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Function of Individual 30S Subunit Proteins of *Escherichia coli*. Effect of Specific Immunoglobulin Fragments (Fab) on Activities of Ribosomal Decoding Sites

(polyphenylalanine synthesis/streptomycin fixation/IF-fMet-tRNA/EF-Tu-Phe-tRNA)

J. C. LELONG*, D. GROS*, F. GROS*, A. BOLLEN†, R. MASCHLER‡, AND G. STÖFFLER‡

* Département de Biologie Moleculaire, Institut Pasteur, 25, rue du Dr. Roux, 75015 Paris, France; † Laboratoire de Génétique, Université Libre de Bruxelles, Rue des chevaux, Rhode Saint-Génèse, Belgium; and ‡ Max-Planck Institut für Molekulare Genetik, D-100 Berlin 33 (Dahlem), Ihnestrasse 63-73, Germany

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ABSTRACT **Specific anti-30S protein immunoglobulin G fragments (Fab) were used to determine the contribution of each of the 30S ribosomal proteins to: (*1*) polyphenylalanine synthesis, (*2*) initiation factor-dependent binding of fMet-tRNA, (*3*) T-factor-dependent binding of phenylalanyl-tRNA, and (*4*) fixation of radioactive dihydrostreptomycin. Twenty of the 21 possible antibodies (antibody against S17 excepted) were used. In conditions where all the 30S proteins were accessible to Fabs, all of these monovalent antibodies strongly inhibited polyphenylalanine synthesis *in vitro*. Antibodies against S4, S6, S7, S12, S15, and S16, however, showed a weaker effect.**

30S proteins can be classified into four categories by their contributions to the function of sites "A" and "P": class I appears nonessential for tRNA positioning at either site (S4, S7, S15, and S16); class II includes proteins whose role in initiation is critical (S2, S5, S6, S12, and S13); class III (S8, S9, S11, and S18) corresponds to proteins whose blockade prevents internal (elongation factor Tu-dependent) positioning; and class IV includes entities that are essential for activities of both "A" and "P" sites (S1, S3, S10, S14, S19, S20, and S21). Dihydrostreptomycin fixation to the 30S or 70S ribosomes was inhibited by antibodies against S1, S10, S11, S18, S19, S20, and S21, but only weakly by the anti-S12 (Str A protein) Fab.

The significance of these results is discussed in relation to 30S protein function, heterogeneity, and topography.

Attempts have been made to identify the individual functions in protein synthesis of some of the ribosomal proteins. Reconstitution of 30S subunits with mixtures of protein lacking individual components (1) or addition of individual components to intact 30S subunits (2) have provided preliminary functional data for some of the 30S proteins. We describe another approach to this problem. Fab immunoglobulin fragments derived from specific antisera raised against individual ribosomal proteins (3–5) are used to inactivate 30S subunit function in several specific steps of protein synthesis. In particular, our data permit a tentative identification of those 30S proteins that constitute or are close to the sites for initiation factor (IF)–fMet-tRNA or elongation factor Tu (EF-Tu)–Phe-tRNA complexes and streptomycin fixation. We find that there are a relatively large number of proteins involved in these functions. Some appear to play a specific role while others have common functions in the various steps.

Abbreviations: Fab, monovalent immunoglobulin F fragment; IF, initiation factor; EF, elongation factor.

MATERIALS AND METHODS

Immunoglobulins were prepared from specific antisera by precipitation with $(NH_4)_2SO_4$. The redissolved precipitate was applied to a Sephadex G-150 column to separate the 7S immunoglobulin Gs. In some preparations, a further purification by DEAE–cellulose column chromatography was performed (5). Fab was prepared as described (6). Only the Fab I fraction was used. A more detailed description of these methods is given elsewhere (5). The precipitation titers (3) of the immunoglobulin Gs used for different Fab preparations estimated with isolated ribosomal proteins were grossly comparable. *In vitro* assays for ribosomal activity are described in the legend of Fig. 1.

RESULTS

Effect of specific Fabs on poly(U)-dependent phenylalanine incorporation

In Fig. 1, the percent inhibition observed in the purified ribosomal system is plotted against the relative concentrations of Fab used. Twenty of the 21 possible Fabs were compared; the exception was the antibody directed against S17. Most of the Fabs tested strongly inhibit polyphenylalanine synthesis, with first-order kinetics. This is particularly true for antibodies against S1, S2, S3, S5, S8, S9, S10, S11, S12, S13, S14, S18, S19, S20, and S21. With these antibodies, inhibition of polyphenylalanine synthesis was near completion at a ratio corresponding to 0.3 A_{280} Fab unit per 0.3 A_{260} ribosome unit. With antibody preparations, directed against S4, S6, S7, S15, and S16, the dose–response curves indicated a significantly smaller effect.

Effect of purified Fabs on aminoacyl tRNA binding to the "P" or "A" sites

Inhibition of polypeptide synthesis by antibody fragments could, in principle, result from an interference with the 30S part of either the "P" or the "A" site, including the sites for the corresponding ribosomal factors (initiation factors and possibly EF-T factors), or any ribosomal conformation whose alteration could indirectly change the properties of these sites, for instance by preventing subunit reassociation. We, therefore, attempted to discriminate between these possibilities by testing antibody effect on two reactions that occur at different ribosomal sites; the AUG- and factor-dependent binding

of initiator tRNA to the "P" site and the EF-TU-dependent binding of phenylalanine tRNA to the "A" site.

Effect on AUG-Dependent [^{3}H]fMet-tRNA Binding to Ribosomes. Fig. 1 and Table 1 summarize the effects of the 20

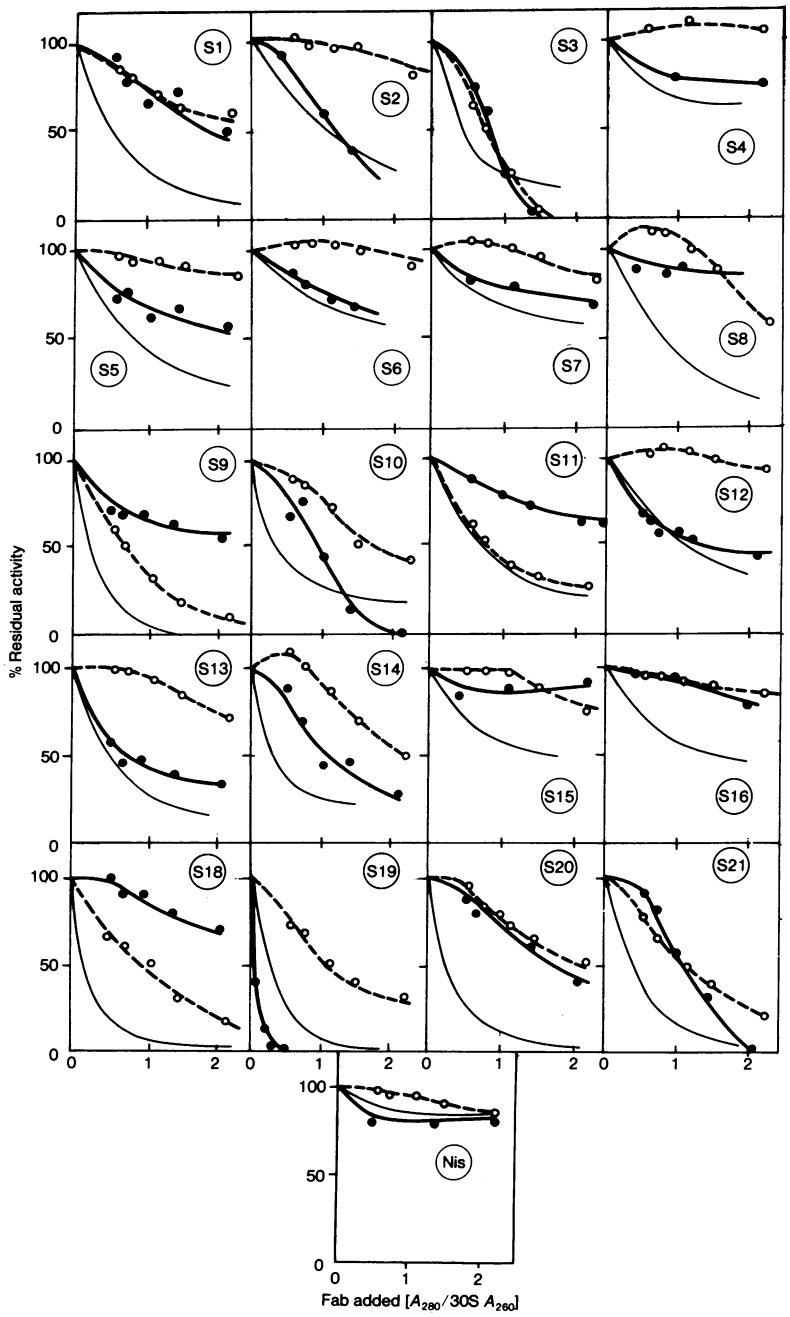

FIG. 1. Effect of Fab upon activity of 30S ribosomal subunits. Comparative effects of specific Fabs on: poly(U)-dependent phenylalanine incorporation (——); fMet-tRNA binding to 30S subunit (●——●); Phe-tRNA binding to 30S + 50S subunits (O- - -O). *Polyphenylalanine synthesis* was measured at a Mg^{++} concentration of 18 mM with saturating amounts of supernatant factors and poly(U), in 100-μl incubation mixtures containing 0.8 A_{260} unit of 70S ribosomes. *fMet-tRNA binding* was assayed by Millipore filtration and performed in 50-μl reaction mixtures containing 0.05 M Tris·HCl, pH 7.5; 0.08 M NH_4Cl; 8 mM Mg acetate; 7 mM 2-mercaptoethanol; 0.2 A_{260} unit of 30S subunits; 1.5 μg of IF-1; 1.3 μg of IF-2; 0.37 μg of IF-3; 0.6 mM GTP; 1.4 A_{260} units of unfractionated [^{3}H]fMet-tRNA (20 000 cpm) prepared as described (8); and 0.15 A_{260} unit of poly(AUG). Incubation time was 10 min at 25°. *For Phe-tRNA binding,* reaction mixtures (100 μl) contained: 0.05 M Tris·HCl, pH 7.5; 8 mM Mg acetate; 0.08 M NH_4Cl; 7 mM 2-mercaptoethanol; 0.137 A_{260} unit of 30S subunit; 0.274 A_{260} unit of 50S subunit; 0.7 mM GTP; 5 μg of EF-T (Tu + Ts) purified according to Ravel (23); 1.4 mM fusidic acid; 2 A_{260} units of unfractionated [^{14}C]Phe-tRNA (40,000 cpm); 50 μg of poly(U). Incubation time was 15 min at 25° before Millipore filtration. Control experiments showed that under these conditions, Phe-tRNA binding was 100% inhibited by 0.5 mM tetracycline and not transferable to puromycin. Percent inhibition is plotted against the relative Fab/ribosome ratios (expressed in A_{260} Fab units per A_{260} unit of 30S). Ribosomal subunits were obtained by zonal centrifugation either from unwashed or 1 M NH_4Cl-washed 70S subunits (7) dissociated by dialysis against 20 mM Tris·HCl, pH 7.5, 0.25 mM Mg acetate, 60 mM KCl, 1 mM dithiothreitol.

TABLE 1. *Effects of antibodies against 30S subunit protein on fMet-tRNA binding, Phe-tRNA binding, and polyphenylalanine synthesis*

Protein neutralized	No. of copies per ribosome	Requirement for assembly	Percent of inhibition		
			fMet-tRNA binding	Phe-tRNA binding	Polyphenylalanine synthesis
S1	0.1	−	50	40	80
S2	0.4	−	60	20	47
S3	0.7	−	100(69)	100(67)	84(52)
S4	1.2	+	24	0	37
S5	1.2	−	50(63)	15(33)	56(50)
S6	0.25	−	50(50)	10(19)	26(10)
S7	1	+	30	15	37
S8	1	+	10	40	63
S9	1	+	40	90	99
S10	0.6	+	100(82)	60(85)	68(88)
S11	0.4	−	30(75)	70(67)	54(76)
S12	0.2	−	60(92)	5(4)	48(57)
S13	0.7	+	65(65)	25(0)	73(23)
S14	0.4	−	75(88)	50(88)	72(93)
S15	1	+	10	25	39
S16	1	+	20	15	46
S18	0.35	−	30(34)	80(46)	89(57)
S19(149) (172)	0.5	−	100(76) 40	nd(67) 70	92(73)
S20	0.8	−	50(78)	50(4)	87(40)
S21	0.3	−	100(83)	75(30)	84(52)
NIs			20	15	

Percent inhibition for an Fab/30S ratio of 2.0 (see legend to Fig. 1) (tRNA binding) or 1.0 (polyphenylalanine synthesis). The values in *parentheses* are taken from ref. 1. The number of copies for various 30S proteins are those indicated by Weber *et al.* (21) and Deusser (22).

different Fab fragments on the AUG-dependent binding of radioactive initiator tRNA to purified 30S subunits, a reaction catalyzed by initiation factors IF-1 + IF-2. From the data obtained, the percent inhibition at a fixed Fab/ribosome ratio (A_{280} unit of Fab per A_{260} unit of 30S or 30S equivalent) has been computed in Table 1. Fabs can be divided into three categories: (*i*) Those directed against S3, S10, S14, S19, and S21 behave as very strong inhibitors (inhibition ranging between 75 and 100%). (*ii*) Antibodies against proteins S1, S2, S5, S6, S12, S13, and S20 also markedly interfere with formation of a ribosomal initiation complex, albeit to a smaller extent (between 50 and 65% inhibition). (*iii*) Fabs against S4, S7, S8, S9, S11, S15, S16, and S18 show only weak or virtually negligible effect (below 50% inhibition).

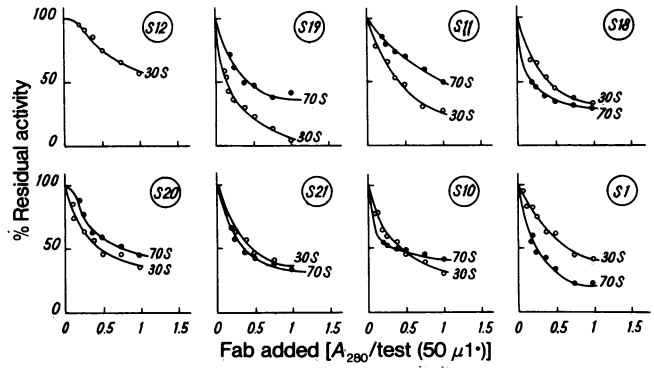

FIG. 2. Effect of Fab upon streptomycin binding to 30S ribosomal subunits and 70S ribosomes of *E. coli*. 30S subunits (1.0 A_{260} unit) or 70S ribosomes (3.0 A_{260} units) of *E. coli* were preincubated at 40° for 30 min. Specific Fabs dissolved in TMA I were added (5 min., 0°). To the incubation mixture (final volume 50 µl) [^{3}H]dihydrostreptomycin (specific activity 750 mCi/mmol) was added (molar ratio streptomycin per ribosome = 100). After incubation at 35° for 35 min, the incubation mixtures were diluted with TMA I and filtered through Millipore filters (0.45 µm). After washing, the filters were dried and counted (0.4 g of POPOP and 7.0 g of PPO/liter of toluene). TMA I: 10 mM Tris·HCl, pH 7.5; 10 mM $MgCl_2$; 30 mM NH_4Cl; 6 mM 2-mercaptoethanol.

When the sensitivity of the initiator tRNA-binding capacity of ribosomes to specific antibodies was investigated by use of 70S or a 30S + 50S mixture in the presence of IF1 + IF2 + IF3, instead of 30S ribosomes, it was observed that, in most cases, the presence of 50S subunits did not significantly change the inhibition pattern.

Effects on the EF-Tu-Dependent Poly(U)-Directed Phe-tRNA Binding. Fig. 1 and Table 1 provide data concerning the comparative antibody effects on tRNA positioning at the "A" site. Fusidic acid was present in the binding reaction mixture to prevent possible translocations. In certain experiments, a pure EF-Tu preparation containing no EF-G activity was used; the results were similar to those obtained with a cruder EF preparation. Moreover, no difference was found whether fusidic acid was present or absent from the system.

Among the monovalent antibody fragments, three categories could be distinguished. One includes antibodies against S9, S11, and S18 which exert a strong inhibition of Phe-tRNA positioning and virtually none on initiator tRNA binding; antibody against S8 showed a less-pronounced effect. Another comprises antibody against S1, S3, S10, S14, S19, S20, and

S21; these antibodies inhibit *both* Phe-tRNA and fMet-tRNA binding. The third corresponds to antibodies showing no effect on Phe-tRNA positioning to the "A" site (antibodies against S2, S5, S6, S7, S12, S13, S15, and S16). In some cases, preincubation of antibodies with dissociated subunits gave rise to stronger inhibition than use of 70S ribosomes. This was particularly so with antibodies against S3, S8, S9, S11, and S21 (data not shown).

Effect of Fabs on the binding of radioactive streptomycin to ribosomes

Several investigators (10–12) have suggested that streptomycin inhibits protein synthesis by causing distortion of the "P" site, thereby inducing rapid release of initiator or peptidyl tRNA. Also, binding of streptomycin and initiation factors to ribosomes is mutually exclusive (7). Therefore, we analyzed the effect of specific antibodies, prepared against the 30S subunit proteins, on the binding of the antibiotic to sensitive ribosomes. Antibodies directed against proteins S1, S10, S11, S18, S19, S20, and S21 inhibited binding of [^{3}H]dihydrostreptomycin to 30S or 70S ribosomes (Fig. 2). The magnitude of effects observed with the other antibodies were not significantly greater than with a nonimmune control preparation.

The results with S12 (P10) are unexpected since work from Nomura's laboratory (1, 20) had clearly implicated this protein (Str A gene product) (24), not only in the ability of the ribosome to initiate protein synthesis but also as a critical component in streptomycin fixation to sensitive 30S subunits. Yet, our data indicate that the monovalent antibody against S12 does not appreciably impair dihydrostreptomycin binding (Fig. 2). However, an antibody against the "fidelity" protein S11 (P7) displayed a positive effect on streptomycin fixation. This suggests that S12 may not represent a real target site for the antibiotic, but rather that it could be involved by long-range effects on neighboring proteins. There is evidence that streptomycin specifically interacts with 16S RNA on the small subunit (30). This situation could account for the present finding that as many as six distinct 30S proteins appear involved in antibiotic fixation, for these could primarily induce on 16S RNA the requisite conformation for streptomycin binding.

DISCUSSION

The use of specific antibodies to delineate functions of the structural ribosomal proteins is subject to several limitations. Lack of effect of a particular species of antibody on ribosomal functions does not necessarily imply that the corresponding protein is not involved in the reaction under study. The possibility that some antibodies do not have access to the protein in its state of association with the ribosome could be considered. This possibility is unlikely since Stöffler *et al.* (3, 4, 9) have shown that the whole series of proteins within the intact 30S subunit could readily complex with the cognate antibodies. Another possible cause for the failure of a particular antibody to inhibit a given ribosomal function can reside in the fact that it does not react with a determinant that is essential for the *in situ* activity of the proteins (9).

However, several important conclusions can be drawn (Table 2):

(*1*) At least 15 of the 21 protein components of the 30S ribosomes appear essential at some stage of polypeptide-chain synthesis. Lack of effect of anti S4, S7, S15, and S16 Fabs cannot be accounted for by a failure to bind the corresponding proteins *in situ*, as shown in independent experiments (3, 4). These proteins belong to the class of entities that directly interact with 16S RNA. RNA-binding proteins do not all appear "inert," however, since S8 and S20, which also can complex specific regions of 16S RNA, belong to the class of protein components that are essential in protein synthesis.

TABLE 2. *A tentative classification of 30S proteins according to their involvement in fMet-tRNA binding, Phe-tRNA binding, and streptomycin fixation to the 30S subunit*

Protein	Binding (a)	(b)	(c)
S1	+	+	+
S2	−	+	−
S3	−	++	++
S4	−	−	−
S5	−	+	−
S6	−	+	−
S7	−	−	−
S8	−	−	+
S9	−	(+)	++
S10	+	++	+
S11	+	−	++
S12	(+)	+	−
S13	−	+	(+)
S14	−	++	+
S15	−	−	−
S16	−	−	−
S18	+	(+)	++
S19	++	++	++
S20	+	+	+
S21	+	++	++

If the number of copies is taken in account for the relative concentration of Fab per 30S protein, antibodies against S3, S9, and S10 would be particularly strong inhibitors whereas antibodies against S1, S5, and S6 would show no significant inhibition. Effect on: (*a*) [^{3}H]dihydrostreptomycin binding; (*b*) fMet-tRNA binding to the "P" site; (*c*) Phe-tRNA binding to the "A" site.

(*2*) A large series of antibodies against 30S subunit protein (antibodies against S1, S3, S10, S14, S19, S20, and S21) exert an inhibitory effect on both fMet-tRNA and Phe-tRNA binding. This result is probably relevant to the high degree of *overlapping* that exists between the two categories of decoding sites ("A" and "P" sites).

(*3*) Certain antibodies appear to be *highly selective* in their effect with respect to reactions mediated by the "A" or "P" site.

(*a*) If one subtracts the background values (% inhibition with a nonimmune Fab), antibodies against S2, S5, S6, S12, and S13 exert a stronger effect on fMet-tRNA binding than on Phe-tRNA binding. This agrees with previous results from reconstitution experiments (1) and confirms Nomura's conclusion concerning the role of protein S12, the Str A gene product, in the initiation step (20, 29). There is very good agreement between our immunochemical data and the reconstitution studies, providing one takes into account, in this latter case, those proteins not considered as "assembly proteins." Our data diverge from previous data (1) only with respect to S1 and S2 (but these entities were possibly lacking in the reconstructed ribosomes) as well as with regard to S20

and S21, since we could not confirm any selective involvement of these latter two proteins in initiation.

(*b*) Antibodies against S9, *S11*, and, to a smaller extent, S8 and S18 preferentially inhibit positioning to the "A" site. This suggests that a few proteins from the small ribosomal subunit are specifically required for a reaction (the EF-Tu-dependent binding of an aminoacyl-tRNA to the "A" site) which is classically regarded as occurring on the 50S subunit. Since the existence of a common binding site for EF-TU and EF-G on the 50S subunit (L7/L12) is well documented (16, 17, 25, 26), our data agree with results (18, 19) that demonstrate the requirement of S9 and S11 for the ribosome-dependent *GTPase* activity of EF-G. Another protein S5 was reported, on the basis of reconstitution experiments, to be also involved in this latter reaction (19). Yet, we have found no effect of antibody against S5 on the EF-Tu-dependent reaction. However, antibodies against S9 and S11 did not inhibit EF-G-dependent *GTP binding* to ribosomes (25). Therefore, these proteins appear to be required after EF-G binding, possibly to induce proper conformation of the GTPase "site" of the 50S subunit. Since GTP hydrolysis is concomitant with EF-G release, and since the binding of EF-G and EF-Tu is mutually exclusive, our data might support the hypothesis that S9 and S11 play a role in the EF-Tu-binding step.

That proteins S9 and S11 from the small subunit are required in a reaction that involves (and takes place on) the large subunit also agrees with the fact that the corresponding antibodies efficiently prevent physical reassociation between subunits, as seen by sucrose gradient experiments (18, 27). Protein S11 binds in a specific manner to 23S RNA (28).

Preincubation of dissociated subunits with antibodies against S9 and S11 promotes significantly greater inhibition of enzymatic Phe-tRNA binding than when 70S ribosomes are used, a result which also fits in well with the presence of S9 and S11 at the interface of the two subunits. This, as we have seen, also applies to S3, S18, and S21.

Our results also agree with data based upon the 30S subunit heterogeneity and obtained by the addition to an *in vitro* system of an excess of each purified entity (2). By this approach S2, S3, and S14 were implicated in *both* fMet-tRNA (14) and Phe-tRNA (15) binding to 30S subunit, and S1 in the capacity to bind poly(U) (13). Protein S21 was reported to be absent from native 30S subunits and to inhibit fMet-tRNA binding when 50S subunits are added (15). Although apparently inconsistent with our data, the latter result at least suggests that S21 might occupy a key position in the initiator tRNA-decoding site.

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