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Peptide Chain Termination: Effect of Protein S on Ribosomal Binding of Release Factors

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Abstract. The protein factor S, previously shown to stimulate polypeptide chain termination in bacterial extracts, has two effects upon the complex formed between ribosomes, release factor, and terminator (trinucleotide) codon: (1) in the absence of GTP or GDP, S stimulates formation of an [R·UAA·ribosome] intermediate, and (2) in the presence of GTP or GDP, S participates in dissociation of this intermediate. Factor S can stimulate fMet release from [fMet-tRNA^f·AUG·ribosome] intermediates in either the presence or absence of GTP or GDP. A model is proposed which relates the *in vitro* effects of S ± GTP (or GDP) on fMet release to the effects of S ± GTP (or GDP) on the binding and dissociation of R factor from ribosomes.

The release of *N*-formylmethionine (fMet) from [fMet-tRNA^f·AUG·ribosome] intermediates can be used to study peptide chain termination *in vitro*.[1,2] In *E. coli*, this release reaction involves at least two events: (1) binding of one of two protein release factors, R1 or R2, to ribosomes upon terminator codon recognition[3] and (2) hydrolysis of ribosomal-bound peptidyl-tRNA.[4] We previously reported that a third protein factor, S, stimulates the rate of fMet release in the absence of GTP.[2,5,6] Since S lowered the K_m for trinucleotide codons without altering the V_{max} of release, we suggested that S acts at the terminator codon recognition event.[6] By the use of radioactive trinucleotide codons which quantitate the formation of [R·[^{3}H]UAA·ribosome] intermediates,[3] terminator codon recognition can be measured without the necessity for hydrolysis of peptidyl-tRNA. In this report, S alone is shown to stimulate the formation of the [R·[^{3}H]UAA·ribosome] intermediate, while in the presence of GTP or GDP, S facilitates dissociation of this intermediate. We also show that S can stimulate the rate of GTP- or GDP-dependent fMet release.

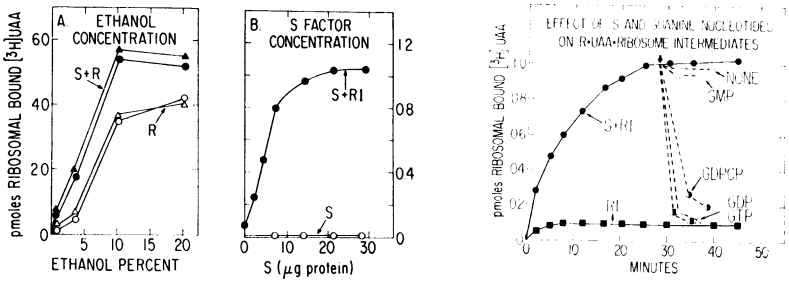
Materials and Methods: Assay of ribosomal-bound [^{3}H]UAA dependent on S: Binding of [^{3}H]UAA to ribosomes dependent on S is determined by a modification of a previously reported procedure.[3] Each reaction contains in a final volume of 0.05 ml: 0.05 M Tris–acetic acid, pH 7.4; 12 mM magnesium acetate; 0.05 M ammonium acetate; 3 mM β-mercaptoethanol; 9 pmol [^{3}H]UAA, 8.0 Ci/mmol; 2–20% ethanol (v/v); 0.82–1.5 A_{260} units *E. coli* MRE 600 ribosomes prepared as previously described;[1] 4.0 μg R1 protein (Fraction VI)[5] or 1.1 μg R2 protein (Fraction VII)[5]; and S (Fraction IV).[6] Reactions are initiated by the addition of ethanol, incubated at 0°C for the indicated time, diluted with 3.0 ml of cold buffer that contains 10% ethanol; 0.05 M Tris–acetic acid, pH 7.4; 0.02 M magnesium acetate; and 0.05 M ammonium acetate; and washed under

gentle suction onto a nitrocellulose filter (HA millipore filter, 25 mm diam, 0.45 μm pore size, lot no. 8568). All measurements of binding, unless indicated, are of extent. Filters are dried and counted as previously described[3] at a counting efficiency of 30%.

fMet release: Both R and S activities are determined by the release of f[^{3}H]-Met from [f[^{3}H]Met-tRNA^f·AUG·ribosome] intermediates as previously described.[1-6] The tRNA^{fMet} is supplied from Oak Ridge National Laboratories through an interagency agreement with the National Institute of General Medical Sciences.

Materials: The [^{3}H]UAA was prepared by enzymic methods as previously described[7] using [^{3}H]ADP (20.3 Ci/mmol, Schwarz BioResearch Corp.). Analysis of the [^{3}H]UAA in two different paper chromatographic systems (H_2O–*n*-propanol–NH_3 35:55:10 by volume; and 40 g ammonium sulfate dissolved in 100 ml of 0.1 M potassium phosphate, pH 7.0) indicated that its R_f was identical with authentic UAA; no contaminating radioactivity was detected. The L-[^{3}H-methyl]-methionine (5.1 Ci/mmol) was obtained from Schwarz BioResearch Corp. Protein determinations were carried out by a modification of the method of Lowry.[8] The R1 (Fraction VI), R2 (Fraction VII), and S (Fraction IV) preparations used in these studies have been characterized.[5,6] Antibiotics were used at a final concentration of 10^{-4} M and were obtained from sources previously reported.[3,4] Purity of GTP, GDP, and GDPCP was determined by thin-layer chromatography.[9] The GDP and GDPCP used had no detectable contaminants (level of sensitivity, 5%). The GTP used contained a 10% contamination of GDP.

Results. In reactions containing ribosomes, radioactive terminator trinucleotide, R factor, and ethanol, a stable [R·radioactive terminator codon·ribosome] intermediate is formed that can be quantitated by its retention on nitrocellulose filters.[3] In the presence of R1 or R2, S stimulates binding of [^{3}H]UAA to ribosomes (Fig. 1*A*). In other studies, we have shown that S stimulates the ribo-



(*Left*) FIG. 1. Stimulation of [^{3}H]UAA binding to ribosomes. (*A*) Each reaction is incubated 30 min at 0°C and contains in 0.05 ml: 1.5 A_{260} units ribosomes; 9 pmol [^{3}H]UAA (3600 cpm/pmol); 4.0 μg R1 (Δ, ▲) or 1.1 μg R2 (○, ●); 16.0 μg S as indicated; ethanol as indicated, and additional components as described in *Materials and Methods*. Binding of [^{3}H]-UAA (0.07 pmol) in the absence of R factor is subtracted from all values. (*B*) Each reaction is incubated 30 min at 0°C and contains in 0.05 ml: 0.82 A_{260} unit ribosomes; 9 pmol [^{3}H]UAA (3000 cpm/pmol); 4.0 μg R1 as indicated; S as indicated; 4% ethanol, and additional components as described in *Materials and Methods*. Binding of [^{3}H]UAA (0.02 pmol) in the absence of R factor is subtracted from all values.

(*Right*) FIG. 2. Dissociation of S-dependent [R·terminator codon·ribosome] intermediates with GTP, GDP, or GDPCP. Each reaction is incubated for the indicated time at 0°C and contains in 0.05 ml: 0.82 A_{260} ml ribosomes; 9 pmol [^{3}H]UAA (2600 cpm/pmol); 4.0 μg R 1; 1.40 μg S as indicated; 4% ethanol; and additional components as described in *Materials and Methods*. At 27 min, as shown by ↓, the indicated nucleotide (buffered in 0.075 M Tris-chloride, pH 7.4; 0.05 M ammonium acetate, 0.012 M magnesium acetate; 4% ethanol) is added at a final concentration of 10^{-6} M to reactions containing S and R1. The quantity of [R·[^{3}H]UAA·ribosome]intermediate is determined as described in *Materials and Methods*. Binding of [^{3}H]UAA in the absence of R factor (0.02 pmol) is subtracted from all values.

somal binding of [^{3}H]UAG with R1 but not R2. S, therefore, stimulates ribosomal binding of trinucleotides in the presence of release factors corresponding to their codon specificity (R1, UAA or UAG; R2, UAA or UGA)[3]. The stimulation by S is optimal in reactions containing 10% ethanol but is observed over the 2–20% range (Fig. 1*A*). We have routinely used 4% ethanol (Fig. 1*B*) to investigate the stimulation of UAA binding by S. UAA binding under these conditions is stimulated 12-fold. The S-dependent UAA binding is inhibited by tetracycline (89%) and streptomycin (100%), antibiotics previously shown to inhibit terminator codon binding in the absence of S[3]. Sparsomycin, amicetin, erythromycin, fusidic acid, and spectinomycin have no effect on S-dependent UAA binding to ribosomes (unpublished data).

The effect of S on the rate of [^{3}H]UAA binding is shown in Fig. 2. The UAA binding is complete in 20 min. When 10^{-6} M GTP, GDP, or GDPCP is added to these reactions, UAA rapidly dissociates from the ribosome. The addition of GMP has little effect. In other experiments where UAA is bound to ribosomes without S (in reactions containing 10% ethanol), GTP, GDP, and GDPCP have no effect on ribosomal-bound UAA. Thus, the ability of these nucleotides to participate in dissociation of UAA from ribosomes requires S. The addition of GTP, GDP, or GDPCP, but not GMP or CTP, at zero time prevents the detection of S-dependent UAA ribosomal binding (Table 1).

TABLE 1. *Effect of nucleotides on S-dependent formation of [R · [^{3}H]UAA · ribosome] intermediates.*

Addition	Ribosomal-bound [^{3}H]UAA (Δ pmol)
Complete	0.54
+GTP	0.05
+GDP	0.05
+GDPCP	0.05
+GMP	0.48
+CTP	0.60
(—S—)	(0.06)

Each reaction mixture is incubated for 15 min at 0°C and contains in 0.05 ml: 0.82 A_{260} unit ribosomes; 9 pmol [^{3}H]UAA (3600 cpm/pmol); 4.0 μg R1; 14.0 μg S; 4% ethanol; 10^{-6} M nucleotide as indicated; and additional components as described in *Materials and Methods*. Binding of [^{3}H]UAA (0.02 pmol) in the absence of R factor is subtracted from all values.

The evidence that S stimulates the binding of both UAA and R to ribosomes is presented in Table 2. The indicated reactions are separated into ribosomal and supernatant fractions by centrifugation and subsequently examined for both R and [^{3}H]UAA content. Since S does not stimulate fMet release under the conditions used for these R determinations, any stimulation in release that is observed represents increased binding of R to ribosomes. The addition of S to reactions containing UAA, R, and ribosomes increased the ribosomal binding of UAA 16-fold and of R 26-fold. Factor S, therefore, stimulates the ribosomal binding of both UAA and R. Since the fMet release assay reflects a catalytic function of R rather than a quantitation of R molecules, these comparisons do not permit us to directly relate the molecules of R and UAA bound to ribosomes. Similar results are obtained by Bio-gel column chromatography (A-1.5 m, 100–200 mesh; BioRad Labs., Richmond, Calif.) which separates ribosomal-bound

TABLE 2. *Effect of S on binding of R and [^{3}H]UAA to ribosomes.*

Binding conditions	Amount in ribosomal pellet: R activity (Δ pmol f[^{3}H]Met)	Amount in ribosomal pellet: [^{3}H]UAA (pmol)
R1 + Ribosomes	0.29	
R1 + Ribosomes + UAA	0.35	0.1 (0.01)
R1 + Ribosomes + UAA + S	1.85	1.2 (0.16)
R1 + Ribosomes + UAA + GTP*	0.37	0.1 (0.01)
R1 + Ribosomes + UAA + S + GTP*	0.44	0.1 (0.01)

* GTP is added after a 25-min incubation at 0°C.

Each reaction is incubated for 25 min at 0°C and contains in 0.50 ml: 14.0 A_{260} units ribosomes; 0.05 M ammonium acetate; 12 mM magnesium acetate; 0.05 M Tris–HCl pH 7.4; 3 mM β-mercaptoethanol; 4% ethanol; 40 μg R1; and, as indicated, 100 pmol [^{3}H]UAA and 225 μg S. All GTP additions (10^{-4}M) are made after the 25 min incubation. The ribosomal and supernatant fractions are separated by centrifugation at 200,000 × *g* for 180 min in a Spinco No. 50 rotor with tube adaptors. The ribosomal pellet is suspended, prior to assay, in 0.15 ml of buffer containing 0.02 M Tris–HCl, pH 7.5; 2 mM β-mercaptoethanol.

Ribosomal R activity: Each release assay is incubated for 15 min at 24°C and contains in 0.05 ml: 3.7 pmol [f[^{3}H]Met-tRNA^f·AUG·ribosomes]; 2.5 nmol UAA; 0.05 M ammonium acetate; 0.05 M Tris-acetate, pH 7.2; 0.03 M magnesium acetate; 0.6 mM GTP; and 2 μl of the indicated ribosomal fraction. The f[^{3}H]Met released (2700 cpm/pmol) is proportional to R. Release in the absence of added ribosomal fractions (0.7 pmol) has been subtracted from all values. Radioactivity associated with the [^{3}H]UAA does not extract into ethyl acetate at pH 1, and hence does not affect the quantitation of f[^{3}H]Met release.

Ribosomal [^{3}H]UAA content: The [^{3}H]UAA (8800 cpm/pmol) content is determined on 15 μl aliquots by scintillation counting in Beckman Bio-Solv. Values in parentheses represent the [^{3}H]-UAA content of 2 μl of each ribosomal fraction.

and free R and [^{3}H]UAA. When GTP is added to the S-dependent [R·UAA·ribosome] intermediate, both R and UAA dissociate from ribosomes (Table 2).

The requirements for the ribosomal binding of S are shown in Table 3. The ribosomal fractions are isolated as described in the legend to Table 1, and S activity in each ribosomal pellet is determined in release reactions containing R2 and UGA. Since the highly purified R1 preparation originally bound to ribosomes does not participate in fMet release with the codon UGA, these release assay conditions estimate ribosomal S, not R content. The ribosomal binding of S is stimulated by addition of both R1 and UAA, which suggests that the intermediate formed with S is a complex composed of R, S, terminator codon, and ribosomes. In experiments not shown, S dissociates from the ribosome when GTP (10^{-4} M) is added to pre-formed intermediates.

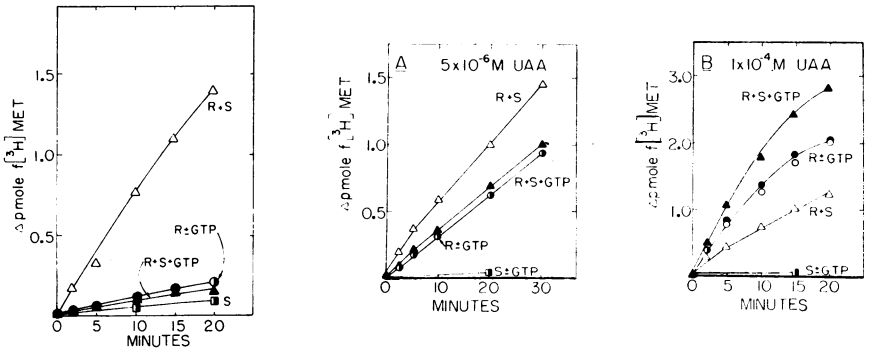
TABLE 3. *Binding of S to ribosomes by R and UAA.*

Binding conditions	Ribosomal S content: S-dependent f[^{3}H]Met (Δ pmol)
S + R1	0.55
S + UAA	0.49
S + R1 + UAA	1.40

Each reaction is incubated for 25 min at 0°C and contains in 0.50 ml: 14.0 A_{260} units ribosomes; 0.05 M ammonium acetate; 12 mM magnesium acetate; 0.05 M Tris–HCl, pH 7.4; 3 mM β-mercaptoethanol; 4% ethanol; 100 μg R1 as indicated; 200 pmol [^{3}H]UAA as indicated; and 225 μg S. The ribosomal and supernatant fractions are separated as described in Table 2. The ribosomal pellet is suspended prior to assay in 0.10 ml buffer containing 0.02 M Tris–HCl, pH 7.5; 2 mM β-mercaptoethanol.

Determination of ribosomal-bound S activity: Each release assay is incubated for 15 min at 30°C and contains in 0.05 ml: 3.7 pmol [f[^{3}H]Met-tRNA·AUG·ribosomes]; 0.5 nmol UGA; 0.05 M potassium acetate; 0.03 M magnesium acetate, 0.05 M Tris–acetic acid, pH 7.2; 1.3 μg R2 and 0.010 ml of the indicated ribosomal fraction. The f[^{3}H]Met (2600 cpm/pmol) released (0.56 pmol) without added ribosomal fractions is subtracted from each value.

We have previously reported that S stimulates fMet release from [fMet-tRNA^f·AUG·ribosome] intermediates in the presence of R and one of its corresponding terminator codons (Fig. 3). Under optimal conditions for this effect (30°C, K^+, and 10^{-5} M UAA),[6] little fMet release occurs with R alone either in the presence or absence of GTP. Factor S stimulates the rate of release 10-fold. This effect of S is eliminated by the addition of GTP (10^{-4} M). Capecchi and Klein[10] have observed a similar activity with a protein factor, which they have designated as α.



(*Left*) FIG. 3. Stimulation of fMet release by S ± GTP. Each reaction is incubated for the indicated time at 30°C and contains in 0.05 ml: 4.0 pmol [f[^{3}H]Met-tRNA^f·AUG·ribosomes]; 0.5 nmol UAA; 0.05 M potassium chloride; 0.05 M Tris-acetate, pH 7.2; 0.03 M magnesium acetate; 10^{-4} M GTP as indicated; 0.28 μg R1 as indicated; and 14.0 μg S as indicated. F[^{3}H]Met (3300 cpm/pmol) extracted at zero time (0.30 pmol) is subtracted from each value.

(*Right*) FIG. 4. Effect of [UAA] on fMet release ± S and GTP. (A and B) Each reaction is incubated for the indicated time at 24°C and contains in 0.05 ml: 4.0 pmol [f[^{3}H]Met-tRNA^f·AUG·ribosomes]: UAA as indicated; 0.05 M ammonium acetate; 0.05 M Tris-acetic acid, pH 7.2; 0.03 M magnesium acetate; 10^{-4} M GTP as indicated; 0.28 μg R1 as indicated; and 14.0 μg S as indicated. f[^{3}H]Met (3300 cpm/pmol) extracted at zero time (0.50 pmol) is subtracted from each value.

Using *in vitro* conditions that differ from those described above, we have recently observed that S can also stimulate fMet release that is dependent on the presence of GTP. The critical variables that influence these paradoxical effects of S and GTP on fMet release appear to be the concentration of terminator trinucleotide and type of monovalent cation (K^+ or NH_4^+) used. Stimulation of fMet release by S alone requires a low level of trinucleotide and occurs in the presence of either K^+ or NH_4^+, whereas stimulation of release by S + GTP requires a high level of trinucleotide and occurs only in the presence of NH_4^+ (cation data not shown). In Fig. 4, the paradoxical effects of S + GTP on fMet release are shown to be a function of the concentration of UAA. Where trinucleotide codon concentration limits the rate of fMet release (Fig. 4*A*), S alone stimulates release and this stimulation is eliminated by the addition of GTP. This data is qualitatively similar to that presented in Fig. 3. In Fig. 4*B*, where trinucleotide codon concentration is 20 times higher, S alone significantly reduces the rate of fMet release. The addition of S and GTP to these reactions stimulates the rate of release over that observed with R1 alone.

These same effects are observed with R2 (unpublished data). While these studies (Fig. 4*B*) show only a 40% stimulation of release by S and GTP, other studies with lower levels of R have shown a 240% stimulation. Thus, under appropriate *in vitro* conditions, S can stimulate peptide chain termination in the presence or absence of GTP. Recently we reported[11] that GTP, but not GDP, stimulated release of fMet from rabbit reticulocyte [fMet-tRNA·ribosome] intermediates with crude reticulocyte R. We are currently investigating this apparent difference in the effect of these nucleotides on bacterial and mammalian termination.

Discussion. The release of fMet from [fMet-tRNA^f·AUG·ribosomes] occurs after the trinucleotide-directed binding of R to ribosomes. The concentration of trinucleotide codon can limit the rate of fMet release, presumably by limiting the rate of formation of [R·trinucleotide codon·ribosome] intermediate. Under such conditions, S alone stimulates fMet release (Figs. 3 and 4*A*) and lowers the K_m for trinucleotide codons.[6] In the studies reported, we demonstrate that S stimulates the binding of R to ribosomes and suggest that S may stimulate fMet release by facilitating the binding of R to the [fMet-tRNA·AUG·ribosome] intermediate.

At higher concentrations of trinucleotide codon the rate of fMet release is increased, presumably by increasing the rate of R binding to ribosomes. Under these conditions, S alone does not stimulate release, but S + GTP (or GDP) is necessary for stimulation of the rate of release (Fig. 4*B*). We show in the present studies that S + GTP (or GDP) can participate in the dissociation of the [R·terminator trinucleotide·ribosome] intermediate. Since the fMet release reaction reflects a catalytic function of R, any process which decreases or increases this catalytic function of R molecules would be reflected in changes in the rate of fMet release. Thus, a possible explanation for the reduced rate of fMet release by S alone, and the increased rate of fMet release by S and GTP (or GDP), is that S confers stability to the [R·terminator codon·ribosome] intermediate and that S, together with either of these nucleotides, actively dissociates this intermediate. The active dissociation of R from ribosomes would increase R turnover and hence, the rate of catalysis.

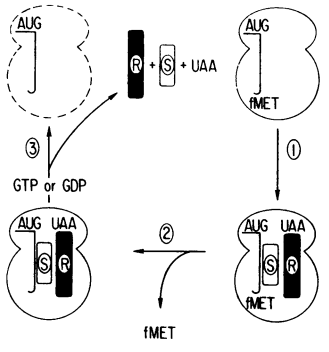


FIG. 5. Schematic model for events in termination.

Our data, therefore, suggest that S affects *in vitro* peptide chain termination in several ways. A schematic model shown in Fig. 5, though not unique, appears to take into account all of the available data. In reaction *1*, S acts to stimulate the binding of R to the aminoacyl site[3] of the 70S ribosome, forming an [R·S·UAA·ribosome·AUG·fMet-tRNA^f] complex. After formation of this complex, hydrolysis of the ribosomal bound fMet-tRNA^f occurs, releasing free fMet, as shown in reaction *2*. In reaction *3*, the interaction of GTP or GDP with the [R·S·UAA·ribosome·AUG·tRNA^f] complex results in the dissociation of R, S, and UAA from the ribosome. The fate of the

[$tRNA^f \cdot AUG \cdot$ribosome] intermediate is not known. This dissociation of R from ribosomes by S and GTP (or GDP) permits the participation of R in additional cycles of fMet release.

While each of the above intermediate reactions involving S can be separately demonstrated *in vitro*, we cannot be certain if each occurs during peptide chain termination *in vivo*. Peptide chain termination *in vivo* differs in at least two important ways from our *in vitro* model system: (1) the natural mRNA containing the terminator codon is bound to ribosomes; therefore, requirements we observe for trinucleotide directed release may be more complex; and (2) the *in vivo* GTP concentration for *E. coli* is estimated to range from 0.16 to 1.3 $\times$ 10^{-3} M,[12] a value adequate to inhibit reaction *1* as determined *in vitro* (Fig. 5). Thus, it is quite possible that the dissociation activity of S, rather than the binding activity, is its physiologic role. Studies with naturally occurring mRNA may help to resolve these questions.

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Abbreviations: GDPCP, 5′-guanylylmethylenediphosphonate; fMet, *N*-formylmethionine.

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