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Coordinate Inhibition of Elongation Factor G Function and Ribosomal Subunit Association by Antibodies to Several Ribosomal Proteins*

(*E. coli*/GTP hydrolysis/translocation)

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ABSTRACT **We previously showed that treatment of *Escherichia coli* ribosomes with antibodies specific for proteins L7 and L12 inhibits both EF-G·GDP binding and ribosome-dependent GTP hydrolysis (Highland *et al.* (1973) *Proc. Nat. Acad. Sci. USA 70*, 142–150; and Kischa *et al.* (1971) *Nature New Biol. 233*, 62–63). We now report that antibodies to six additional proteins also inhibit GTP hydrolysis, but do not inhibit EF-G·GDP binding. Moreover, inhibition by these antibodies is dependent on the state of association of the treated ribosomes. When 70S couples are treated, only antibodies to proteins L14 and L23 are inhibitory and then only partially. However, when separated ribosomal subunits are treated individually and then mixed with the complementary untreated subunits, inhibition by antibodies L14 and L23 is complete, and antibodies to proteins L19, L27, S9, and S11 now show an inhibitory effect. In addition, treatment of subunits with any of these six antibodies (but not those to L7 or L12) results in inhibition of reassociation, which is presumably responsible for the inhibition of hydrolysis. These data suggest that the area of interaction between EF-G and the ribosome is restricted to proteins L7 and L12, and that antibodies to proteins L14, L19, L23, L27, S9, and S11, but not L7 and L12, block the physical association of the subunits.**

During protein synthesis elongation factor G (EF-G) promotes the translocation of peptidyl-tRNA from the acceptor site to the donor site on the ribosome (4, 5). Concomitant with this translocation is the expulsion of deacylated tRNA from the donor site, coordinate movement of the mRNA along the ribosome, and hydrolysis of GTP. Although they are mechanistically describable, little is known about the actual nature of the interactions that occur between EF-G and the ribosome, and because of the complexity of the protein-synthesizing system these interactions are difficult to study directly. However, two partial reactions involving EF-G and the ribosome offer the possibility for such study. One of these reactions involves the hydrolysis of GTP to GDP and P_i, dependent on both ribosomes and EF-G (6) and the other the binding of EF-G and GDP to the ribosome in a complex stabilized by fusidic acid (7–9).

Previously, we reported that antibodies to proteins L7 and L12 inhibited GTPase activity of EF-G (3) and that only these two antibodies inhibited ribosome·EF-G·GDP complex formation when 70S ribosomes were treated with antibodies (2). Moreover, exposure of ribosomes to high salt and methanol caused a selective loss of proteins L7 and L12 and a concomitant loss of EF-G activity, and the rebinding of these proteins restored the lost activity (10, 11). More recently, it has been suggested that proteins S5 and S9 (12) and L6 and L10 (13) also play a role in EF-G activity.

In order to more fully examine the question of which ribosomal proteins are involved in EF-G function, we treated 70S ribosomes and ribosomal subunits with antibodies to individual ribosomal proteins and examined the effects on subsequent EF-G binding and hydrolysis of GTP.

MATERIALS AND METHODS

Preparation of Materials. The preparation of antisera against individual ribosomal proteins, and the purification and characterization of IgGs and their corresponding Fab fragments from these sera, were as described (14, 15)**. *Escherichia coli* EF-G, ribosomes, and ribosomal subunits were prepared as previously described (16, 17). In all cases 30S subunits were heat-activated (18) for 1 hr at 37° in a solution that contained 20 mM Tris·HCl (pH 7.4), 10 mM $MgCl_2$, 60 mM NH_4Cl, and 1 mM dithiothreitol. [^{3}H]GTP (specific activity 12.0 Ci/mmol) and [γ-^{32}P]GTP (specific activity 10 Ci/mmol) were obtained from the Radiochemical Centre, Amersham. Fusidic acid was a generous gift of either Ms. Barbara Stearns of E. R. Squibb or Dr. W. Godtfredsen of Leo Pharmaceutical Products.

Reaction of Antibodies with Ribosomes. The reaction of antibodies with 70S ribosomes, as described previously (2), was for 3 min at 0° in a solution (final volume, 20 μl) that contained 10 mM Tris·HCl (pH 7.4), 10 mM magnesium acetate, 60 mM NH_4Cl, 1 mM dithiothreitol, 11 pmol of ribosomes, and the indicated amount of IgG or Fab. The reaction with 30S or 50S subunits was identical except that either 9 pmol of 30S ribosomes or 9 pmol of 50S ribosomes were used.

Formation of the 50S Ribosome·EF-G·GDP Complex. Complex formation was as previously described with 70S couples (2) except that 50S subunits (4.4 pmol) were used.

Hydrolysis of GTP Dependent on EF-G and Ribosomes. Ribosomes or ribosomal subunits (4.4 pmol) in buffer with or

Abbreviation: EF-G, elongation factor G.

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TABLE 1. *The effect of antibody binding on the ribosome·G-factor-dependent hydrolysis of GTP*

Ribosomal protein specificity of the antibody preparation	GTP hydrolysis with treated 70S ribosomes (% of control)	GTP hydrolysis with treated 50S subunit (% of control)
L7 or L12*	2	2
L19	110	48
L27	102	33
L14	71	14
L23	67	15
L1–L6, L8–L11, L13, L15–L18, L20–L22, L24, L25, L28–L30, L33	98–148	98–171

The reaction conditions were as described in *Materials and Methods*. IgG (0.15 A_{280} units) was present in the initial reaction with ribosomes, yielding an IgG/ribosome molar ratio of 180. The amount of hydrolysis observed in the absence of IgG (310 pmol) is defined as 100%.

* These two proteins are immunologically identical. See *Discussion*.

without antibody were added to a reaction mixture whose final volume was 50 μl and which contained 50 mM Tris·HCl (pH 7.4), 10 mM $MgCl_2$, 160 mM NH_4Cl, 1 mM dithiothreitol, and 2.4 units of EF-G (16). The reaction mixture was incubated 10 min at 37° and 1.0 nmol of [γ-^{32}P]GTP (approximately 30,000 cpm) was added. Incubation was continued for another 10 min and hydrolysis of [γ-^{32}P]GTP was determined as described (19).

Analysis of Subunit Association. 30S or 50S subunits were first reacted with an appropriate Fab as described above. Nine pmoles of the complementary subunit were then added followed by a glutaraldehyde-containing solution which produced a final volume of 100 μl and contained: 10 mM Tris·HCl (pH 7.4), 10 mM $MgCl_2$, 50 mM NH_4Cl, 1 mM dithiothreitol, and 2.5% glutaraldehyde. This mixture was then incubated for 10 min at 0° in order to fix ribosomal couples so as to prevent their dissociation during sedimentation (20). Aliquots (90 μl) of such reaction mixtures were layered on 5–28.8% isokinetic sucrose gradients, 10 mM Tris·HCl (pH 7.4), 10 mM $MgCl_2$, 50 mM NH_4Cl, 1 mM dithiothreitol, and were centrifuged for 60 min at 56,000 rpm and 4° in an SW56 rotor. The gradients were then collected and analyzed as described by Noll *et al.* (21). Control experiments were also carried out in which 30S or 50S subunits separately were fixed with glutaraldehyde and the resulting products analyzed. Some aggregation occurred, but the sedimentation of these products did not interfere with the analysis of couple formation.

TABLE 2. *The effect of antibody binding on the ribosome·G-factor-dependent hydrolysis of GTP*

Ribosomal protein specificity of the antibody preparation	GTP hydrolysis with treated 70S ribosomes (% of control)	GTP hydrolysis with treated 30S subunit (% of control)
S9	100	15
S11	100	2
S1–S8, S10 S12–S16, S18–S21	100–165	106–161

The reaction conditions were as described in Table 1.

TABLE 3. *The effect of antibody binding on subunit association and GTP hydrolysis*

Ribosomal protein specificity of the antibody preparation	70S ribosomes formed (% of control)	GTP hydrolysis (% of control)
None	100	100
NIS*	111	106
L15	107	102
S5	100	110
L14	34	25
L19	12	<1
L23	21	10
L27	40	34
S9	42	30
S11	1	3
L7	102	<1

The reaction conditions for measurement of GTP hydrolysis were as described in Table 1 except Fabs (Fab/ribosome ratio equal to 250) were used. Analysis of subunit association was as described in *Materials and Methods*. The 30S subunit caused no detectable GTP hydrolysis with EF-G. Hydrolysis with the 50S subunit was stimulated 10 to 20-fold by the addition of the 30S subunit and all of the values reported here reflect the effect of antibodies on this stimulation. The 100% values were obtained under identical experimental conditions but without Fab. 100% association equalled 5.4 pmol 70S and 100% GTP hydrolysis was 220 pmol.

* Nonimmune serum.

RESULTS

Effect of Antibodies on GTP Hydrolysis. We first treated 70S ribosomes with antibodies prepared against proteins of the 50S subunit and measured their subsequent ability to hydrolyze GTP in the presence of EF-G. The results (Table 1) indicate that in addition to antibodies to proteins L7 and L12, antibodies to proteins L14 and L23 inhibited GTP hydrolysis. However, this inhibition was only partial. We reasoned that the access of the antibody to its antigenic determinant might be limited by the association of the ribosomal subunits, and inhibition might be more complete if 50S subunits were treated with antibody prior to the addition of 30S subunits. The results of such an experiment are also shown in Table 1. The inhibition by antibodies to proteins L14 and L23 was now essentially complete and, in addition, antibodies to proteins L19 and L27 were now inhibitory. Clearly, subunit separation increased sensitivity to these antibodies.

In analogous experiments both 70S couples and 30S subunits were treated with antibodies specific for 30S proteins. As Table 2 shows, only antibodies to proteins S9 and S11 inhibited GTP hydrolysis, and only when 30S subunits were treated. In addition, tests were run in which the molar ratio of antibody to ribosomes was varied. It can be seen (Fig. 1) that with all the inhibitory antibodies, but not with the control, essentially complete inhibition was achieved.

TABLE 4. *The effect of antibody binding on the formation of the 50S ribosome·EF-G·[³H]GDP complex*

Ribosomal protein specificity of the antibody preparation	[³H] GDP binding (% of control)
L14, L19, L23, L27	106–109
L7 or L12	4
L1–L6, L8–L11, L13 L15–L18, L20–L22, L24, L25, L28–L30, L33	98–121

The reaction conditions were as described in the *text*. For reactions with proteins L7, L12, L14, L19, L23, and L27, Fabs (Fab/ribosome ratio equal to 350) were used. In all other cases, IgGs (IgG/ribosome ratio equal to 180) were employed. Under the conditions used, the binding of [³H]GDP to Millipore filters was limited by ribosomes; in the absence of antibody, about 1 pmol was bound.

Effect of Antibodies on Subunit Association. The greater effect of antibodies (other than those to proteins L7 and L12) on separated subunits suggested that they acted indirectly by preventing couple formation. Hence, 50S subunits were treated with antibodies L14, L19, L23, and L27, and 30S subunits were treated with antibodies S9 and S11. The ability of these treated subunits to associate with their complementary untreated subunit and to participate in GTP hydrolysis was then examined. The degree of association was analyzed on sucrose gradients after glutaraldehyde fixation in order to minimize the dissociating effects of centrifugation.

As Table 3 shows, those antibodies whose inhibition of GTP hydrolysis is manifest or increased by subunit dissociation (L14, L19, L23, L27, S9, and S11) prevented subunit association. The control antibodies (nonimmune serum and antibodies to L15 and S5) prevented neither subunit association nor GTP hydrolysis. Antibody to protein L7, which completely inhibits GTP hydrolysis, did not inhibit subunit association, suggesting that this protein is not at the subunit interface.

Effect of Antibodies on EF-G·GDP Binding to the 50S Subunit. Since subunit dissociation increased inhibition of GTP hydrolysis by antibodies to proteins L14, L23, L19, and L27, it seemed possible that inhibition of EF-G binding might also be observed if 50S subunits were treated with these antibodies instead of the 70S couples tested previously. As with the treatment of 70S ribosomes (2), only antibodies to proteins L7 and L12 inhibited the formation of the 50S·EF-G·GDP complex (Table 4). This result reinforces our previous suggestion that the area of interaction between EF-G and the ribosome is restricted to proteins L7 and L12.

DISCUSSION

The hydrolysis of GTP by the 50S subunit and EF-G is stimulated by the 30S subunit but is not dependent on it (6, 22). Neither the cause nor the nature of this stimulation is presently known. To gain insight into these areas we have examined the effect of antibodies, prepared against individual ribosomal proteins, on the physical interaction of the subunits and also on the hydrolysis of GTP dependent on EF-G and both ribosomal subunits.

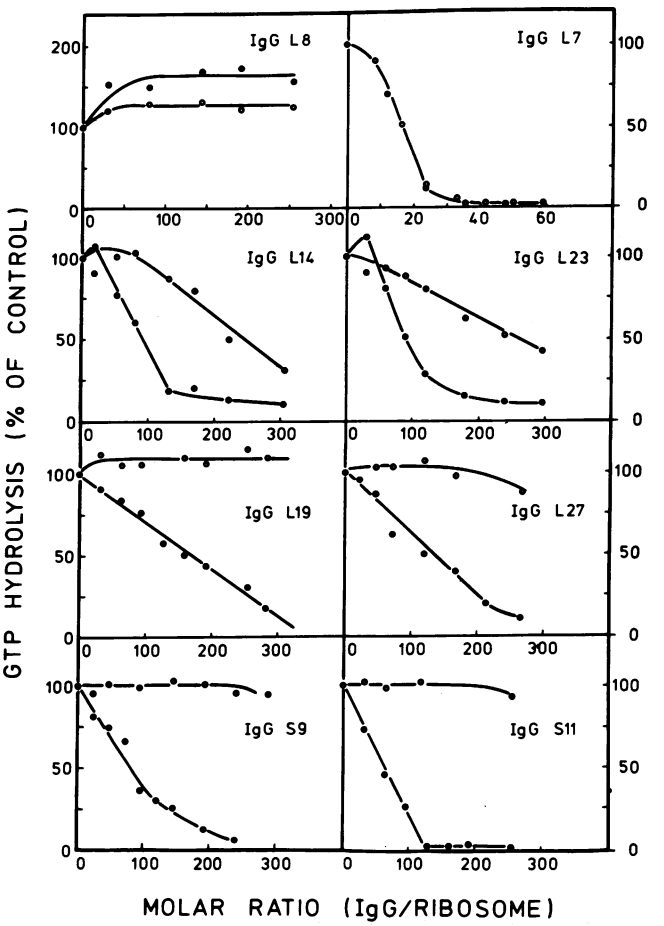

FIG. 1. Concentration dependence of the effect of IgG on the hydrolysis of GTP by ribosomes and EF-G. The reaction conditions were as described in the *text*. O——O indicates treatment of 70S couples, ●——● indicates treatment of the appropriate subunit.

The data summarized in Tables 1 and 2 and Fig. 1 indicate that two classes of antibodies inhibit this GTP hydrolysis. The first is limited to antibody produced against either protein L7 or L12. [These two proteins differ only by the acetylation of one amino acid (23, 24)†† and are immunologically identical (15).] The inhibition caused by this antibody is unaffected by the state of association of the treated ribosomes. Moreover, of the 50 individual antibodies tested, only this antibody, when bound, blocks the subsequent binding of EF-G (ref. 2; Table 4).

The inhibition of GTP hydrolysis by the second class of antibodies, those to proteins L14, L19, L23, L27, S9, and S11, differs from that of the first in two respects. First, none of these antibodies inhibits EF-G binding either to 70S couples (2) or to 50S subunits (Table 4). Second, the extent of inhibition of GTP hydrolysis is dependent on the state of association of the ribosomes during treatment. When individual subunits are treated all antibodies in this group inhibit GTP hydrolysis, but when 70S couples are treated only antibodies to proteins L14 and L23 are inhibitory and then only partially (Tables 1, 2, and Fig. 1).

On the basis of arguments lodged earlier (2) and the results reported here (Table 4), it is suggested that only proteins L7

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and L12 comprise the physical region of contact between EF-G and the ribosome during GTP hydrolysis, and so only antibodies to these proteins directly interfere with GTP hydrolysis and EF-G binding. It seemed likely, therefore, that the inhibition of GTP hydrolysis by the second class of antibodies might result from an indirect effect, namely the inhibition of subunit interaction. Indeed, antibodies to proteins L14, L19, L23, L27, S9, and S11 did inhibit subunit association, while antibody to protein L7 did not. In addition, two randomly selected antibodies that inhibited neither hydrolysis nor binding were without effect on subunit association. A complete analysis of the effect of antibodies on subunit association is currently under way‡‡. Although we cannot rule out the possibility that the observations result from allosteric alterations of subunit conformation, alterations which perhaps fortuitously prevent both GTP hydrolysis and subunit association, this seems quite unlikely.

As much as the present data indicate that proteins L14, L19, L23, L27, S9, and S11 are at or near the subunit interface and perhaps directly involved in 70S couple formation, they equally indicate that proteins L7, L12, and S5 are not. This is of interest in light of a recent report (12) implicating protein S9 as well as S5 in both GTP hydrolysis and couple formation. In work with 30S cores that lacked proteins S5 and S9 as well as other proteins, it was found that the re-addition of both S5 and S9 was necessary and sufficient to restore both GTP hydrolysis and couple formation. In light of the present evidence, that antibody to protein S5 inhibits neither GTP hydrolysis nor couple formation, the results of Marsh and Parmeggiani (12) may indicate that S5 is required for the binding of S9 to the 30S core and is not directly involved in couple formation of GTP hydrolysis.

Similar experiments involving partial reconstitution of ribosomal subunits, reported by Schrier *et al.* (13), suggest a possible role for proteins L6 and L10 in GTP hydrolysis. It seems possible that a situation analogous to that of S5 exists and that these proteins are not directly involved in GTP hydrolysis, but rather are required for the binding of L7 and L12 to 50S cores. Indeed, experiments to be reported elsewhere§§ support this suggestion.

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