A POLYPEPTIDE CHAIN RELEASE FACTOR FROM THE UNDEVELOPED CYST OF THE BRINE SHRIMP, *ARTEMIA SALINA*

M. Anne REDDINGTON and Warren P. TATE
Department of Biochemistry, University of Otago, P.O. Box 56, Dunedin, New Zealand

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1. Introduction

Release factors have been well characterised from *Escherichia coli* [1] and more recently from rabbit reticulocytes [2]. There are some marked differences between the corresponding bacterial factors (RF-1 or RF-2) and the reticulocyte factor. Each of the bacterial factors recognises two of the three possible termination codons (RF-1; UAA or UAG and RF-2, UAA or UGA) [1] while the reticulocyte factor recognises all three codons at the same site [3]. The reticulocyte factor exhibits a ribosome-dependent GTPase activity; GTP and its hydrolysis appear to be involved directly in the interaction of the eukaryotic factor with the ribosome [3]. In contrast there is evidence that GTP may play a role in the interaction of the bacterial factor RF-1 and RF-2 with the ribosome through an association with the stimulatory factor, RF-3 [4]. While the bacterial factors are single polypeptides of 50 000 mol. wt [5] the reticulocyte factor is a dimeric protein of 110 000 mol. wt [2].

We have shown that extracts of the ametabolic cyst of the brine shrimp, *Artemia salina* are able to catalyse the elongation and release of the subunit of rabbit globin from reticulocyte polysomes [6]. This indicated that the cysts of the brine shrimp because of their abundance could be a good source of eukaryotic factors to study the mechanism of polypeptide chain termination in higher organisms.

2. Experimental

Dry cysts of *Artemia salina* (100 g) after treatment with 1% (v/v) sodium hypochlorite and extensive washing in deionised water were suspended in a buffer suitable for isolation of both ribosomes and release factor, (10 mmol/l Tris–HCl, pH 7.6, 50 mmol/l KCl, 9 mmol/l $MgCl_2$, 1 mmol/l dithiothreitol, 5% (v/v) glycerol). The cysts were broken at 8000 p.s.i. in a Manton-Gualin laboratory Homogenizer, debris removed by centrifuging and 100 000 × *g* supernatant obtained. The protein fraction precipitated by ammonium sulphate between 30% and 75% saturation was fractionated on a column of DEAE–Sephadex A-50 (50 × 2.5 cm) with 500 ml of the buffer containing a KCl gradient from 50–600 mmol/l. The column fractions containing a GTP-binding protein presumed to be elongation factor-1 (EF-1) and suspected of containing release factor activity (RF) eluting at 275 mmol/l KCl were further chromatographed on Sephacryl S-200 (50 × 2.5 cm) in the buffer, and then phosphocellulose (30 × 1.5 cm) and eluted with 150 ml of a KCl gradient (50–400 mmol/l). The RF activity was measured using a f[^{3}H]Met-$tRNA_f^{Met}$/AUG/ribosome substrate prepared as in [7] where the ribosomes were derived either from undeveloped cysts or from developing embryos (18 h) of *Artemia salina.* Except where indicated each reaction contained within 0.05 ml: 4 pmol substrate, 0.01 ml of a fraction containing RF; polynucleotide or oligonucleotide as indicated at a concentration of 2.0 A_{260}; 0.1 mmol/l GTP and a buffer containing 20 mmol/l Tris–Cl, pH 7.5, 60 mmol/l KCl. The f[^{3}H]Met was extracted at pH 1 into ethylacetate. The f[^{3}H]Met obtained in the absence of release factor was subtracted in each case. The release factor was stored at −80°C and was stable

for at least 6 months. The [^{3}H]methionine (11 mCi/mmol) was purchased from Amersham/Seale and purified $tRNA_f^{Met}$, UAA, and AUG were obtained from Miles Laboratories. *Artemia salina* dehydrated cysts were marketed as San Francisco Bay Brand, a division of Metaframe Corporation, California.

3. Results and discussion

A release factor was not detected in extracts of the *Artemia salina* cysts until a late stage in the purification because of an activity which interfered with the product analysis for the factor. In contrast the RF derived from reticulocyte preparations is observed after chromatography on DEAE Sephadex A50 of the protein fraction obtained by ammonium sulphate fractionation [8]. In this case EF-1 elutes at a similar KCl concentration to that of the RF. The analogous GTP binding fraction in the *Artemia salina* extracts, also presumed to be EF-1, was taken therefore, through further purification steps until the RF was identified. The release factor eluted from phosphocellulose at 250 mmol/l KCl and was resolved from both the GTP binding activity and the interfering activity which did not absorb to the column (fig.1).

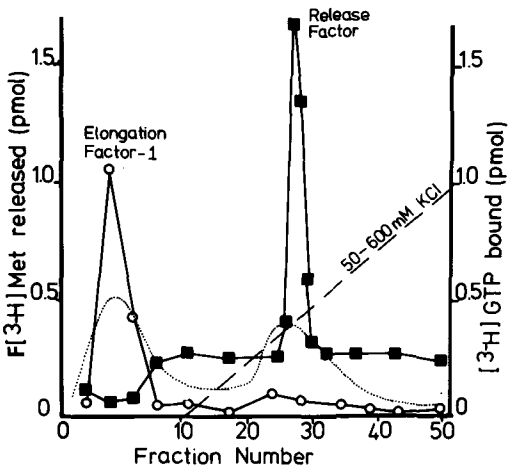

Fig.1. Fractionation of *Artemia salina* release factor. *Artemia salina* release factor was resolved on chromatography of the EF-1 fraction on phosphocellulose as described in Experimental. The release factor was assayed as described using Poly (U,G,A) for the termination codon. EF-1 was determined from its ability to form a stable complex with [^{3}H]GTP (2070 cpm/pmol) [12]. A_{254} (. . .).

The latter is shown in fig.1 as a decrease in the free f[^{3}H]Met extracted, significantly below the background levels in the substrate used. If the phosphocellulose chromatography is carried out as the first column step in the procedure the release factor co-chromatographs with a different interfering activity which hydrolyses the substrate independent of termination codon. We find that the partial purification of EF-1 by the procedure outlined and the resolution of the RF from this fraction is a convenient method of obtaining a purified release factor essentially free of EF-1 and EF-2. At this stage, the preparation contains several bands when analysed by electrophoresis on polyacrylamide gels.

An RF was not detected in initial studies which used salt washed ribosomes from the undeveloped cyst to prepare the f[^{3}H]Met-tRNA/AUG/ribosome substrate. Ribosomes from developing embryos (18 h) had similar activities to those from the cyst in the binding of fMet-tRNA at the 'P' site and the formation of fMet-puromycin (a measure of peptidyl transferase). However, when they were used to prepare substrate for in vitro termination, the RF was identified. A comparison the activity of the substrates containing ribosomes from the cysts or from developing embryos in response to increasing amounts of RF is shown in fig.2. The RF from the cyst functions very poorly on ribosomes from the cyst but significantly better on ribosomes from the later stage of development. This suggests that the factor involvement in the termination

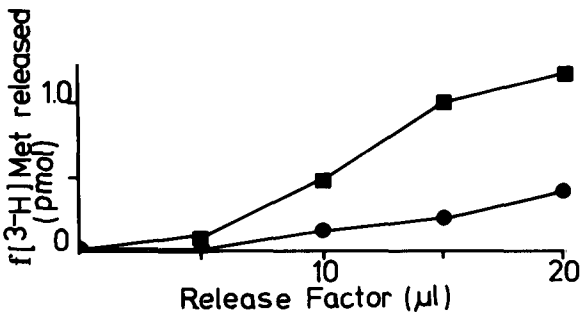

Fig.2. Effect of ribosomes from different stages of development of *Artemia salina* on the assay for release factor. Ribosomes from undeveloped cysts (●—●) or developing embryos (18 h) (■—■) were used to make f[^{3}H]Met-tRNA/AUG/ribosome substrate as described [7]. The relative abilities of each substrate to detect release factor was determined. The f[^{3}H]Met extracted in the absence of release factor (0.2 pmol) was subtracted in each case.

Table 1
Codon recognition by the release factor from *Artemia salina*

Artificial mRNA or oligonucleotide added	f[^{3}H]Met released (Δpmol)
Poly(U,G,A)	0.70
Poly(U,G),poly(U)	0.02, 0
UAA	0
UAAA	0.55
UGAA	0.64
UAGA	0.29
UAGG	0.15

Each reaction contained within 0.05 ml; 3.6 pmol f[^{3}H]Met-tRNA/AUG/ribosome substrate, the polynucleotides at a concentration of 2.0 A_{260}, the oligonucleotides at a concentration of 1.2 A_{260} and other components as described in Experimental. The f[^{3}H]Met extracted in the absence of termination codon (0.6) was subtracted in each case. Incubations were for 20 min at 24°C

reaction is limited by a property of the cyst ribosomes and this possibility is currently under investigation. Ribosomes from the cyst are known to be less active in translating Poly U than those from developing embryos [9]. It has been reported that wash fractions of the ribosome from the cyst contain a small inhibitory RNA [10] and an inhibitory protein [11]. The same fractions from late developing embryos contains an additional RNA which neutralises the inhibitor RNA [10].

As shown in table 1 the RF will release f[^{3}H]Met in response to an artificial mRNA, Poly (U, G, A), which contains all three termination codons (UAG, UGA, UAA). It does not respond to the other artificial mRNA's, Poly U and Poly (U, G) which contain no possible termination codons. Unlike the *E. coli* release factors it is not active with the trinucleotide termination codon, UAA. It does, however, respond to tetranucleotides containing any one of the three termination codons. At several concentrations UAAA or UGAA support in vitro termination better than UAGA or UAGG. Studies with the reticulocyte factor and reticulocyte ribosomes using the same preparations of the tetranucleotides did not show such marked differences (Tate – unpublished). However this may reflect the ability of the *Artemia salina* ribosomes to bind the tetranucleotides in a termination complex rather than a difference in the codon recognition properties of the release factors.

The release of f[^{3}H]Met from the termination complex is stimulated 4-fold by GTP (0.1 mmol/l) only marginally by ATP (0.1 mmol/l) and the stimulatory effect is abolished by equimolar amounts of the β-γ methylene analogue, GMP-PCP. The RF exhibits a ribosome dependent GTPase activity either on ribosomes from developing embryos of the brine shrimp or rabbit reticulocytes. The activity is stimulated by artificial mRNA, Poly (U, G, A) although the effect is greater on reticulocyte ribosomes. The stimulation of the GTPase is not limited to mRNA's containing the termination codon since Poly U and Poly (U, G) enhance the activity to a somewhat lesser extent than Poly (U, G, A) (table 2). These properties indicate that the *Artemia salina* RF is similar to the codon recognition factor from rabbit reticulocytes.

The molecular weight of the brine shrimp RF has been estimated from sedimentation studies to be 50 000 and from Sephadex gel elution to be about 80 000. However, studies with the reticulocyte factor have indicated that apparent molecular weights from these techniques for this protein are quite variable depending on the conditions used. A complete characterisation is necessary, therefore, before

Table 2
Ribosome-dependent GTPase of *Artemia salina* release factor

Additions	Ribosomes	
	Artemia salina	Reticulocytes
	(Δpmol [γ-^{32}P]GTP hydrolysed)	
None	20	22
Release factor	51	54
Release factor poly(U,G,A)	70	110
Release factor poly(U,G)	46	92
Release factor poly(U)	60	94

Each reaction contained within 0.05 ml: 400 pmol [γ-^{32}P]GTP (specific activity 220 cpm/pmol), ribosomes (18 h embryos of *Artemia salina* or rabbit reticulocytes, as indicated) at a concentration of 1.2 A_{260}, polynucleotides as indicated and a salt-buffer mixture as in table 1. Incubations were for 10 min at 30°C

conclusions on the molecular structure of the factor can be made.

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