimulates GTPase activity while its omission from 30S particles produces no loss of activity.

This peculiar behavior of S2 and S5 could be explained in various ways. It has been shown that the ability of 30S particles to stimulate the GTPase activity of 50S subunits correlates with their ability to associate with 50S subunits to form 70S complexes (2, 13). The stimulation may be due to an overall conformational change of the 50S subunits involving direct interactions of several 30S proteins at the intersubunit interface rather than a few interactions of one or two proteins. In this case S2, or S5, would be one of several proteins directly involved in the association with 50S subunits, so that the single omission of either would still leave sufficient interactions with 50S subunits to produce the required conformational change. However, the removal of all the split proteins would considerably weaken the interaction between subunits, so that the addition of S2 or S5 alone to core particles would significantly stimulate the association. Alternatively, S2, or S5, might not interact directly with 50S subunits but might be one of several proteins whose combined presence cooperatively induces the correct conformation of another 30S protein (or 16S RNA) which is directly involved in such interaction.

The total reconstitution system showed a requirement for six proteins—S4, S7, S8, S9, S11, and S15--and some loss of activity when S9 was omitted was also seen with partial reconstitution. Four of these proteins--S4, S7, S8, and S9--are required for the physical assembly of 30S subunits in that their omission from reconstitution mixtures leads to particles sedimenting much more slowly than native 30S subunits (11, 14). The observation that S9 stimulates the GTPase activity of core particles suggests that for this protein the loss of activity may be related to a specific functional impairment. But the absence of GTPase activity when S4, S7, or S8 was omitted could be due simply to faulty assembly, and we can draw no conclusion from these experiments as to a possible involvement of these proteins in the GTPase function. Particles reconstituted in the absence of S11 and S15, on the other hand, have sedimentation coefficients close to

30S (11, 14, and W. Held, S. Mizushima, and M. Nomura, unpublished experiments) and neither S11 nor S15 is strongly required for the heat-dependent conformational rearrangement of the intermediate "RI particle" during reconstitution (15). Hence it is likely that these proteins play some role in the GTPase function. They may be involved directly in the GTPase reaction or they could act indirectly by affecting other proteins and/or ribosomal RNA which directly participate in this function.

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