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Identification of a soluble protein that stimulates peptide bond synthesis

(peptidyl transferase/ribosomes/elongation factor/protein synthesis)

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ABSTRACT **A soluble protein factor was isolated, free of elongation factor (EF)-T and EF-G, based on its ability to stimulate the synthesis of peptide bonds using ribosomal bound 70S·AUG·*N*-formyl-[^{35}S]methionyl-tRNA complex and added puromycin as substrates. Over 90% of this activity was found in the ribosome-free cytoplasm of *Escherichia coli* extracts. Other features such as molecular weight, purification properties, and catalytic activities distinguish this factor from ribosomal proteins and known activators of translation. The factor requires all components needed for peptide bond synthesis and is inhibited by antibiotics known to specifically block the peptidyl transferase activity of ribosomes. The factor increases the binding affinity of the ribosome for the aminoacyl-tRNA analog puromycin about 10-fold. We suggest that this extraribosomal factor modulates the intrinsic activity of ribosomes to catalyze peptide-bond synthesis, and regard it as a new factor required for peptide chain elongation, which we call EF-P.**

A functional topology of ribosomes has been constructed to accommodate our intuition of how these particles might catalyze peptide bond synthesis. Peptide chain synthesis, as presently conceived, is a cyclical process that takes place on the ribosome. It involves a site specific alignment between peptidyl-tRNA and aminoacyl-tRNA, peptide bond formation, and translocation of mRNA. The binding of aminoacyl-tRNA and the translocation of mRNA are mediated by the soluble proteins elongation factor (EF)-T (Tu + Ts) and EF-G, respectively, and require GTP hydrolysis (1). Until now the formation of peptide bonds has been thought to proceed "spontaneously" without the requirement for soluble factors or exogenous energy sources (1, 2). However, we now report that the reaction between ribosomal bound *N*-formyl-Met-tRNA and puromycin, the classical model of peptide bond synthesis, is indeed stimulated by a soluble protein factor, which we have called EF-P. Thus peptide bond synthesis need not be restricted to an autonomous function of the ribosome.

The relationship of this protein, EF-P, to component X, a factor needed for translation with messengers other than poly(U) (3–5), is discussed.

RESULTS AND DISCUSSION

Peptide bond synthesis between puromycin, an analog of the amino acid terminus of aminoacyl-tRNA, and ribosomal bound *N*-formyl-Met-tRNA is catalyzed by 70S or 50S particles, and is reported to occur without soluble factors or GTP (2, 6). However, as shown in Table 1, when the puromycin concentration is lowered to 1–3 μM, adding the soluble factor results in a marked stimulation of *N*-formyl-[^{35}S]Met-puromycin synthesis. To simplify discussion we refer to this factor as EF-P, i.e., a factor that stimulates the peptidyl transferase reaction.

Abbreviation: EF, elongation factor.

EF-P activity may have gone undetected thus far (6) because at the levels of puromycin generally used to assay peptidyl transferase activity the ribosomes are saturated with this analog, and the "spontaneous" formation of *N*-formyl-methionyl-puromycin proceeds at its maximal velocity, effectively depleting the system of the substrate, ribosomal bound *N*-formyl-[^{35}S]Met-tRNA. In addition, crude EF-P (steps 1 and 2; see legend to Fig. 2) contains an inhibitor that causes ribosome-bound *N*-formyl-Met-tRNA to fall off the ribosome (data not shown). This inhibitor could be EF-G, which has been reported to block binding of *N*-formyl-Met-tRNA (12, 13). This effect of EF-G is apparently inoperative during peptide-chain elongation.

The time course of the EF-P stimulated formation of *N*-formyl-[^{35}S]Met-puromycin is shown in Fig. 1. After 10 min, approximately 80% of the ribosomal bound *N*-formyl-[^{35}S]Met-tRNA reacts with puromycin. Under these conditions the reaction is pseudo-first order since puromycin is present in more than a ten-fold molar excess over ribosomal bound *N*-formyl-Met-tRNA.

Table 2 indicates that the formation of *N*-formyl-[^{35}S]Met-puromycin in the presence and absence of partially purified EF-P is absolutely dependent on added Mg^{++}. Both reactions proceed to an appreciable extent in the absence of added Tris or NH_4Cl. The 20% reaction in the absence of AUG corresponds to the fraction of *N*-formyl-[^{35}S]Met-tRNA which binds to ribosomes in the absence of this codon. The small amount of *N*-formyl-[^{35}S]Met-puromycin formed in the presence of EF-P but without added ribosomes is unexplained. Exogenous GTP is not required for the reaction.

The data of Table 3 show that both the "spontaneous" and the EF-P stimulated formation of *N*-formyl-[^{35}S]Met-puromycin are inhibited specifically by antibiotics known to inhibit the "fragment reaction" (2) and not by other protein synthesis inhibitors. The small stimulation observed with erythromycin has been previously reported (14). The fact that the "spontaneous" and EF-P mediated synthesis of *N*-formyl-[^{35}S]methionyl-puromycin requires all components needed for peptide bond synthesis and is blocked by the same spectrum of antibiotic inhibitors, suggests that both reactions occur at the same or overlapping ribosomal loci.

It is argued here that EF-P is not a loosely bound ribosomal protein nor is it a hitherto undiscovered activity of EF-T or EF-G.

Several lines of evidence suggest that EF-P is not a ribosomal protein. Upon fractionation of the S-30 extract into S-100 and ribosomes, more than 90% of the EF-P activity is found in the S-100 fraction (10% of the activity is found in the ribosomal wash). With the exception of S1, all *E. coli* ribosomal proteins have molecular weights of 28,000 or less (15). This is considerably lower than the molecular weight of 50,000 ± 3,000 determined for EF-P by chromatography on

Table 1. Effect of puromycin concentration on the synthesis of *N*-formyl-[^{35}S]Met-puromycin

Puromycin (μM)	% *N*-formyl-[^{35}S]Met-puromycin formed	
	+EF-P	—EF-P
1.2	27	1.5
3.5	58	6
7.1	73	22
17.7	76	39
35.4	77	44
70.8	86	69
177.0	88	—
354.0	92	88

One hundred percent reaction is defined as the amount of *N*-formyl-[^{35}S]Met-puromycin formed in 30 min at 35° with 354 μM puromycin. This was 52,000 dpm of *N*-formyl-[^{35}S]Met-puromycin.

Assay for EF-P: *N*-Formyl-[^{35}S]Met-tRNA was bound to *E. coli* Q13 ribosomes with AUG, as described (7, 8). Binding was measured on nitrocellulose filters (9). At zero time, 5 μl of the fraction to be assayed was added to 5 μl of 7 μM puromycin at 0°. This mixture was incubated for 30 sec at 35° before adding 20 μl of *N*-formyl-[^{35}S]Met-tRNA·AUG·ribosome complex. After incubation at 35° for 3 min, 1 ml of 1 M phosphate (pH 7.2) was added to stop the reaction and the mixture was extracted for 15 sec with 1.8 ml of ethyl acetate (10) (extraction efficiency was 46%). After separation of phases, 1 ml of the ethyl acetate layer was added to 10 ml of Bray's solution (11). Radioactivity was determined in a Packard Tri-Carb scintillation counter. Counting efficiency was 85% for ^{35}S, 50% for ^{14}C, and 20% for ^{3}H.

Sephadex G-75 (Fig. 2). In addition, the majority of ribosomal proteins are basic and are not retarded by DEAE-cellulose. EF-P, on the other hand, binds strongly to DEAE-cellulose and elutes at a much higher KCl concentration than that required to extract those ribosomal proteins that bind to this resin (15).

Experiments in Table 4 indicate that purified EF-P and purified EF-G are unable to substitute for one another. This indicates that each is free of the other. Also, fusidic acid, an inhibitor of EF-G (1), does not affect the EF-P catalyzed formation of *N*-formyl-[^{35}S]Met-puromycin (Table 3). The reported molecular weight for EF-G is 74,500 (18), whereas that for EF-P is 50,000 (Fig. 2).

Table 2. Requirements for the stimulation of *N*-formyl-[^{35}S]Met-puromycin formation by EF-P

Component	*N*-Formyl-[^{35}S]Met-puromycin formed, dpm	
	+EF-P	—EF-P
Complete system	10,350	785
—Mg	0	0
—Tris	8,675	300
—NH_4Cl	4,275	160
—Ribosomes	1,050	0
—AUG	1,975	165
$+10^{-3}$ M GTP	6,190	600
$+10^{-6}$ M GTP	10,340	600
$+10^{-3}$ M GDPCP	10,020	725
$+10^{-6}$ M GDPCP	10,280	760

The complete system consists of 8 mM Mg^{++}, 15 mM Tris (pH 7.4), 55 mM NH_4Cl, approximately 30 pmol of ribosomes, and approximately 75 pmol of AUG. 27,000 dpm of *N*-formyl-[^{35}S]Met-tRNA were bound to ribosomes prior to puromycin addition. The complete system minus NH_4Cl contains 18 mM NH_4Cl due to the fact that the ribosomes are suspended in a buffer which contains NH_4Cl. Similarly, the complete system without Tris contains 0.37 mM Tris. GDPCP is β,γ-methylene-guanosine triphosphate.

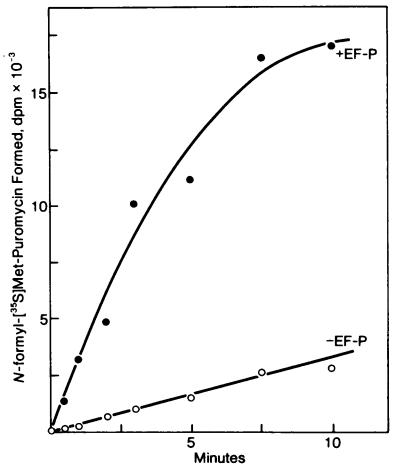

FIG. 1. Time course for EF-P catalyzed formation of *N*-formyl-[^{35}S]Met-puromycin. Assay was performed as described in the legend to Table 1 except that the time of the incubation at 35° was varied. Each incubation (+EF-P) contained 70 μg of EF-P (step 4 fraction). *N*-Formyl-[^{35}S]Met-tRNA bound to ribosomes available for reaction with puromycin was 20,900 dpm.

As can be seen in Table 4, purified EF-P does not catalyze the exchange of [^{3}H]GDP at 35° while EF-T does. Similarly, EF-T does not stimulate *N*-formyl-[^{35}S]Met-puromycin synthesis.

In addition, treatment of EF-P with 1×10^{-3} M *N*-ethylmaleimide for 30 min at 35° caused a 25% reduction in *N*-formyl-[^{35}S]Met-puromycin synthesis. This loss could result from reaction of *N*-ethylmaleimide with amino acids other than cysteine or with a relatively unreactive cysteine (19). EF-G, EF-Tu, and EF-Ts should be totally inactivated under these conditions (18, 20).

EF-P shares a number of features in common with component X, a factor needed for synthesis with messengers other than poly(U) (3–5). These include properties during purification, stability to temperature, and molecular weight. However, purification of both activities to homogeneity is

Table 3. Effect of antibiotics on the formation of *N*-formyl-[^{35}S]Met-puromycin in the presence and absence of added factor (Step 4)

Antibiotic	*N*-Formyl-[^{35}S]Met-puromycin formed (% of control)	
	+EF-P	—EF-P
Fusidic acid	100	110
Erythromycin	100	130–150
Streptomycin	100	105
Gougerotin	0	0
Chloramphenicol (*1*)	45	60
Chloramphenicol (*2*)	10	20

Final concentrations are: fusidic acid 1.7×10^{-3} M, erythromycin 2×10^{-5} M, streptomycin 2×10^{-5} M, gougerotin 8.5×10^{-5} M, chloramphenicol (*1*) 1.6×10^{-5} M, and chloramphenicol (*2*) 6.4×10^{-5} M. *N*-Formyl-[^{35}S]Met-puromycin formed in the absence of antibiotic minus EF-P was 950 dpm and plus EF-P (12.5 μg of Step 4) was 5425 dpm.

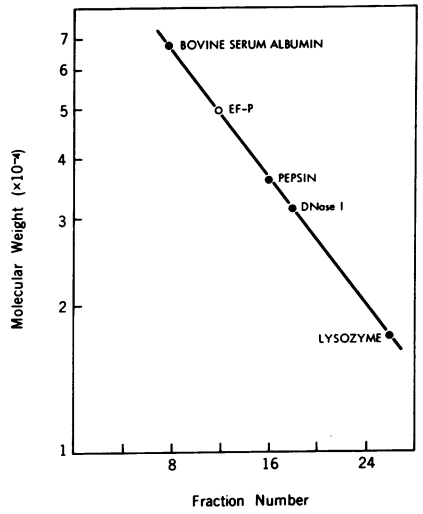

FIG. 2. Molecular weight of EF-P estimated by chromatography on Sephadex G-75. Approximately 3 mg of step 4 EF-P was applied to a 1.5 × 16 cm Sephadex G-75 column equilibrated with 10 mM Tris (pH 7.4), 10 mM KCl, and 1 mM L-cysteine. EF-P was isolated from the ribosome-free supernatant (S-240) of *E. coli* K-12 (Step 1) prepared as described (3). Fractions precipitated with ammonium sulfate (35–55%) (Step 2) were dialyzed and then chromatographed on DEAE-cellulose columns which were eluted batchwise with 0.2 M KCl (Step 3), concentrated (Step 4), and chromatographed on Sephadex G-75 or hydroxylapatite (Step 5). Details of this procedure will be published elsewhere.

required to establish the relationship between these proteins.

In spite of these uncertainties, the simpler assay of peptide bond synthesis has allowed us to probe some questions regarding the mechanism of the EF-P stimulated reaction.

We find that the purified factor has no effect on the binding of *N*-formyl-Met-tRNA to ribosomes, with or without the AUG codon. In fact, crude preparations of EF-P contaminated with EF-G prevent the binding of *N*-formyl-Met-tRNA. We have also examined whether EF-P enhances the association of 30S and 50S subunits into 70S ribosomes. To test this possibility the *N*-formyl-[^{35}S]Met-tRNA bound to ribosomes in the presence of AUG was analyzed on sucrose density gradients. All of the *N*-formyl-[^{35}S]Met-tRNA bound, with or without added EF-P, was found in the 70S fraction, suggesting that this factor does not promote ribosome association (Fig. 3).

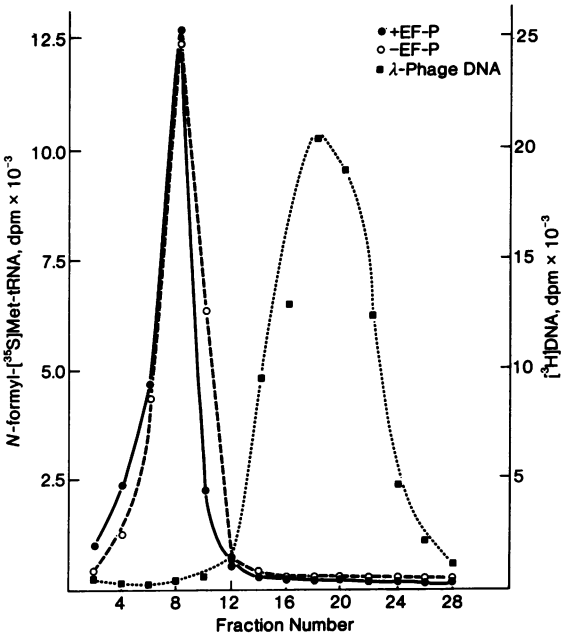

FIG. 3. Lack of effect of EF-P on the distribution of *N*-formyl-[^{35}S]Met-tRNA bound to ribosomes. Gradients are 3.3 ml of 5–20% sucrose run in 7.5 mM Mg^{++}, 15 mM Tris (pH 7.4), 37 mM NH_4Cl. EF-P and ribosome-bound *N*-formyl-[^{35}S]Met-tRNA were incubated together for 5 min at 35° before being applied to the top of the gradient. λ phage [^{3}H]DNA (32 S) was a gift of Dr. A. J. Becker.

We have also asked whether EF-P binds puromycin directly and then transports it to ribosomes. In this case purified EF-P was incubated with [^{3}H]puromycin at either 4° or 35° and then applied to a Sephadex G-25 column equilibrat-

Table 4. Lack of EF-G and EF-T activity in EF-P preparations

Exp. no.	Factor added	Product formed, dpm: *N*-Formyl-[^{35}S]Met-puromycin	[^{14}C]Poly-(Phe)	[^{3}H]GDP bound
1	None	775	0	—
	EF-G (5 μg)	1,034	6,330	—
2	None	1,460	0	—
	EF-P (1.5 μg)	4,440	130	—
3	None	1,950	—	730
	EF-T (5 μg)	2,250	—	28,190
	(10 μg)	—	—	36,590
	EF-P (1.5 μg)	6,680	—	635
	(3 μg)	—	—	825

Step 5 EF-P was used. EF-T and EF-G were purified as described by Gordon *et al.* (16). EF-T was assayed by its ability to exchange [^{3}H]GDP (17). EF-G was assayed by its ability, together with EF-T, to promote the synthesis of polyphenylalanyl-tRNA directed by poly(U) (16). Incorporation due to 5 μg of EF-T (slightly contaminated by EF-G), which was present in every tube in the poly(U) assay, was subtracted from all values.

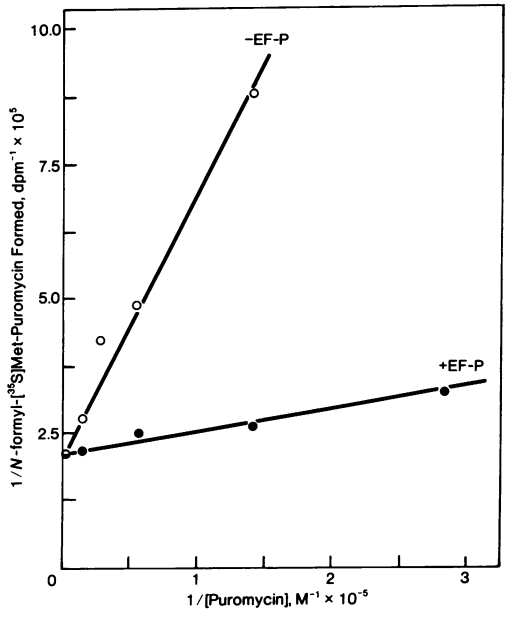

FIG. 4. Double reciprocal plot of *N*-formyl-[^{35}S]Met-puromycin synthesis as a function of the puromycin concentration. Data are shown in Table 1.

ed at either 4° or 35° with 7.5 mM $MgCl_2$, 15 mM Tris (pH 7.4), and 37 mM NH_4Cl. If EF-P had bound to puromycin, then some of the radioactivity should have been found in the void volume. However, all of the [^{3}H]puromycin eluted in a single peak, well separated from the void volume at both 4° and 35°, in the presence and absence of added factor (data not shown).

Finally, we have considered the possibility that EF-P alters the affinity of the ribosome for puromycin. Fig. 4 shows a double reciprocal plot of the reaction product as a function of puromycin concentration in the presence and absence of the factor. The apparent affinity constant of the ribosome for puromycin calculated from this experiment is 2.4×10^{-5} M without, and 2.0×10^{-6} M with EF-P.

Taken together, these data suggest that EF-P stimulates peptide bond synthesis by increasing the affinity of ribosomes for puromycin. The K_m for the binding of puromycin in the presence of EF-P is exactly the same as the K_m reported for the binding of puromycin to *E. coli* polyribosomes (21). On the other hand, the K_m for the binding of this antibiotic to the 50S ribosomal subunit in the "fragment reaction" is approximately 2×10^{-4} M (22). It is quite possible that the presence of EF-P induces a conformation in native polyribosomes that is responsible for their high affinity for puromycin.

Several reports indicate that inclusion of a variety of agents, extraneous to the cellular milieu, such as alcohols and very high cation concentrations, unmask the activity of ribosomes to catalyze decoding and translocation (23, 24). *In vitro* this "nonenzymatic" translation is 100-fold less efficient than translation in the presence of EF-T and EF-G. *In vivo* EF-T and EF-G are indispensable (25, 26) and may be viewed as allosteric effectors regulating the intrinsic activity of ribosomes to catalyze decoding and translocation. EF-P could act in an analogous manner to these proteins regulating steps that are temporally between them. EF-P could act to stimulate the efficiency of all peptide bond synthesis or, alternatively, it could be limited to stimulating the synthesis of the first peptide bond. If EF-P stimulates synthesis of most peptide bonds, then it could act as postulated for factor X (3–5).

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