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Complete purification of tRNA, charged or modified with hydrophobic groups, by reversed-phase high-performance liquid chromatography on a C_4/C_{18} column system

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Abstract

Phe-tRNA^{Phe}, N-acetyl-Phe-tRNA^{Phe} and Leu-tRNA^{Leu-4} (from brewer's yeast and *Escherichia coli*) were each separated with baseline resolution from the uncharged tRNA species by using a wide-pore C_4 column and inverse salt gradient elution. The alterations at the 3′ end of the tRNAs result in a considerable shift of retention time on this column. The method is useful not only for obtaining tRNA preparations as required for poly(U) translational studies, but also for producing 20–50-mg amounts of tRNA for NMR and X-ray analysis. These aminoacylated species (charged by crude synthetase mixtures) can be purified from the crude tRNA mixtures in a one-step procedure.

1. Introduction

In protein synthesis, the aminoacyl-tRNA (aa-tRNA) molecules are the key to the decoding of the RNA messenger. A proper and productive interaction of aa-tRNA with the A site on the ribosome–mRNA complex is mediated by elongation factor (EF) Tu · GTP. The formation of the ternary complex EF-Tu · GTP · aa-tRNA and the tRNA–mRNA–ribosome interactions are of critical importance for an accurate peptide-chain elongation. Most studies on polypeptide-chain elongation are performed in poly(U) translating systems and require pure preparations of N-acetyl-Phe-tRNA^{Phe}, Phe-tRNA^{Phe}, deacylated tRNA^{Phe} and Leu-tRNA^{Leu-4} (for error frequency analysis). Large quantities are needed for NMR or crystallographic analysis of complexes such as EF-Tu · GTP · aa-tRNA. Purification of wild-type and mutant EF · Tu species for such purposes is fairly easy (for recent approaches see [1–4]). In contrast, it is more complicated to completely purify large quantities of specifically charged aminoacyl-tRNA as single species and in the literature we could not find an efficient and suitable procedure. We here describe a reversed-phase high-performance liquid chromatographic method with a wide-pore C_4 column for the efficient and large-scale purification of these charged and modified tRNA species from a crude mixture. This is the first comprehensive report on a methodology that can provide all the tRNA species necessary for

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poly(U) translation and error frequency analysis studies.

2. Materials and methods

2.1. HPLC instrumentation and methods

As HPLC-columns we used 250 × 4.6 mm I.D. Hypersil with 300 Å pore-size and 5 μm particles C_4 and C_{18} columns from Phenomenex (Torrance, CA, USA). As guard columns we used 30 × 4.6 mm I.D. C_4 and C_{18} Nucleosil columns from Macherey–Nagel (Düren, Germany) with the same particle and pore sizes.

The columns were operated at room temperature (about 20°C) on a Beckman System Gold HPLC system (Beckman Instruments, Fullerton, CA, USA). Gradient elution was performed with buffer A and buffer B, both containing 0.1 *M* potassium phosphate (final pH of 5.7), 1 m*M* sodium azide, and either 1.5 *M* ammonium sulphate or 4% 2-propanol, respectively. They were filtered through a Millipore HVLP filter (Millipore Intertech, Bedford, MA, USA), pore size 0.45 μm, prior to use. To increase reproducibility, each day buffer B was freshly prepared and 2-propanol was added *after* filtering. For HPLC analysis the samples were dissolved in 0.1 *M* potassium phosphate (pH 5.7), 1 m*M* sodium azide and 2 *M* ammonium sulphate prior to injection. The samples were eluted at a flow-rate of 0.75 ml/min using gradients as depicted in Figs. 1–3.

2.2. Isolation of crude tRNA synthetase mixtures

Crude tRNA synthetase mixtures were isolated from fresh baker's yeast (a kind gift of P.H.J. Mesters, Royal Gist Brocades, Delft, Netherlands) and *Escherichia coli* MRE600 (purchased from Porton Products, Maidenhead, UK). Cells were disrupted by grinding them in a mortar at 4°C with a double mass portion of alumina type A-5 (Sigma, St. Louis, MO, USA) and a trace of bovine pancreas deoxyribonuclease I (P-L Biochemicals, Milwaukee, WI, USA). Thereafter, the paste was diluted with standard buffer (64 m*M* TrisHCl pH 7.6, 5 m*M* $MgCl_2$, 60 m*M* NH_4Cl, 10 m*M* 2-mercaptoethanol, 1 m*M* sodium azide) and the alumina was removed by centrifugation at 3000 *g* for 30 min. The supernatant was subjected to ammonium sulphate fractionation at 4°C: the fraction of interest, between 45 and 68% saturation, was collected by centrifugation at 14 000 *g* for 30 min. The precipitate was redissolved in, and dialysed against standard buffer. This fraction was applied to a DEAE-Sephadex column in standard buffer. After thorough washing with standard buffer, the column was eluted with 300 m*M* NaCl in standard buffer. Fractions with absorbance at 280 nm were pooled and the tRNA-free crude synthetases were precipitated (70% saturated ammonium sulphate), redissolved and dialysed against standard buffer with 5% glycerol and stored at −80°C. For both preparations the protein concentration was about 15 μg/μl as determined with the "Coomassie Protein Assay Reagent" kit from Pierce (Rockford, IL, USA).

2.3. Aminoacylation of tRNA

Crude tRNA mixtures from *E. coli* MRE600 and brewer's yeast were bought from Boehringer Mannheim (Mannheim, Germany) whereas pure *E. coli* $tRNA^{Leu-4}$ (NAA), with 1.5 nmol of amino acid esterified per absorbance (260 nm) unit of tRNA, was bought from Subriden RNA (Rollingbay, Washington, DC, USA). Partially purified $tRNA^{Phe}$ from brewer's yeast was obtained as described in the Results section.

The crude tRNA mixtures were aminoacylated at 37°C for 20 min, using 0.75 mg of the homologous crude synthetase mixtures per 300 absorbance (260 nm) units of crude tRNA mixture in 1 ml standard buffer containing 1 m*M* ATP, 0.1 m*M* CTP, 10 μg/ml pyruvate kinase, 5 m*M* phosphoenolpyruvate and 0.1 m*M* of each of the 17 amino acid as present in the "Amino Acid Standard H" mixture from Pierce.

The (partially) pure $tRNA^{Phe}$ and $tRNA^{Leu}$ preparations were aminoacylated at 37°C for 20

min, using 0.75 mg of the homologous crude synthetase mixture per 10 absorbance (260 nm) units of tRNA in 0.5 ml of the same reaction mixture as above but with 0.15 m*M* Phe or Leu instead of the amino acid mixture. The aminoacylated tRNAs were subjected to one phenol extraction, followed by three times ethanol precipitation at −80°C for 30 min. The precipitates were lyophilised, in order to avoid interference by traces of ethanol with the proper elution of the tRNAs from the C_4 column.

Acetylation of the α-amino group of Phe-tRNA was carried out as described in Ref. [5].

3. Results

3.1. The effect of aminoacylation and acetylation on the retardation of tRNA on a C_4 column

Aminoacylation or modification of tRNA with hydrophobic groups might significantly influence its retention on a C_4 column. Accordingly Fig. 1 shows optimized separation of charged and acetylated partially purified brewer's yeast $tRNA^{Phe}$. In a control experiment, the acetylation procedure itself has no effect on the retention time of uncharged $tRNA^{Phe}$. In the same way, aminoacylation of $tRNA^{Leu}$ retards its migration significantly (Fig. 2). The elution order is $tRNA^{Phe}$, $tRNA^{Leu}$, Phe-$tRNA^{Phe}$, Leu-$tRNA^{Leu}$ and N-acetyl-Phe-$tRNA^{Phe}$, according to increasing hydrophobicity. To further investigate the effect of aminoacylation on the retardation of tRNA, we also compared crude uncharged *E. coli* tRNA with the same tRNA mixture charged in the presence of only phenylalanine, only leucine or the "Amino Acid Standard H" mixture (17 different amino acids). All the elution patterns are shown in Fig. 3. The Phe-$tRNA^{Phe}$ is clearly visible as a new peak beyond the bulk of uncharged tRNAs. The same is true for the aminoacylation of two out of the six iso-acceptors of $tRNA^{Leu}$. Similar results were obtained with a crude brewer's yeast tRNA mixture and phenylalanine (not shown).

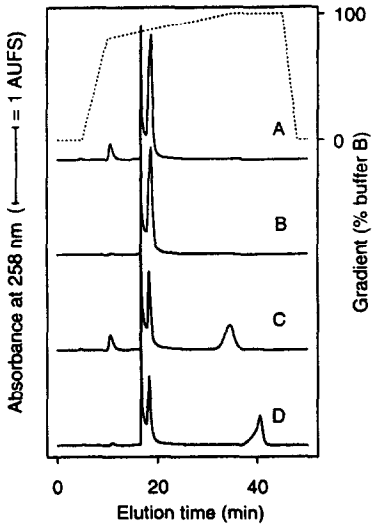

Fig. 1. The effect of aminoacylation and acetylation on the retention time of $tRNA^{Phe}$ from brewer's yeast. Elution patterns of non-aminoacylated (A, B) and aminoacylated (C, D) tRNA of about 2 absorbance (258 nm) units are shown, before (A, C) or after (B, D) acetylation with acetic anhydride. The retention times of residual traces of ATP and phenol are about 7 and 11 min, respectively. The dotted line indicates the elution gradient of buffers A and B. For further details see Materials and methods.

3.2. Collecting the C_4 column effluent directly on an in-line C_{18} column

The tRNA peaks cannot simply be recovered by ethanol precipitation because of coprecipitation of considerable amounts of sulphate and phosphate salts that are present in the column effluent. We therefore developed a new strategy

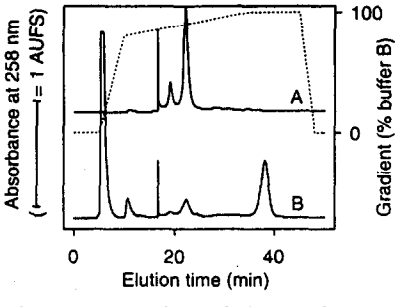

Fig. 2. The influence of aminoacylation on the retention time of $tRNA^{Leu-4}$ from *E. coli*. Elution patterns of non-aminoacylated (A) and aminoacylated (B) tRNA samples of about 1 absorbance (258 nm) unit are shown (see caption to Fig. 1).

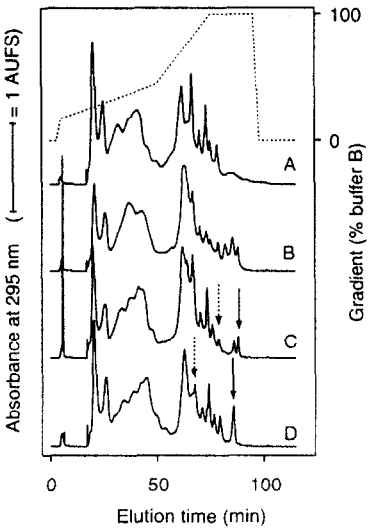

Fig. 3. The resolution of aminoacylated tRNAs from *E. coli*. Elution patterns are shown of samples [about 300 absorbance (258 nm) units each] of non-aminoacylated crude tRNAs (A) or tRNA aminoacylated in the presence of either the "Amino Acid Standard H" mixture (B), leucine (C) or phenylalanine (D). For $tRNA^{Leu-4}$ (C) and $tRNA^{Phe}$ (D), the positions of uncharged (dotted arrow) and charged (solid arrow) species are indicated (see caption to Fig. 1).

by connecting one or several wide-pore C_{18} columns via a switching valve to the outlet of the detector (Fig. 4). In this way, the peak (or peaks) of interest can be loaded in-line onto the C_{18} column(s). The macromolecules in the C_4 column effluent will again bind to the C_{18} column because its matrix is much more hydrophobic. One or more other peaks can be collected onto a subsequent C_{18} column and used for other purposes. After the C_4 run is finished, each C_{18} column is in turn directly connected to the HPLC pump, thoroughly washed with distilled water, and the absorbed tRNA is eluted with 35% (v/v) ethanol. Finally, the collected tRNA can be concentrated by simple ethanol precipitation with 70% (v/v) ethanol or by

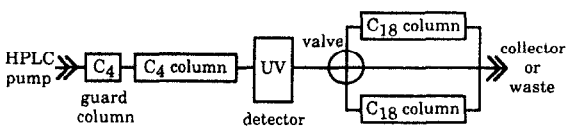

Fig. 4. Schematic illustration of the C_4/C_{18} chromatographic system for the preparative isolation of tRNAs.

lyophilisation, whichever is more convenient. Both aminoacyl- and acetylaminoacyl-tRNA can be recovered without significant deacylation as judged by an analytical run on a C_4 column. Very satisfying results are also obtained with a preparative C_4 column (250 × 22.5 mm I.D.) for large-scale isolations of $tRNA^{Phe}$. For example, the uncharged but partially purified brewer's yeast $tRNA^{Phe}$ [of about 0.9 nmol of phenylalanine esterified per absorbance (260 nm) unit] that we used in this paper, was isolated from crude brewer's yeast tRNA [about 55 pmol of phenylalanine esterified per absorbance (260 nm) unit] with a yield of 80% according to the methodology described above (see Fig. 1).

4. Discussion and conclusions

Although the isolation of some species of non-aminoacylated tRNA is relatively easy and well documented (see for example [6–8] and references cited therein), this is less so for aminoacylated tRNA, whereas for certain applications the availability of pure and fully aminoacylated tRNA preparations is essential. In some methods of aa-tRNA purification, affinity chromatography on a column with immobilized EF-Tu is used [9,10] and in other cases use is made of mixed-mode columns [7,11] or C_{18} columns [12]. However, there are several important drawbacks to these procedures. Except for the C_{18} support, the columns are not commercially available and laborious to prepare. In the mixed-mode methodologies the tRNA cannot simply be recovered by ethanol precipitation (as discussed further on). Additional drawbacks of the affinity chromatography methodology are (a) the large amounts of expensive EF-Tu that are needed for the coupling to the matrix, (b) the relatively short durability of the affinity column (several months), (c) the inability to separate iso-accepting tRNA species after aminoacylation, and (d) the inability to separate uncharged from N-acetylated aminoacyl-tRNA. The normal-pore (100 Å) C_{18} HPLC columns lack sufficient tRNA-binding capacity [for a 300 × 3.9 mm I.D. column this is only 50 absorbance (260 nm)

units], although they can separate complex mixtures of uncharged tRNA species as shown recently [13].

In our view the wide-pore C_4 column is the optimal choice for the purification of tRNA charged or modified with hydrophobic groups (see also [14]). In addition to the high level of resolution (see Figs. 1–3), the wide-pore C_4 column is durable and has a relatively large binding capacity: the 250 × 4.6 mm I.D. Hypersil 300-5 C_4 column can be loaded with 600 absorbance (260 nm) units of tRNA. This is much more than the 10–20 absorbance (260 nm) units of tRNA a 100 Å C_4 column of the same dimensions can handle [15]. This can be explained by the diameter of the tRNA molecule which is about 75 Å (see also the 100 Å pore C_{18} column mentioned above).

The main bottleneck of previous mixed-mode and C_4 HPLC methods is the somewhat inefficient and time-consuming diafiltration or precipitation procedure [7,14,15] of the pooled tRNA fractions. Especially for aa-tRNA with its labile ester bound, a fast isolation procedure is commendatory. The in-line C_{18} column (see Fig. 4) provides an adequate solution. Now, Phe-$tRNA^{Phe}$, N-acetyl-Phe-$tRNA^{Phe}$ and Leu-$tRNA^{Leu-4}$ can individually be separated from the bulk of tRNAs (Figs. 2 and 3) and immediately loaded onto the C_{18} column. This dramatically simplifies the purification of amounts up to 1 mg of these charged species. For NMR and X-ray applications large amounts (20–50 mg) of aa-tRNA are needed and the desired species of non-aminoacylated tRNA can be collected on an in-line C_{18} column with repetitive sample applications of the crude tRNA mixture on the C_4 column. The collected enriched tRNA fraction is afterwards aminoacylated and separated from non-aminoacylated tRNA as described. The method may be of more general use, because the relatively large effects of aminoacylation and N-acetylation on the retention time point to an exposed position of both the amino acid side chain and the N-acetyl group. For example, tyrosine has a similar effect as phenylalanine (results not shown) and also Trp-tRNA was recently found at a retarded position on a 100 Å C_4 column [15]. The change in retention time may also reflect changes in the overall three-dimensional structure of the tRNA molecule upon charging and modification.

It is important to check the technical specifications of the detector cell for the increased back pressures. A proper choice of the dimensions and specifications (pore and particle size) of the C_{18} column can thus be made. In our case the C_{18} column has a back pressure of about 4.5 MPa at a flow-rate of 0.75 ml/min whereas the Beckman 166 detector module can withstand pressures up to 7 MPa.

In conclusion, the in-line combination of both a wide-pore C_4 and C_{18} column provides an efficient system for the purification of tRNA charged or modified with hydrophobic groups. The above described methodology can provide *all* the tRNA species necessary for poly(U) translation studies. The same approach may be successful for the purification of other tRNA species as well. It can easily be scaled up to meet the quantitative and qualitative requirements of NMR or X-ray experiments.

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