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Peptide Chain Termination, VII. The Ribosomal and Release Factor Requirements for Peptide Release

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Abstract. Release factors participate in release of fMet from fMet-tRNA·AUG·ribosome intermediates upon binding to ribosomes. This release requires R factor and occurs in the absence of terminator codon in reactions containing 20 per cent ethanol. Release occurs only when both 30*S* and 50*S* ribosomal subunits are present and when fMet-tRNA is located in the ribosomal P site. Release factor-dependent deacylation of fMet-tRNA is inhibited by sparsomycin, amicetin, lincocin, and chloramphenicol, antibiotics which have little effect on binding of R factor to ribosomes. The possible role of peptidyl transferase in the release reaction is discussed.

Introduction. Peptide chain termination consists of at least two events which can now be studied independently: terminator codon recognition and peptide release. *In vitro* release of fmethionine from fMet-tRNA·AUG·ribosome intermediates requires one of three terminator codons and its corresponding protein release factor, R1 (UAA or UAG) or R2 (UAA or UGA).[1, 2] More recently R1 and R2 were found to bind to ribosomes with this same codon specificity.[3] We report in this communication conditions which permit R factor to mediate peptide release independent of terminator codon and investigate the ribosomal subunit[4] and site requirements[5] for peptide release.

Materials and Methods. Preparation of ribosomes and protein factors: *Escherichia coli* B or MRE 600 ribosomes washed in 1.0 *M* ammonium chloride were prepared as described previously.[6] Ribosomal subunits from MRE 600 ribosomes were isolated by sucrose gradient centrifugation.[7] Initiation factors F_1 and F_2 were partially purified from the 1.0 *M* ammonium chloride wash of MRE 600 ribosomes by DEAE-cellulose column chromatography.[8] The F_1 preparation, which was free of R factor activity, was heated at 65°C for 10 min prior to use in order to inactivate an enzyme responsible for deacylation of fMet-tRNA.[9] Purified release factors R1 (Fraction VI) and R2 (Fraction VII); transfer factors Ts, Tu, and G, and stimulatory protein (S) were prepared as previously described.[10]

Preparation of ribosomal intermediates: The fMet-tRNA·AUG·70*S* ribosome intermediate was prepared as previously described.[1] Formation of fMet-tRNA·AUG·30*S* and [30*S* + 50*S*] ribosomal intermediates was performed at 24°C for 15 min in 0.05 ml reactions containing: 0.65 A_{260} units 30*S* ribosomes and/or 0.85 A_{260} 50*S* subunits; saturating levels of initiation factors F_1 (12.0 μg) and F_2 (9.0 μg); 19.0 pmoles f[^{3}H]Met-tRNA; 0.001 *M* GTP; 0.015 *M* magnesium acetate; 0.1 *M* ammonium acetate; and 0.05 *M* Tris-acetate, pH 7.2. Ribosomal subunits were preincubated at 37°C for 15 min in 0.025 *M* magnesium acetate, 0.170 *M* ammonium acetate, 0.085 *M* Tris-acetate, pH

7.2, prior to use. Any modification of binding reactions are detailed in the legends. Poly[^{14}C]phenylalanyl-tRNA·50*S* ribosomal subunit intermediates were prepared as previously reported[11] with MRE 600 ribosomes. Ribosomal binding of aa-tRNA was assayed by the method of Nirenberg and Leder.[12]

Release assay: Release reactions without terminator codon were incubated at 4° or 24°C and contained in 0.050 ml: 0.03 *M* magnesium acetate; 0.1 *M* ammonium acetate; 0.05 *M* Tris acetate, pH 7.2; 5.1 pmoles f[^{3}H]Met-tRNA·AUG·ribosomal intermediate or 1.4 A_{260} ribosomes and 5.6 pmoles f[^{3}H]Met-tRNA; R1 or R2; and initiated by the addition of ethanol to 20% (v/v). Optimal monovalent cation concentration with fMet-tRNA·AUG·ribosome intermediate is 0.05 to 0.1 *M;* the rate of release is 1.7 times greater with NH_4^+ than with K^+. This cation optimum is higher (0.25 *M*) for reactions using fMet-tRNA and ribosomes and NH_4^+ or K^+ are equally effective. Optimal ethanol concentration for release in the absence of codon is 20 to 25%. Release of f[^{3}H]methionine and f[^{3}H]Met-ethyl ester were assayed as previously described.[1, 13] Formyl[^{3}H]methionine released in the presence of R and ethanol was identified by paper electrophoresis (53 v/cm; 75 min) on Whatman no. 1 in 0.05 *M* pyridine-acetate, pH 3.5.[8, 14] Binding of UA[^{3}H]G to ribosomes in the presence of R factor was assayed as previously described.[3] Formyl[^{3}H]Met-puromycin and released poly[^{14}C]phenylalanine were determined by ethyl acetate extraction at pH 8.0. Extraction efficiency was 50[15] and 75%, respectively; corrections were applied so that reported values represent total product formed.

Materials: The [^{14}C]-L-phenylalanine (513 mCi/mm) and [^{3}H]methylmethionine (5.1 Ci/mm) were purchased from Schwarz BioResearch and GOPOPCP* was purchased from Miles Laboratory. The UA[^{3}H]G synthesis, sequence, and base composition were previously described.[3] Sparsomycin was furnished by the Cancer Chemotherapy National Service Center; spectinomycin was the gift of Dr. Julian Davies. Radioactivity was determined by scintillation counting.

Results. Ribosomal subunit requirements for codon-directed release: The ribosomal subunit requirements for peptide chain termination are examined in two ways. In the first instance, f[^{3}H]Met-tRNA is bound to 30*S*, and 30*S* + 50*S* ribosomal subunits by AUG in the presence of partially purified initiation factors and GTP. In reactions containing UAG and R1 (Fig. 1), release of fmethionine occurs only when both 30*S* and 50*S* ribosomal subunits are present; no release occurs from the fMet-tRNA·AUG·30*S* ribosome intermediate alone. Similar results were obtained with R2 and UGA. The small amount of release occurring with 50*S* subunits alone is due to a 5 per cent contamination with 30*S* ribosomes. Since fMet-tRNA does not bind to 50*S* subunits under these con-

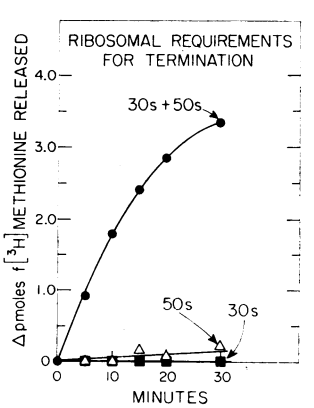


FIG. 1.—*Ribosomal requirements for termination:* Each reaction was incubated at 24°C and contained in 0.05 ml: 0.015 *M* magnesium acetate; 0.1 *M* ammonium acetate; 0.05 *M* Tris-acetate, pH 7.2; 0.88 μg R1; 2.5 nmoles UAG; and, as indicated, 4.1 pmoles f[^{3}H]Met-tRNA·AUG·(30*S* + 50*S*) ribosomal intermediate; 4.6 pmoles f[^{3}H]Met-tRNA·AUG·30*S* ribosomal intermediate; 5.0 pmoles f[^{3}H]Met-tRNA and 0.85 A_{260} 50*S* ribosomal subunits. The amount of f[^{3}H]methionine present at zero time was subtracted from each value (0.31 pmole in reactions containing f[^{3}H]Met-tRNA·AUG·[30*S*] or [30*S* + 50*S*] ribosomal intermediates and 0.63 pmole in those containing 50*S* ribosomal subunits alone).

ditions, release of peptides was investigated with poly [^{14}C]phenylalanyl-tRNA·50*S* ribosomal intermediates. Release of polyphenylalanine occurs only when 30*S* subunits are added to the 50*S* ribosomal intermediates (Table 1). No release is observed in the absence of 30*S* ribosomes although polyphenylalanyl-tRNA·50*S* ribosomal intermediates are reactive with puromycin, as demonstrated previously.[11] These results indicate that both ribosomal subunits are required for codon-directed peptide release.

The ribosomal site requirements for peptide chain termination is investigated by an alternate approach. Neither fMet release nor fMet-puromycin formation occur when fMet-tRNA is bound to ribosomes at 5 mM Mg^{++} in the presence of GOPOPCP and initiation factors (Fig. 2). Both processes do occur, however, when GTP is substituted for GOPOPCP.[16] These results indicate that peptidyl-tRNA must be in the ribosomal P site for both peptide release and peptide bond formation. Control studies assured that GOPOPCP does not inhibit peptide release.

TABLE 1. *Release requirements for poly Phe-tRNA·50S ribosomal intermediates.*

Additions	Poly Phe Released (pmoles) 50*S*	30*S* + 50*S*
None	1.37	—
R1	1.00	1.39
R1, UAG	1.11	2.68
Puromycin	3.48	7.19

Each reaction was incubated 60 min at 24°C and contained in 0.05 ml: 8.6 pmole poly[^{14}C]-Phe-tRNA·50*S* ribosome intermediate; 0.1 *M* ammonium acetate; 0.03 *M* magnesium acetate; 0.05 *M* Tris-acetate, pH 7.2; and where indicated, 4.0 μg R1; 7.5 nmoles UAG; 0.3 A_{260} 30*S* ribosomal subunits; or 10^{-3} *M* puromycin.

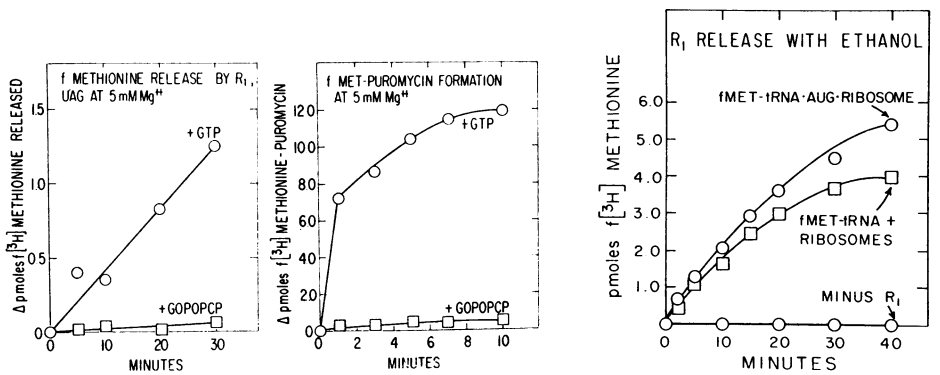
TABLE 2. *Requirements for codon-independent release.*

Components	f[^{3}H]-Methionine (pmoles)
Complete	1.25
− Ribosomes	0.10
− R factor	0.08
− Ethanol	0.06
− Ribosomes, R factor	0.10

Each reaction was incubated 30 min at 4°C and contained in 0.05 ml: 5.6 pmoles f[^{3}H]Met-tRNA; 1.4 A_{260} ribosomes; 4.4 μg R1; 20% ethanol (v/v); and 0.03 *M* magnesium acetate; 0.25 *M* ammonium chloride; and 0.10 *M* Tris-acetate, pH 7.4.

Release of fMet independent of codon recognition: Both R1 and R2 promote the release of fMet without terminator codon (Fig. 3) in reactions containing ethanol and either f[^{3}H]Met-tRNA·AUG·ribosome intermediate or ribosomes and f[^{3}H]Met-tRNA. The initial rate of release is proportional to R factor concentration at either 4° or 24°C (unpublished data).

The requirements for codon-independent release are shown in Table 2. Formyl-methionine release is dependent upon release factor, ribosomes, and ethanol. Thus fMet release can occur in the absence of initiator (AUG) and terminator codons if reactions contain ethanol. Monroe has previously shown an interaction of fMet-tRNA with the P site of the 50*S* ribosomal subunit in reactions containing ethanol.[17] Ethanol stimulates the formation of an R·ribosome intermediate, as shown in Table 3, suggesting that it eliminates the requirement for trinucleotide codons in promoting formation of an R·ribosome·fMet-tRNA intermediate. It is unresolved whether ethanol has additional effects in the release reaction.



(*Left*) FIG. 2—*Ribosomal site requirements for termination*: Each reaction was incubated at 24°C for the indicated time and contained in 0.05 ml: 3.4 pmoles f[^{3}H]Met-tRNA·AUG·ribosome formed in the presence of partially purified F_1, F_2, and 10^{-3} *M* GTP or GOPOPCP; 0.66 μg R1; 2.5 nmole UAG; 0.1 *M* ammonium acetate; 0.005 *M* magnesium acetate; and 0.05 *M* Tris-acetate, pH 7.2. Reactions for the formation of fMet-puromycin contained 10^{-3} *M* puromycin with R1 and UAG omitted. Values represent the f[^{3}H]Met release because of added terminator codon or puromycin.

(*Right*) FIG. 3.—*Release with R1 and ethanol*: Each reaction was incubated at 4°C and contained in 0.05 ml: 0.03 *M* magnesium acetate, 0.2 *M* ammonium acetate, 0.05 *M* Tris-acetate, pH 7.2; 4.0 μg R1; 20% ethanol (v/v); and, as indicated, 0.64 A_{260} ribosomes, and 9.4 pmoles f[^{3}H]Met-tRNA or 10.9 pmoles f[^{3}H]Met-tRNA·AUG·ribosomal intermediate. The f[^{3}H]-Met present at zero time has been subtracted from all values (0.4 pmole in reactions with f[^{3}H]Met-tRNA·AUG·ribosome intermediate and 0.65 pmole in those containing ribosomes and f[^{3}H]Met-tRNA).

In other studies,[5] codon-independent release of fMet was shown to be specific for R1 and R2 and was not found with other protein factors involved in peptide chain elongation (Tu, Ts, G) or termination (S). Release activity determined either in the presence of ethanol or terminator codon was identical in column fractions containing R1 (Fraction VI) and R2 (Fraction V). Since cruder preparations of release factor contain tRNA and components that inhibit codon-independent release, this method has not been used for purification of R. Only purified R1 or R2 have been used in the reported studies.

In an earlier report[13] it was demonstrated that tRNA added to these reactions results in the formation of fMet-ethyl ester. Purified R1 and R2 release only fMet, not fMet-ethyl ester (Table 4), and do not convert fMet-ethyl ester to fMet (unpublished data). Electrophoretic analysis of the products of the codon-independent release reaction reveals only fMet.

The ribosomal subunit requirements for the codon-independent release reaction are shown in Table 5. Release occurs only when both 30*S* and 50*S* ribosomes are present. Thus the ribosomal requirements for codon-directed and codon-independent release are identical and differ from the formation of fMet-puromycin (Table 5) and fMet-ethyl ester[13] which occur with 50*S* subunits alone.

Antibiotic inhibition of codon-independent release and terminator codon recognition by R1: Codon-independent release of fMet with R1 and ribosomal binding of UA[^{3}H]G by R1 measure two aspects of chain termination and are differentially affected by antibiotics. As shown in Table 6, sparsomycin,

TABLE 3. *Ethanol-dependent binding of R2 to ribosomes.*

Additions to R binding reaction	UAA Dependent R2 Activity (pmoles f[^{3}H]Met fraction) Supernatant	Pellet
R2 + ribosomes	1.51	0.57
R2 + ethanol	1.98	0.18
R2 + ribosomes + ethanol	0.07	1.91

Each reaction was incubated for 15 min at 4°C and contained in 0.10 ml: 9.9 μg R2; 0.03 *M* magnesium acetate; 0.1 *M* ammonium acetate; 0.05 *M* Tris-acetate, pH 7.2; 0.006 *M* β-mercaptoethanol; and where indicated, 6.0 A_{260} units ribosomes; and 20% ethanol (v/v). Reactions were centrifuged at 200,000 *g* for 60 min at 4°C, after which the supernatant fraction was aspirated. The pellet was suspended in 0.1 ml buffer containing 0.25 *M* ammonium acetate; 0.05 *M* Tris-acetate, pH 7.1; and 0.006 *M* β-mercaptoethanol. Each fraction was assayed for R2 activity at 24°C for 15 min in 0.05 ml reactions containing: 5.1 pmoles f[^{3}H]Met-tRNA·AUG·ribosome intermediate; 2.5 nmoles UAA; 0.03 *M* magnesium acetate; 0.05 *M* ammonium acetate; 0.05 *M* Tris-acetate, pH 7.2; and 0.005 ml of the supernatant or ribosomal fraction.

lincocin, chloramphenicol, amicetin, and erythromycin inhibit release more than codon recognition. Streptomycin and tetracycline, on the other hand, inhibit codon recognition but have less effect on fMet release. Thus, several antibiotics that inhibit peptidyl transferase[18] also affect the release event. Conversely, those antibiotics which inhibit aa-tRNA binding to the ribosomal A site[19] preferentially affect terminator codon recognition.

Discussion. Peptide chain termination consists of at least two events: recognition of terminator codons and hydrolysis of peptidyl-tRNA. These events can now be studied in concert[1] or as partial reactions.[3, 5] Terminator codon recognition requires either R1 (UAA or UAG) or R2 (UAA or UGA) and results in the formation of an R·terminator codon·ribosome intermediate.[3] In this communication, the hydrolysis of fMet-tRNA in the absence of terminator codons is shown to require R factor, ribosomes, and ethanol and is suggested to occur via an R·ribosome·fMet-tRNA intermediate. Release factor, therefore, initially binds to ribosomes upon codon recognition[3] and subsequently participates in peptidyl-tRNA hydrolysis. The inhibition of UA[^{3}H]G binding to ribosomes by tetracycline and streptomycin suggests that terminator codon recognition occurs at the 30*S* ribosomal A site.[19]

The release of peptides may occur by one of several mechanisms. Since R factors are proteins, they may directly catalyze release of peptides. Alternately,

TABLE 4. *Products of codon-independent release reaction.*

Addition	Picomoles Released f[^{3}H]Met	f[^{3}H]Met-ethyl ester
R1	3.63	0.11
R2	4.21	0.09
None	0.38	0.09

Each reaction was incubated at 0°C for 20 min and contained in 0.05 ml: 5.1 pmoles f[^{3}H]Met-tRNA·AUG·ribosomal intermediate; 0.03 *M* magnesium acetate; 0.1 *M* ammonium acetate; 0.05 *M* Tris-acetate, pH 7.2; 20% ethanol (v/v); and as indicated, R1 (10.0 μg) or R2 (3.3 μg). In identical reactions containing 0.02 A_{260} tRNA 3.45 pmoles f[^{3}H]Met-ethyl ester was formed. Assays for f[^{3}H]methionine and f[^{3}H]Met-ethyl ester were performed as described.[1, 5]

TABLE 5. *Ribosomal subunit requirements for codon-independent release.*

Addition	pmoles f[^{3}H]Met	f[^{3}H]Met-puromycin
30*S*	0.12	0.13
50*S*	0.11	3.26
30*S* + 50*S*	0.84	—

Each reaction was incubated at 4°C for 45 min and contained in 0.05 ml: 5.65 pmoles f[^{3}H]Met-tRNA; 0.03 *M* magnesium acetate; 0.25 *M* ammonium acetate; 0.1 *M* Tris-acetate, pH 7.4; 20% ethanol (v/v); and, as indicated, 10^{-3} *M* puromycin; 4.4 μg R1; 0.45 A_{260} 30*S* ribosomal subunits and/or 1.01 A_{260} 50*S* subunits. Ribosomal subunits were preincubated at 40°C for 10 min in 0.015 *M* magnesium acetate; 0.125 *M* ammonium acetate; and 0.05 *M* Tris-acetate, pH 7.4, prior to use.

TABLE 6. *Effects of antibiotics on codon recognition and release by R1.*

Addition	Codon-Independent fMet Release		UA[^{3}H]G Binding to Ribosomes	
	pmoles	Control (%)	pmoles	Control (%)
None	1.50	100	0.95	100
Tetracycline	1.06	71	0.22	23
Streptomycin	0.70	47	0.16	17
Spectinomycin	1.40	93	0.90	94
Erythromycin	0.96	64	0.76	80
Amicetin	0.64	43	0.90	94
Sparsomycin	0	0	0.83	87
Chloramphenicol	0.38	26	0.81	84
Lincocin	0.39	26	0.89	94

Each release reaction was incubated at 4°C for 5 min and contained in 0.05 ml: 0.03 *M* magnesium acetate; 0.1 *M* ammonium acetate; 0.05 *M* Tris-acetate, pH 7.1; 5.46 pmoles f[^{3}H]Met-tRNA·AUG·ribosomal intermediate (0.32 A_{260} ribosomes); 4.0 μg R1; and 20% ethanol (v/v). All values are measurements of the rate of fMet release.

Each binding reaction was incubated at 4°C for 15 min and contained in 0.02 ml: 0.02 *M* magnesium acetate; 0.1 *M* ammonium acetate; 0.1 *M* Tris-acetate, pH 7.2; 0.33 A_{260} ribosomes; 25 pmoles UA[^{3}H]G (spec. act. = 1.0 Ci/mmole); 10.0 μg R2; and 10% ethanol (v/v). Reactions were plated on nitrocellulose filters as previously described.[3] All values are measurements of the extent of UA[^{3}H]G binding.

Final antibiotic concentration was 1×10^{-4} *M*. The values obtained in the absence of R1, (0.35 pmole f[^{3}H]methionine, 0.03 pmole UA[^{3}H]G) were subtracted from all determinations.

release factors may activate and/or interact with a ribosomal constituent that catalyzes the hydrolytic event. Peptidyl transferase, a 50*S* ribosomal subunit enzyme, can catalyze formation of peptide bonds or under special conditions, ester bonds.[13] On the basis of these observations we suggested that the function of peptidyl transferase was to facilitate nucleophilic attack on ribosomal bound peptidyl-tRNA, usually resulting in peptide bond formation. Peptide release could result from nucleophilic attack on peptidyl-tRNA if the nucleophile were a hydroxyl (OH^-) group. Peptide release and peptidyl transferase activities share common characteristics apart from these theoretical considerations: both are inhibited by sparsomycin, amicetin, lincocin, and chloramphenicol;[18] and peptidyl-tRNA must be located in the ribosomal P site for either release or peptide bond formation. Although these studies and observations by others[20] indirectly suggest that peptidyl transferase participates in peptide chain termination, they do not explain why in reactions containing ethanol and R, where the preferred nucleophile is ethanol not hydroxyl (OH^-), the product of the reaction is fMet not formyl-methionyl-ethyl ester. One possible explanation is that protein release factors participate enzymatically in the hydrolytic reaction. Available information does not permit a definitive statement on the mechanism of peptide release at this time, but the methods described in this communication provide an experimental approach to the problem.

Termination of protein synthesis occurs when release factors recognize a terminator codon at the ribosomal A site, resulting in formation of an R·ribosome intermediate. An additional protein factor, S, facilitates the recognition of terminator codons.[21] Deacylation of peptidyl-tRNA bound to the ribosomal P site follows formation of the intermediate.

* Abbreviation used: GOPOPCP, 5′-guanylylmethylenediphosphonate.

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