ISOLATION OF A FACTOR THAT STIMULATES CLEAVAGE OF RIBOSOMAL BOUND *N*-ACETYL OR *N*-FORMYL METHIONYL $tRNA_f^{met}$

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1. Introduction

During the code-dependent condensation of amino acids on ribosomes the amino group of an incoming amino acyl-tRNA attacks the ester linkage at the carboxyl end of the nascent peptide and the 3′ OH group of the ribose of the terminal adenosine of tRNA [1]. This reaction is catalyzed by peptidyl synthetase, a part of the 50S subunit [2], but the active center of this enzyme has not been isolated free of this particle [3,4]. Instead, indirect approaches with antibiotics that specifically block peptide bond synthesis have been used as probes to study the reaction [1–3]. Other observations suggest that the peptidyl transferase synthesizes ester bonds with a suitable acceptor [6,7], and catalyzes ester hydrolysis if one of the release (R) factors and a cognate nonsense codon [8,9] or a suitable nucleophile [10] are included. These combined observations suggest that ribosomes have the inherent capacity to hydrolyze ester linkages. We report here that the hydrolytic activity of ribosomes is uncoupled when a high molecular factor from the ribosome-free cytoplasm is added. This reaction is nonsense-codon independent and is blocked by inhibitors of peptide bond formation when the initiator fMet-tRNA is bound to ribosomes.

2. Materials and methods

Ribosomes from *E. coli* Q_{13}, mid log cells (General Biochemicals), were washed twice with 0.01 M Tris, buffered to pH 7.4, 0.01 $MgCl_2$, 0.5 M NH_4Cl [12].

Assays for factor activity were conducted in two stages: in Stage I, either *N*-acetyl-[^{3}H] Met-$tRNA_f^{met}$ or *N*-formyl-[^{35}S] Met-$tRNA_f$ was bound to ribosomes with AUG; in Stage II, factor was added to Stage I reactions and after incubation, either *N*-acetyl-[^{3}H] methionine or *N*-formyl-[^{35}S] methionine was extracted into acid ethyl acetate [12]. A small level of non-enzymatic hydrolysis was subtracted from all values. At Stage I, reactions (0.045 ml) were for 15 min at 24°C and contained 25 to 30 pmol *N*-acetyl-[^{3}H] or *N*-formyl-[^{35}S] Met-$tRNA_f^{met}$ [12], 5 nmol AUG, 11 mM $MgCl_2$, 20 mM Tris, pH 7.4, and 10 A_{260} units of ribosomes. Binding was measured on Millipore filter membranes [13]. About 70% of the input fMet-$tRNA_f$ was bound with AUG and less than 10% bound without added AUG. For Stage II (0.065 ml reaction vol), the indicated levels of factor and/or antibiotics were incubated at 24 or 35°C, for the specified times.

Factor was purified as follows: The S-150 fraction, made from 20 g of Q_{13} cells [12], was precipitated with 55% ammonium sulfate at pH 7.0 (As fraction). After 6.5 hr dialysis against 0.01 M Tris, pH 7.4, 0.002 M dithiothreitol, (buffer I), the As fraction was applied to DEAE cellulose columns (0.9 × 20 cm) equilibrated with buffer I. The bulk of the factor was found in the void volume. These steps gave about a one hundred-fold enrichment of the factor over crude extracts. RF_1, and RF_2 were purified free of the factor described here by step 3 of the purification scheme of Ganoza and Tompkins [16]. EF-Tu, EF-Ts, EF-G, were prepared and assayed according to Gordon et al. [17]. Peptidyl-tRNA hydrolase was purified about 500-fold by polymer partitioning, DEAE [14], and CM cellulose chromatography [15]. After the CM step, the hydrolase preparations were essentially free of the factor described here. Proteins were estimated as cited [19].

Sparsomycin, and fusidic acid were a gift of Dr S. Pestka and of Dr H. P. Ghosh. Chloroamphenicol came from Sigma Chemical Corp., streptomycin from Glaxo Ltd., England. UAG, UGA and AUG were synthesized with polynucleotide phosphorylase [18] using UpA and UpG (Miles Chemical Co.) as primers. For all assays, pure $tRNA_f^{met}$ was acylated, acetylated, and formylated as previously described [12].

3. Results and discussion

Fig. 1A shows that sparsomycin blocks the hydrolysis of ribosomal bound *N*-acetyl-[^{3}H]Met-tRNA catalyzed by different levels of the factor isolated from the supernatant fraction of *E. coli* Q_{13} cells. The sparsomycin sensitive cleavage depends on addition of the partially purified factor, ribosomes, and all the components needed to bind the *N*-acetyl-Met-tRNA to ribosomes, i.e. AUG, NH_4^+ and Mg^{++} ions (table 1), Sparsomycin therefore inhibits hydrolysis only when the *N*-acetyl-Met-tRNA is bound to these particles. Other antibiotics, thought to block translocation [5] or messenger-tRNA interactions, i.e. fusidic acid and streptomycin, have little or no effect on this reaction (table 2). A small and variable amount of ribosome-independent hydrolysis was observed, and was not sensitive to antibiotics (see Tables 1 and 2).

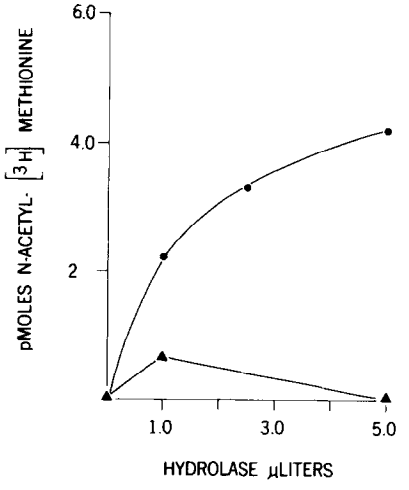

Fig. 1. Effect of factor concentration on the sparsomycin sensitive hydrolysis of *N*-acetyl-[^{3}H]Met-$tRNA_f$. The indicated levels of partially purified factor were added to 17 pmol (58 μg/μl) of *N*-acetyl-[^{3}H]Met-tRNA·AUG. ribosome complex (see Materials and methods). Reaction was for 5 min at 35°C. Reactions without sparsomycin (○——○) or with 2.8×10^{-4} M sparsomycin (▲——▲).

Table 1
Requirements for the sparsomycin-sensitive cleavage of *N*-acetyl-Met-tRNA

Reaction	*N*-acetyl-[^{3}H] methionine released, pmol
Complete	3.35
+ sparsomycin	− 0.45
− ribosomes	0.39
− ribosomes + sparsomycin	0.41
− AUG	− 0.09
− AUG + sparsomycin	0.35
− $MgCl_2$ (0.001 M)	0.15
− $MgCl_2$ (0.001 M) + sparsomycin	0.10
− NH_4Cl (0.02 M)	1.50
− NH_4Cl (0.02 M) + sparsomycin	− 0.35
− factor	0.43
− factor + sparsomycin	0.37

The complete system had 35 pmol *N*-acetyl [^{3}H]Met-$tRNA_f^{met}$ bound to ribosomes at Stage I, and 50 μg As fraction at Stage II (see Materials and methods). Incubation was for 5 min at 35°C.

Previous studies from this [20–23] and other laboratories [8,9,10] indicate that hydrolysis of ribosomal bound f-Met-tRNA, occurs only when the release proteins and the codons UAA, or UAG (for RF_1) or UAA or UGA (for RF_2) [8] are added together. However, a large background of nonsense-codon independent hydrolysis is generally observed prior to purification. Therefore, one possibility is that this activity is due to an R protein complexed to an RNA bearing a nonsense codon. Several experiments suggest that this is not the case. For example, all the release factors can be separated on DEAE-cellulose, DEAE-Sephadex-A-50 or by PEG-dextran partitioning from the factor (see [8–10,14,16] and Materials and methods). The separation of RF_1 and RF_2 from the factor on aluminum Cγ gels which also remove RNA is illustrated in fig. 2. The ribosome-dependent activity has an apparent mol. wt of about 200 000 on Sephadex G-200 (see fig. 3), Sepharose 6B, and sucrose density gradients. Each of the release

Table 2
Effect of antibiotics on the ribosome-dependent cleavage of *N*-acetyl [^{3}H]-Met-tRNA$_f^{met}$ stimulated by the soluble factor

Antibiotics		% Inhibition of *N*-acetyl [^{3}H] methionine release + Ribosomes	– Ribosomes
None		0.00 (7)	0.00 (7)
+ sparsomycin,	2.8×10^{-4} M	69.0 (7)	6.4 (7)
	2.8×10^{-5} M	72.0 (2)	0.0 (2)
	2.8×10^{-6} M	73.0 (2)	0.0 (2)
+ chloroamphenicol,	0.8×10^{-3} M	40.9 (4)	8.1 (4)
	0.8×10^{-4} M	43.3 (2)	10.8 (2)
+ fusidic acid,	0.8×10^{-3} M	8.5 (3)	12.8 (3)
	0.8×10^{-4} M	0.0 (2)	10.5 (2)
+ streptomycin,	0.8×10^{-3} M	23.7 (3)	16.1 (3)
	0.8×10^{-4} M	0.0 (2)	12.5 (2)

Experimental conditions are described in Materials and methods. Incubation was for 30 min at 24°C. The number of experiments is in brackets. The values are averages of the experiments. Variation between determinations was 10%.

factors, on the other hand, has an approx. mol. wt of 40 000. [9,10]. The partially purified factor was found insensitive to RNAse treatment. Since bulk RNA was removed by the purification, and the activity differed in physical properties from the R factors, it is unlikely that the ribosome-dependent hydrolysis is due to a complex of RNA and R proteins.

The factor described here purifies as a protein (see Materials and methods), is destroyed by 4 min incubation at 50°C and by digestion with trypsin. The preparations are also free of the known elongation or initiation proteins. Also, homogeneous EFTu, EFTs, EFG or partially purified IF_1, IF_2 or IF_3, or the recently isolated rescue protein [25], do not stimulate ribosome-dependent hydrolysis.

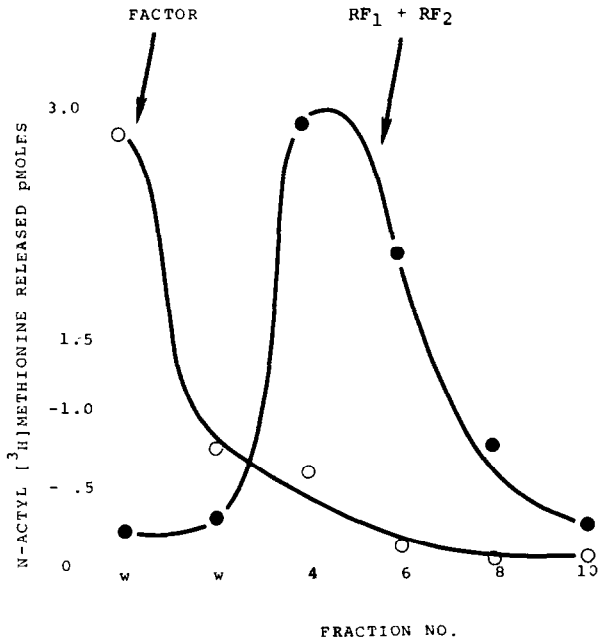

Fig.2. Separation of R factors from the factor that stimulates the ribosome-dependent cleavage of *N*-acetyl-[^{3}H]Met-tRNA. To each 5 A_{235} absorbing units of the 55% As fractions (see Methods) were added 1 mg of alumina Cγ. After 10 min stirring at 0°C, the gel and adsorbed protein were removed by centrifugation at 15 000 *g* for 10 min. The unadsorbed fraction is W. The gel was re-extracted with the following phosphate buffers, pH 7.0, containing 0.002 M dithiothreitol; 0.005 M for fraction 1, 0.025 M for 2 to 4, 0.05 M for 5 to 8 and 0.1 M for the remaining fractions. RF_1 and RF_2 were assayed with UAA (see Methods and [8]). In determinations not included UAG (for RF_1) or UGA (for RF_2) were used to verify that both proteins were present. Codon-independent hydrolysis of *N*-acetyl-[^{3}H] methionyl-tRNA, sensitive to sparsomycin (see Methods) was assayed on 5 μl aliquots of each fraction.

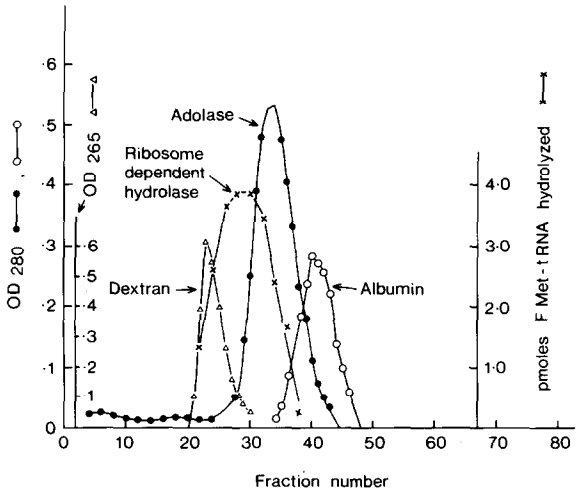

Fig.3. Mol. wt determination of the factor by Sephadex G-200 chromatography. 18 mg of the DEAE fraction (see Methods) were loaded on a 55 × 0.9 cm column equilibrated in buffer I with 0.01 M KCl; 0.5 ml fractions were collected every 4 min and 20 μl were assayed for 60 min at 24°C as in Methods. Markers were run with dextran blue or KCl.

The data of table 3 show that the ribosome-dependent hydrolysis is not due to peptidyl-tRNA hydrolase [15,16] because a temperature-sensitive mutant of this enzyme [24] catalyses the reaction even at non-permissive temperatures. Also the purification followed for peptidyl tRNA hydrolase [14,15] either separates or selectively destroys the normal factor.

It has been shown that a hydrolytic activity of the 50S particle can be induced by addition of nucleophilic agents, foreign to the cellular milieu [10]. The factor we have isolated may well be a natural agent that triggers this process in the cell. Although the function of this reaction is unknown, its biological relevance and as that of other factors that interact with ribosomes may be uncovered by appropriate screening of conditionally lethal mutants defective in translation [25].

Acknowledgements

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Table 3
Activity of mutant and wild-type peptidyl-tRNA hydrolase

Strain	Treatment	pmol substrate released *N*-formyl-[^{35}S] methionine		
	N-acetyl-[^{14}C] phenylalanine		+ sparsomycin	− sparsomycin
DP	None	2.07	1.87	5.48
	43°C	2.41	1.87	5.57
AA	None	0	0.87	3.38
	43°C	0	0.92	2.49

Assays were as stated in Materials and methods. Where shown, 1.4×10^{-6} M sparsomycin was added. AA is a temperature-sensitive mutant with a thermolabile inactive peptidyl-tRNA hydrolase, CP is the parental strain. Growth conditions and assay of the hydrolase, with *N*-acetyl-[^{14}C] phe-tRNA, were as described in [24].

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