Heterologous mischarging as a means of tRNA fractionation. I. Behaviour of *E.coli* phenylalanyl-tRNA^{Val} on BD-cellulose

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ABSTRACT

The chromatographic behaviour of *E.coli* tRNA^{Val}, Val-tRNA^{Val} and Phe-tRNA^{Val} was studied on BD-cellulose columns. At pH 4.0 and 4°C the elution position of Phe-tRNA^{Val} was not affected by the presence or absence of Mg²⁺, whereas Val-tRNA^{Val} was slightly retarded when Mg²⁺ was omitted. It is postulated that the amino acid and its nature influence the structure that the aminoacyl-tRNA assumes. Under suitable conditions the heteroaminoacylated Phe-tRNA^{Val} eluted significantly later than other tRNAs. This fact showed that heterologous mischarging can be a useful step in tRNA purification methods.

INTRODUCTION

Esterification of an aromatic amino acid to a cognate tRNA causes the tRNA to be more strongly bound to BD-cellulose¹, a property that has been used to purify yeast tRNA^{Tyr}, tRNA^{Trp} and tRNA^{Phe}³. A more general procedure for the isolation of tRNA species which are aminoacylated by non aromatic amino acids, is accomplished by the procedure developed by Gillam et al.⁴. The method involves enzymatic aminoacylation, chemical phenoxyacetylation and isolation of the derivatized material by chromatography on BD-cellulose columns. By this method *E.coli* tRNA^{Met}_f, tRNA^{Met}⁵, tRNA^{Val}⁶, yeast tRNA^{Asp}, tRNA^{Arg}⁴, and yeast tRNA^{Met}⁷, have been purified as well as many other tRNA species.

Within the past few years, several cases of incorrect aminoacylation involving a tRNA from one organism and an aminoacyl-tRNA synthetase from another, have been reported⁸. It has been

shown that Neurospora crassa and yeast phenylalanyl-tRNA synthetase can charge phenylalanine to several E.coli tRNA species⁹⁻¹¹. Using different mischarging conditions, Kern et al.¹¹ have shown that almost all E.coli tRNAs can be heteroaminoacylated with yeast phenylalanyl-tRNA synthetase. These authors used phenoxy-acetylation of the mischarged E.coli Phe-tRNAs, in order to characterize the species involved, by separating the derivatized material from the non-aminoacylated molecules on BD-cellulose column chromatography.

These findings induced us to test whether E.coli tRNA species, which are not natural acceptors of aromatic amino acids but are able to accept phenylalanine, could be separated from unacylated tRNAs on BD-cellulose columns by the incorporation of an aromatic moiety.

This report describes the chromatographic behaviour of E.coli tRNA₁^{Val}, Val-tRNA₁^{Val}, and Phe-tRNA₁^{Val}.

EXPERIMENTAL

¹⁴C-Valine (10 mCi/m mole) was a product from Amersham-Searle, ¹⁴C-Phenylalanine (5 and 492 mCi/m mole) and ³H-Valine (126 mCi/m mole) were from Schwarz-Mann, and ¹⁴C-Alanine (163 mCi/m mole) from CEA-France. BD-cellulose (50-100 mesh) Lot. N° 6902 and E.coli tRNA were products from Schwarz BioResearch. N.crassa (74 OR31-16A) was a generous gift from Dr. J.L. Eppler.

Assays for E.coli homologous amino acid acceptance activity were carried out in 0.1 ml reaction mixtures containing 100 mM 3,3-dimethylglutaric acid buffer pH 7.0, 10 mM Mg(OAc)₂, 10 mM KCl, 2 mM ATP, 0.02 mM ¹⁴C-amino acid, tRNA and enzymes⁵. Incubations were at 37° for 30 min. Within this time, all reactions were complete. Reactions were stopped and processed as described¹². Heterologous aminoacylation was performed according to Strickland and Jacobson¹³. The standard assays were carried out in 0.1 ml reaction mixtures containing 50 mM potassium cacodylate buffer

pH 6.3, 7.5 mM Mg(OAc)₂, 0.5 mM ATP (Mg²⁺/ATP = 15), 0.025 mM ¹⁴C-Phenylalanine and E.coli tRNA. The mixtures were incubated at 22° for 30 min using a partially purified preparation of Neurospora crassa phenylalanyl-tRNA synthetase⁹ in saturating amounts.

Large scale charging of enriched E.coli tRNA^{Val} was done scaling up the standard assay systems, except that ³H-valine was used for the homologous reaction. Each ml of the heterologous incubation mixture contained 58 A₂₆₀ units of enriched E.coli tRNA^{Val}, and 2 to 3-fold the amount of N.crassa phenylalanyl-tRNA synthetase required to reach the plateau of the heteroaminoacylated product. The tRNA and enzyme concentrations were chosen after studying conditions in which the aminoacylated product plotted against time of reaction resulted in a rapid rise to a stable plateau¹⁶ and that the net aminoacylation value resulted proportional to the amount of tRNA present. The other components of the reaction mixture, time and temperature of incubation, are the same described for the standard assays.

The aminoacylated tRNAs were isolated by phenol extraction and repeated ethanol precipitation.

All the columns were performed with the same BD-cellulose batch and the salt gradients were measured by conductivity with a Radiometer conductivity meter CDM3 and by the Mohr method. No differences with either of the two procedures were found.

RESULTS

Total E.coli tRNA was chromatographed at 4° on BD-cellulose. Two peaks of valine (Fig. 1A), three peaks of alanine and one peak of phenylalanine (Fig. 1B) acceptance activity were detected. The fractions were assayed for phenylalanine acceptor activity using the standard assays for heterologous aminoacylation with N.crassa phenylalanyl-tRNA synthetase described under Experimental. Under the given reactions conditions

tRNA₁^{Val}⁶ and the alanine accepting tRNA fractions were mischarged to at least 70% of that achieved with the homologous *E.coli* enzyme. The enriched tRNA₁^{Val} fractions were pooled and precipitated with 2 vol of ETOH to yield an 8-fold purified product with a specific activity of 385 pmoles/A₂₆₀ unit. This tRNA₁^{Val} enriched fraction was used to study the chromatographic behaviour of Phe-tRNA₁^{Val}. Using the conditions described under Fig. 1, the elution of hetero-aminoacylated ¹⁴C-Phe-tRNA₁^{Val} occurred at a higher salt concentration (0.83 M NaCl) than that of the uncharged enriched *E.coli* tRNA₁^{Val} (0.57 M NaCl, see Fig. 1A).

To test whether the separation of ¹⁴C-Phe-tRNA₁^{Val} could be increased, chromatography at a lower pH in the presence of EDTA was performed¹. To compare the elution positions of tRNA₁^{Val}, Val-tRNA₁^{Val} and Phe-tRNA₁^{Val}, a sample of uncharged enriched tRNA₁^{Val}, ³H-Val-tRNA₁^{Val} and ¹⁴C-Phe-tRNA₁^{Val} (obtained by charging portions of the enriched tRNA₁^{Val} peak from the first BD-cellulose column as described in Experimental, Fig. 1A), was freed from Mg²⁺ by Sephadex G-25 gel filtration. Elution was carried out with 0.005 M sodium formate buffer pH 4.0, 0.005 M EDTA. The recovered material was loaded on a column of BD-cellulose previously equilibrated with the same buffer containing 0.3 M NaCl. As seen in Fig. 2A, the interaction between ¹⁴C-Phe-tRNA₁^{Val} and BD-cellulose was greatly enhanced since under this conditions its elution occurred at 0.97 M NaCl. The uncharged tRNA₁^{Val} shifted to an elution point of 0.74 M NaCl and the homologously aminoacylated Val-tRNA₁^{Val} to 0.77 M NaCl. It should be noted that, in general, the bulk of the A₂₆₀ profile was also retarded.

In order to see how the presence of Mg²⁺ would affect the elution of the heteroaminoacylated ¹⁴C-Phe-tRNA₁^{Val} as compared to its effect on tRNA₁^{Val}, Val-tRNA₁^{Val}, and the bulk tRNA, another chromatography was carried out with a sample similar to the one used in the experiment shown in Fig. 2A, except that Sephadex G-25 gel filtration was performed in 0.005 M sodium formate buffer, pH 4.0, 0.01 M MgSO₄. As shown in Fig. 2B, Mg²⁺ ions did not change the elution position of ¹⁴C-Phe-tRNA₁^{Val} (0.97 M NaCl) but decreased considerably the secondary interactions between the bulk tRNA and BD-cellulose. tRNA₁^{Val} eluted at 0.68 M NaCl and Val-tRNA₁^{Val} at 0.73 M NaCl.

Figure 1: Chromatography of uncharged *E. coli* tRNA. A sample of 6930 A₂₆₀ units dissolved in 50 ml of equilibrating buffer, was loaded on a 0.9 x 90 cm column of BD-cellulose equilibrated with 300 mM NaCl, 10 mM NaOAc buffer pH 5.5, 10 mM MgSO₄, and 10 mM mercaptoethanol. Elution was performed in the same buffer with a linear NaCl gradient as indicated. Fractions (2 ml) were collected at 4° and 25 μl aliquots were assayed for homologous and heterologous acceptance activity as described under Experimental.

△—△ Heterologous ¹⁴C-Phe acceptance, represented also by the shaded area. A. ●—● homologous ¹⁴C-Val acceptance. B. ●—● homologous ¹⁴C-Ala and ○—○ ¹⁴C-Phe acceptance.

Figure 2: Elution profiles of Phe-tRNA^{Val}₁ in the presence and absence of Mg²⁺ ions. The BD-cellulose chromatographic runs were performed in 0.9 x 100 cm columns at 4°. Fractions of 2.4 ml were collected. Assays for homologous valine acceptance were carried out as described in Experimental. As for radioactivity measurements, aliquots were dissolved in Bray's solution¹⁸ and counted at 70-80% efficiency. The cpm were corrected for the spill over. **A.** A sample containing uncharged enriched tRNA^{Val}₁, ³H-Val-tRNA^{Val}₁ (31500 cpm) and ¹⁴C-Phe-tRNA^{Val}₁ (26500 cpm), freed from Mg²⁺ as described in the text was chromatographed in 5 mM sodium formate buffer pH 4.0, 5 mM EDTA with a salt gradient as indicated. **B.** Chromatography of a sample containing uncharged tRNA^{Val}₁, ³H-Val-tRNA^{Val}₁ (21000 cpm) and ¹⁴C-Phe-tRNA^{Val}₁ (44500 cpm). Elution was performed with a salt gradient as indicated in 5 mM sodium formate buffer pH 4.0, 10 mM MgSO₄. Recovery of radioactivity was 86-88% for ³H and 100% for ¹⁴C (for both columns)

The profitable retention of heteroaminoacylated *E. coli* Phe-tRNA_{Val}¹ presents a new way to purify tRNA_{Val}¹. Fig. 3 shows a BD-cellulose chromatography of 19 A₂₆₀ units of enriched *E. coli* tRNA_{Val}¹ mischarged with ¹⁴C-Phenylalanine as described under Experimental. Stepwise elution was sufficient to attain a good separation from the bulk tRNA. As shown in Fig. 3, the achieved specific activity was about 1500 pmoles/A₂₆₀ units.

Figure 3: Purification of tRNA_{Val}¹. A sample of ¹⁴C-Phe-tRNA_{Val}¹ (see text) was loaded on a 0.9 x 20 cm column of BD-cellulose¹ previously equilibrated in 300 mM NaCl, 5 mM sodium formate buffer pH 4.0, 10 mM MgSO₄. Elution was stepwise, using the equilibrating buffer and the indicated salt concentrations. The A₂₆₀ profile is represented by two different scales, as indicated. Radioactivity was measured as in Figure 2. Overall recovery was 91%.

DISCUSSION

Fractionation of total *E.coli* tRNA in BD-cellulose results in the separation and enrichment of several tRNA species (Fig. 1A and B) and has been used as a first step in several tRNA purification procedures^{1,6,15}. Our results, in agreement with the original reports of Barnett^{9,14}, show that valine₁ and alanine *E.coli* tRNAs are heteroaminoacylated by the *N.crassa* Phe-tRNA synthetase. Although the extent of mischarging was 70% as calculated from the assays of the column fractions, with the recovered enriched tRNA^{Val}₁ peak (Fig. 1A) the attained level of heteroaminoacylation reached 88%. The lower extent detected during the scanning of the column was probably due to the salt present in the fractions¹⁶.

The chromatographic properties of enriched *E.coli* tRNA^{Val}₁ and its mischarged form here described, show that esterification with the aromatic amino acid was sufficient to retard significantly the elution of this tRNA. *Halobacterium cutirubrum* tRNA presents two different phenylalanine accepting species when chromatographed on BD-cellulose¹⁷. According to Griffiths¹⁷, with one of them the charged form does not shift its elution position, thus postulating that the properties of the tRNA itself are as important as the nature of the amino acid in determining the affinity with the BD-cellulose.

Our results (Fig. 2A and B) showed that at pH 4.0 and 4°C, Pnc-tRNA^{Val}₁ was not affected by the presence or absence of Mg²⁺, whereas Val-tRNA^{Val}₁ was slightly retarded when Mg²⁺ was omitted, changing its elution position from 0.73 M NaCl to 0.77 M NaCl. The uncharged tRNA^{Val}₁ changed from 0.68 M to 0.73 M NaCl in the absence of Mg²⁺. The differences in the chromatographic behaviour between aminoacylated and heteroaminoacylated *E.coli* tRNA^{Val}₁ whether in the presence of Mg²⁺ or not, may reflect a difference in the conformation assumed by the tRNA when esterified with valine or phenylalanine. Several reports have suggested that unacylated and aminoacylated tRNA may have different conformations¹⁹⁻²². Examination of this point by various methods has yielded conflicting results²¹, although negative results cannot exclude conformational changes which may be undetectable by the assay method⁶.

Our results suggest that not only the esterification of the amino acid, but also its nature influence the structure that the aminoacyl-

tRNA assumes.

The chromatographic properties of enriched *E.coli* tRNA₁^{Val} and its mischarged form were utilized to obtain the separation of a highly purified tRNA₁^{Val} by two simple chromatographic steps. In fact, the combination of two BD-cellulose cycles (Fig. 1 and Fig. 3), show that separation and extensive purification of tRNA₁^{Val} can be readily obtained.

This method is an attractive alternative to the one reported previously⁶. With the introduction of the mischarged intermediate, the derivatization step^{1,4,6} can be avoided. Difficulties in obtaining complete derivatization have been observed¹, which in some cases can decrease considerably the yield of the tRNA product⁶. For some tRNA species the use of a phenoxyacetyl derivative has resulted in a less active final product^{6,12}, possibly due to chemical damage^{15,23}. Friedman²⁴ has recently demonstrated that the N-hydroxysuccinimide ester of phenoxyacetic acid reacts with some minor nucleosides of certain species of tRNAs.

It is known that several other tRNAs can also be esterified with phenylalanine upon reaction with an heterologous enzyme^{10,11}. It would be interesting to extend these studies to tRNA species other than the one reported here. Studies on preparative-scale procedures, for the fractionation and isolation of several tRNAs, are currently under investigation.

FOOTNOTES

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Abbreviations: BD-cellulose, benzoylated DEAE-cellulose.

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