N-FORMYLMETHIONYL TRANSFER RIBONUCLEIC ACID IN *PSEUDOMONAS AERUGINOSA*

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1. Introduction

N-Formylmethionyl transfer ribonucleic acid (fMet-tRNA) has been demonstrated in extracts from *Escherichia coli* [1] and subsequently from other sources, including Ehrlich ascites cells [2], chloroplasts [3] and mitochondria of rat liver [4], yeast [4] and Hela cells [5]. It is not certain how extensively fMet-tRNA is found in other bacterial species, since recent reviews [6–8] give no reports of it other than in *E. coli*. It has been suggested that intiation of protein biosynthesis, a role performed by fMet-tRNA in *E. coli* [9], may involve alanine derivatives in *Bacillus subtilis* [6]. By comparison with several other bacterial species, two pseudomonads have been found to show an infrequent occurrence of methionine as the *N*-terminal residue of proteins [10]. This might indicate that methionine residues are readily removed from the *N*-terminal position of proteins after synthesis, or that methionine derivatives participate only to a minor degree in the initiation process, in pseudomonas species. In view of this result and an observation [11] of two separable Met-tRNA synthetase activities in *Pseudomonas aeruginosa*, we have examined this organism to determine whether it contains fMet-tRNA.

We find that fMet-tRNA and Met-tRNA are both present in growing cultures. After esterification of *Ps. aeruginosa* tRNA with radioactive methionine through the *in vitro* action of either of two partially purified Met-tRNA synthetases, as least two species of Met-tRNA are separable by chromatography on benzoylated DEAE cellulose (BDC) columns. The Met-tRNA species which elutes from the column more readily apparently corresponds to Met-tRNA$_F^{Met}$ of *E. coli* in that it is susceptible to enzymic formylation. This species is hardly detectable with *in vivo* extracts, but an approximately equivalent amount of fMet-tRNA$_F^{Met}$ elutes at higher salt concentration in an overlapping peak with the second Met-tRNA species (Met-tRNA$_M^{Met}$). This observation suggests that the Met-tRNA$_F^{Met}$ is almost completely formylated *in vivo*.

2. Results and discussion

The existence of fMet-tRNA in extracts of *Ps. aeruginosa* labelled *in vivo* with ^{35}S-methionine was demonstrated by application of the ionophoretic and radioautographic techniques of Marcker and Sanger [1]. Log phase cells growing in minimal medium were incubated with ^{35}S-methionine (6 μM, 140 μCi/μmole) for 2 min and then chilled by pouring on to crushed, frozen medium. The cells were harvested by centrifugation and resuspended in buffer. The suspension was shaken with an equal volume of phenol to extract tRNA which was then precipitated from the aqueous phase with 70% ethanol [12].

Alkaline digests of the preparation were ionophoresed with carrier methionine and *N*-formyl methionine. A radioautograph is shown in fig. 1 (a). The radioactive spots L and M coincided with those of *N*-formylmethionine and methionine respectively, as detected by the platinic iodide reaction [13]. The transience of the spot produced in this reaction did not allow a photographic record to be made. Coincidence was also shown in chromatography on Whatman No. 1 paper with a butanol : acetic acid : water (4 : 1 : 1) solvent. Samples of the digest were

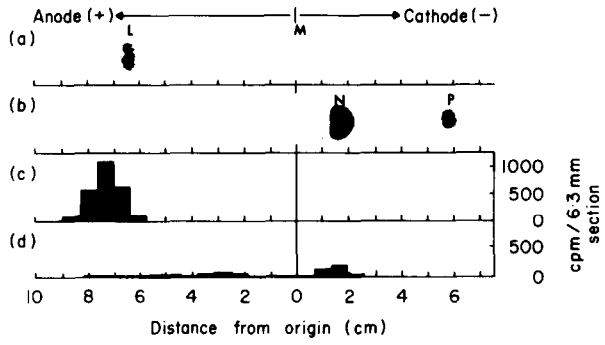

Fig. 1. Ionophoresis of alkaline and ribonuclease digests of *Ps. aeruginosa* tRNA labelled *in vivo* with ^{35}S-methionine.
(a) Alkaline digest
(b) Ribonuclease digest
(c) Alkaline digest of eluate from N in fig. 1(b)
(d) Alkaline digest of eluate from P in fig. 1(b).
Experimental details as described in text.

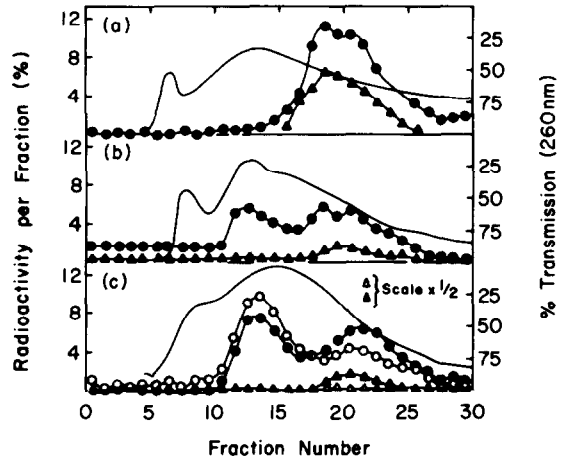

Fig. 2. BDC-column chromatography of *Ps. aeruginosa* tRNA labelled with radioactive methionine. Columns (3 cm × 1 cm diameter) were loaded with tRNA samples in 0.3 M NaCl, washed with 10–15 ml of 0.3 M NaCl, 0.02 M Na acetate (pH 5.0) and 0.01 M $MgCl_2$ and then eluted with a gradient of 0.3 to 0.8 M NaCl, containing 0.02 M Na acetate (pH 5.0) and 0.01 M $MgCl_2$. The eluate was monitored continuously for % transmission at 260 nm and collected in 1 ml fractions. On completion of the gradient, the columns were washed with 0.65 M NaCl, 0.02 M Na acetate pH 5.0 and 0.01 M $MgCl_2$ in 25% ethanol and fractions collected until elution of a further peak of UV absorbing material was complete. For (c) the gradient was taken to 1.0 M NaCl. Radioactivity in the fractions was meaured by drying 50 µl samples on glass paper filters which were then transferred to vials for liquid scintillation counting. Radioactivity present in the fractions as fMet-tRNA was determined on 0.95 ml samples which were hydrolysed by incubation at 37° for 1 hr with 50 µl of M NaOH. The solution was acidified with 150 µl of M HCl and extracted with 5 ml of ethylacetate. 4 ml of the ethylacetate phase was evaporated to dryness at 70° in a scintillation vial and the residue subjected to liquid scintillation counting. Results were calculated on the basis of 70% extraction of *N*-formylmethionine into ethyl acetate under these conditions. Radioactivity in individual fractions is expressed as the percentage of the sum of the cpm for the given isotope in all fractions from the gradient, including those eluted with 25% ethanol.

Continuous line: % transmission at 260 nm
●—● : ^{35}S-radioactivity
○—○ : ^{3}H-radioactivity
▲—▲ : ^{35}S-fMet-tRNA radioactivity
△—△ : ^{3}H-fMet-tRNA radioactivity

(a) Chromatograph of tRNA extracted from a log-phase culture of *Ps. aeruginosa* growing in minimal medium after 5 min of labelling with ^{35}S-methionine (2 µM, 260 µCi/µmole).

(b) Chromatograph of *Ps. aeruginosa* tRNA from a 10 min incubation at 25° *in vitro* with ^{35}S-methionine plus Met-tRNA synthetase and a post-ribosomal supernatant from *Ps. aeruginosa*. The incubation contained 125 mM tris (pH 7.7), 1 mM Na_2MgATP, 5 mM $MgCl_2$, 50 µM ^{35}S-methionine, 0.1 mg/ml bovine serum albumin, 1 mM 2-mercaptoethanol, 0.5 mg/ml tRNA [16] from *Ps. aeruginosa*, plus *Ps.aeruginosa* Met-tRNA synthetase (sufficient to give maximal methionylation of the tRNA) and a post-ribosomal supernatant from log phase *Ps. aeruginosa*. The supernatant was prepared from cells broken in an Aminco pressure cell at 10 tons/sq in while suspended in a medium of 1 mM tris (pH 7.7), 10 mM $MgCl_2$, 5 mM 2-mercaptoethanol and 2 µg/ml of crystalline pancreatic deoxyribonuclease (Sigma).

(c) Co-chromatograph of a preparation of ^{3}H-Met-tRNA with a similar preparation of ^{35}S-Met-tRNA, which had been partially formylated by reaction with *p*-nitrophenylformate. The labelled Met-tRNA's were prepared in separate incubations similar to that described under (b), with the omission of the post-ribosomal supernatant and isolated by phenol extraction and ethanol precipitation. The ^{35}S-Met-tRNA was then reacted with *p*-nitrophenylformate as described by Marcker and Sanger [1]. After reisolation it was mixed with the ^{3}H-Met-tRNA preparation for chromatography.

acidified with HCl and subjected to extraction with ethyl acetate [14] followed by ionophoresis of both organic and aqueous phases. The radioactivity coinciding with methionine remained almost exclusively in the aqueous phase whereas that coinciding with *N*-formylmethionine partitioned between the phases approximately as quoted by Caskey et al. [14].

To show evidence of attachment of the radioactivity to tRNA, ribonuclease digests were prepared and subjected to ionophoresis to separate Met-adenosine and fMet-adenosine [1]. The radioautograph in fig. 1(b) shows spots N and P with relative mobilities appropriate to fMet-adenosine and Met-adenosine, respectively. The radioactive compounds were separately eluted from the ionophoretograms with distilled water and treated with NaOH to hydrolyse acyladenosine esters. The hydrolysates were then ionophoresed with carrier methionine and *N*-formylmethionine. The ionophoretograms were cut into 1/4 inch (6.3 mm) sections for counting in a liquid scintillation spectrometer. The distribution of radioactivity for the products from the eluates of N and P is shown in fig. 1(c) and 1(d), respectively. The area of radioactivity shown in fig. 1(c) concided with the position of *N*-formylmethionine as indicated by the platinic iodide reaction. Similarly, the main area of radioactivity (to the right of the origin) shown in fig. 1(d) coincided with the position of carrier methionine. The cause of the radioactivity to the left of the origin in fig. 1(d) was not identified, but might possibly be oxidation products of methionine. The tentative respective identification of N and P with fMet-adenosine and Met-adenosine is thus strongly supported by the ionophoretic properties of their alkaline hydrolysates.

To find whether *N*-formylmethionine was attached to a unique tRNA species, studies were made using fractionation on BDC-columns [15]. The tRNA labelled *in vivo* with ^{35}S-methionine for 5 min (or 2 min) gave the profile shown in fig. 2(a) with no clear indication of more than one peak of labelled tRNA. Little radioactivity was eluted by subsequent washing of the column with 0.65 M NaCl in 25% ethanol. Ethyl acetate extracts from hydrolysates of the fractions indicated that radioactivity in the peak was in the form of both Met-tRNA and fMet-tRNA. When tRNA, prepared [16] from *Ps. aeruginosa,* was esterified *in vitro* with radioactive methionine by the action of a partially purified Met-tRNA synthetase, two distinct peaks were observed to elute with the NaCl gradient (see results defined by open symbols in fig. 2(c)). No production of fMet-tRNA occurred under these conditions. Two Met-tRNA synthetase preparations, clearly separated on passage of an extract of *Ps. aeruginosa* through G-150 Sephadex [11], gave identical profiles from the aminoacylation reaction, as shown by co-chromatography of their products in a double labelling experiment with ^{3}H-methionine and ^{14}C-methionine. A similar profile also resulted when Met-tRNA synthetase from *E. coli* catalysed the amino-acylation. The observations of two peaks of Met-tRNA from *in vitro* aminoacylation and one containing both Met-tRNA and fMet-tRNA from the *in vivo* experiments, suggest that Met-tRNA$_{F}^{Met}$ is almost completely formylated *in vivo* to fMet-tRNA$_{F}^{Met}$ which chromatographs in the same region as the other species of Met-tRNA (Met-tRNA$_{M}^{Met}$ if it does not become formylated).

With tRNA from *E. coli*, Marcker and Sanger [1] showed that *in vitro* incubation with a fresh post-ribosomal supernatant extract from *E. coli* effected the formylation of some Met-tRNA. Using their conditions we performed a similar experiment incubating tRNA, Met-tRNA synthetase and supernatant extract, all from *Ps. aeruginosa,* with ^{35}S-methionine and then re-isolating the tRNA. Fractionation of the tRNA on a BDC column gave the results shown in fig. 2(b). Here the two peaks are still observed, with fMet-tRNA detected only in the one eluted at higher salt concentration. The proportion of total radioactivity present as fMet-tRNA is considerably smaller than for the *in vivo* labelling shown in fig. 2(a). The results in figs. 2(a) and (b) are explicable by a hypothesis that the species of Met-tRNA to elute earlier is Met-tRNA$_{F}^{Met}$, whilst the species eluting at higher salt concentration may be Met-tRNA$_{M}^{Met}$. The *in vitro* formylation is far from complete so that much of the Met-tRNA$_{F}^{Met}$ remains to give the early peak and only a small amount of fMet-tRNA$_{F}^{Met}$ is produced to overlap with Met-tRNA$_{F}^{Met}$ in the later peak.

Fig. 2(c) presents the results of an experiment in which the formylation of the Met-tRNA was non-enzymic. ^{35}S-Met-tRNA was subjected to reaction with *p*-nitrophenyl formate to formylate the methionyl-residue [1] and then re-isolated and co-chromatographed on an BDC-column with unformylated ^{3}H-Met-tRNA (fig. 2(c)). Once again, fMet-tRNA was found only in the later peak.

The results in fig. 2 show that fMet-tRNA always elutes at higher salt concentration than the most readily eluted Met-tRNA and that the amount of the latter is reduced in proportion to the amount of fMet-tRNA formed. Possibly the absence of a positive charge on the methionine amino group causes fMet-tRNA$_F^{Met}$ to be retained on the column more strongly than Met-tRNA$_F^{Met}$. It would be desirable to confirm the nature of the species of Met-tRNA from the separate peaks by isolating them to demonstrate directly that the material from the early peak can be enzymically formylated *in vitro* whereas that from the second peak can not.

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