Isolation and chromatographic behaviour of phenylalanine tRNA from barley embryos

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Received 2 October 1974

ABSTRACT

Two fractions of phenylalanine tRNA ($\text{tRNA}_1^{\text{Phe}}$ and $\text{tRNA}_2^{\text{Phe}}$) were purified by BD-cellulose and RPC-5 chromatography of crude tRNA isolated from barley embryos. Successive RPC-5 rechromatography runs of $\text{tRNA}_2^{\text{Phe}}$ showed its conversion into more stable $\text{tRNA}_1^{\text{Phe}}$, suggesting that the two fractions have essentially the same primary structure. Both $\text{tRNA}_1^{\text{Phe}}$ and $\text{tRNA}_2^{\text{Phe}}$ had about the same acceptor activity, but $\text{tRNA}_2^{\text{Phe}}$ was aminoacylated much faster than $\text{tRNA}_1^{\text{Phe}}$. RPC-5 chromatography of crude aminoacylated tRNA showed higher contents of phe- $\text{tRNA}_2^{\text{Phe}}$ than of phe- $\text{tRNA}_1^{\text{Phe}}$ but the ratio of these two fractions estimated by relative fluorescence intensity was about 1. Fluorescence spectra of $\text{tRNA}_1^{\text{Phe}}$ from barley embryos suggest that it contains Y base similar to Y_w from wheat $\text{tRNA}_1^{\text{Phe}}$.

INTRODUCTION

Differences in number and levels of isoaccepting tRNAs in diverse tissues and at various physiological states of the same organism indicate that transfer RNAs are involved in cell differentiation and translational regulation (for review see 1, 2). Protein biosynthesis in dormant biological forms (spores etc.) may be controlled by the absence of 3'-terminal residues in tRNA molecules (3, 4). However, the precise mechanism of tRNA regulatory functions is still not clear.

Recently Hiatt and Snyder have reported nine phenylalanine tRNA species in developing barley seedlings (5). One may assume that some of these isoacceptors derive from a common precursor and differ only by its posttranscriptional modifications, which can be related with the control functions of these tRNAs. This assumption can be partly answered by comparison of purified barley phenylalanine tRNA. The present paper describes isolation and some properties of $\text{tRNA}_1^{\text{Phe}}$ from barley embryos

MATERIALS AND METHODS

BD-cellulose and DEAE-cellulose were from Serva, Sephadex G-200

from Pharmacia, [^{14}C]-L-phenylalanine (sp. act. 522 mCi/mole) and [^3H]-L-phenylalanine (sp. act. 1 Ci/mole) were from Amersham. Plaskon CTFE 2300 powder and Adogen 464 were generous gifts from Dr. B. S. Dudock. Other chemicals were commercially available reagent grade.

Crude tRNA from barley embryos (commercial brans sieved to remove grain covers) was obtained as described by B. S. Dudock et al. (6). Details of the procedure are given elsewhere (7). Polyacrylamide electrophoresis (8) in 7.5% gel showed that the tRNA preparation contained 10-15% of 5S RNA and no high molecular weight material. Phenylalanine acceptor activity varied from 25 to 30 pmoles per A_{260} unit depending on tRNA preparation batch.

Aminoacylation of tRNA was carried out with baker's yeast aminoacyl-tRNA synthetase and/or with barley enzyme. Crude yeast aminoacyl-tRNA synthetase was prepared by toluenolysis of yeast paste according to (9), centrifugation of the obtained lysate at $105,000 \times g$ for 1.5 hour and ammonium sulfate fractionation according to (10) followed by stepwise elution from DEAE-cellulose. Fraction containing synthetase activity was dialysed and stored as described (9). Crude barley phenylalanine-tRNA synthetase was obtained as follows: ground barley grains (100 g) were extracted in cold room for 3 hours with 300 ml of 0.01M potassium phosphate buffer, pH 7.2, containing 2 mM MgCl_2 , 5 mM 2-mercaptoethanol and 10% glycerol. The resulting extract was filtered through a mira-cloth, centrifuged at $10,000 \times g$ for 15 min, and the supernatant was fractionated with ammonium sulfate. The fraction precipitating between 40 and 70% of ammonium sulfate saturation was dissolved in extraction buffer and applied on a Sephadex G-200 column (5 x 34 cm) equilibrated with the same buffer. The fractions emerging from the column at $V_e/V_o = 1.44$ were collected, dialysed against 60% glycerol in extraction buffer and stored at -18° for at least one month without noticeable loss of activity.

The incubation mixture for aminoacylation assay with the yeast enzyme contained in 100 μl final volume: 10 μmoles Tris-HCl buffer, pH 8.0, 1 μmole ATP, 0.1 μmole CTP, 5 μmoles MgCl_2 , 1 μmole 2-mercaptoethanol, 5 nmoles of radioactive phenylalanine, 1 A_{260} unit of crude tRNA (or corresponding amount of purified tRNA^{Phe}) and sufficient enzyme preparation (10-50 μg of protein). The mixture was incubated for 10 min at 37° . When barley enzyme was used, 100 μl of the incubation mixture contained 12.5 μmoles of Tris-HCl buffer, pH 8.5, 0.4 μmole MgCl_2 , 0.4 μmole ATP,

4 nmoles of radioactive phenylalanine, 1 A₂₆₀ unit of crude tRNA and about 100 µg of crude enzyme preparation. The incubated aliquots were applied on Whatman 3 MM paper discs and processed as in (12). Radioactivity was measured in toluene based scintillation solution with Packard Tri-Carb Model 2425 scintillation spectrometer.

The samples for analytical RPC-5 chromatography of [³H]-Phe-tRNA^{Phe} were prepared by scaled up aminoacylation procedure with incubation time up to 30 min. Radioactive aminoacyl-tRNA was recovered from the incubation mixture on a small DEAE-cellulose column as described (11).

BD-cellulose chromatography was carried out according to (13), and RPC-5 chromatography according to (14, 15). Details are given in legends to the figures. The pumps and hydraulic system of an aminoacid analyser were used for running RPC-5 columns. Eluates from the columns were tested for A₂₆₀, fluorescence at 420 nm (excitation 310 nm) and phenylalanine acceptance. Barley and yeast enzymes gave essentially the same results, however, the yeast enzyme, being less salt sensitive, was preferred.

Fluorescence measurements were done with Hitachi MPF-4 fluorescence spectrophotometer equipped with 300 W xenon lamp. Measurements were carried out in 1 x 1 cm quartz cells at room temperature. Emission and excitation slits were set at 12 nm.

RESULTS

RPC-5 chromatography pattern of crude tRNA charged with [³H]-phenylalanine is shown in fig. 1. Two radioactivity peaks were observed, a major one (containing roughly 90% of radioactivity) following the minor one, independently on the enzyme used for aminoacylation.

Purification of tRNA^{Phe} on preparative scale involved three steps: BD-cellulose chromatography of crude tRNA, RPC-5 chromatography at pH 5.0 and rechromatography on RPC-5 columns at pH 7.5. Phenylalanine acceptor activity was eluted from BD-cellulose columns in two peaks (fig. 2), one at the end of the salt gradient (fraction S) and the second one with the ethanol containing buffer (fraction Et). Proportion of S to Et fractions varied in different BD-cellulose runs. Fractions S and Et accepted 150-200 pmoles of phenylalanine per 1 A₂₆₀ unit and showed significant fluorescence when excited at 310 nm. Maximum of this fluorescence measured directly in fractions eluted from BD-cellulose column was at 370-400 nm, due probably

to some contaminations accompanying tRNA^{Phe}. When tRNA was freed from the contaminations (by passing it through DEAE-cellulose, RPC-5 or by precipitation with ethanol) both S and Et fractions showed identical fluorescence spectra with emission.

Fig.1. 5 A₂₆₀ units of crude tRNA charged with [³H]-phenylalanine were mixed with 25 units of carrier crude tRNA, applied on a RPC-5 column (1 x 40 cm) and eluted with 300 ml of linear NaCl (0.4-0.9M) gradient in 0.01M sodium acetate buffer, pH 5.0, containing 10 mM MgCl₂, 2 mM EDTA, 2 mM 2-mercaptoethanol and 0.01% NaN₃.
 — [³H] cpm, A₂₆₀.

Fig. 2. BD-cellulose chromatography of crude barley tRNA. On a 2.5 x 130 cm column 15,000 A₂₆₀ units were applied and eluted with NaCl gradient (0.35-1.5M) in 0.05M sodium acetate buffer, pH 5.0 (containing 10 mM MgCl₂, 2 mM EDTA, 2 mM 2-mercaptoethanol and 0.01% NaN₃) followed by 1.5M NaCl in the same buffer containing 20% ethanol. — [³H]cpm, A₂₆₀

Fig. 3. Uncorrected fluorescence spectra of barley tRNA^{Phe}. — emission (excitation 310 nm), excitation (emission 420 nm).

maximum at 430 nm. The uncorrected excitation and emission spectra measured directly after RPC-5 chromatography are shown in fig. 3. RPC-5 chromatography of the fractions S and Et from BD-cellulose resulted in further purification of tRNA^{Phe} and separation of each of the chromatographed fractions into two peaks of phenylalanine acceptance and 420 nm fluorescence, designated respectively S-1, S-2, Et-1 and Et-2 (fig. 4). Fraction S-1 was eluted from RPC-5 columns at the same NaCl concentration as Et-1. Also, fractions S-2 and Et-2 were eluted at identical NaCl molarity. As judged by fluorescence and A₂₆₀, fraction S from BD-cellulose gave after RPC-5 chromatography more S-1 than S-2, while fraction Et gave more Et-2 than Et-1. Aminoacylation test showed always that the later eluting peak was the main acceptor of phenylalanine. Rechromatography of the functions S-1 or Et-1 on RPC-5 at pH 7.5 gave essentially identical patterns. As shown in fig. 5, phenylalanine acceptance

Fig. 4. RPC-5 chromatography at pH 5.0 of 700 A₂₆₀ units of the fraction S from BD-cellulose. RPC-5 column (1.5 x 100 cm) was eluted with 1200 ml of linear NaCl gradient (0.5-0.75M) in buffer as in fig. 1. — [3H]cpm, ---- 420 nm fluorescence, absorbance at 260 nm.

Fig. 5. Rechromatography of fraction Et.1 on RPC-5 at pH 7.5. 50 A_{260} units were applied on a 1 x 40 cm column and eluted with NaCl gradient (0.5-0.7M) in 0.01M Tris-HCl pH 7.5 buffer containing 10 mM $MgCl_2$, 2 mM EDTA and 2 mM mercaptoethanol. — $[^3H]$ cpm, ---- 420 nm fluorescence, absorbance at 260 nm. Essentially the same pattern was obtained with fraction S-1.

Fig. 6. Rechromatography of fraction S-2 on RPC-5 at pH 7.5. Conditions as in fig. 5. — $[^3H]$ cpm, ---- 420 nm fluorescence, absorbance at 260 nm. Essentially the same pattern was obtained with fraction Et-2.

and fluorescence of these fractions overlapped with the peak of A_{260} , indicating their apparent homogeneity. RPC-5 rechromatography at pH 7.5 of the fractions S-2 or Et-2 resulted in their separation, again into two closely eluting peaks of A_{260} and fluorescence (fig. 6) with the maximum of phenylalanine acceptor activity appearing in the second peak.

Cochromatography of fractions S-1 and Et-1 on RPC-5 column at pH 7.5 gave the same pattern as shown in fig. 5. By a similar cochromatography experiment the fraction S-2 could not be distinguished from the fraction Et-2.

The described chromatographic properties of barley tRNA^{Phe} indicated identity of fractions S-1 with Et-1 and S-2 with Et-2. Therefore the appropriate fractions were combined. The pooled fractions number 2 (tRNA₂^{Phe}) had the acceptor activity of 1 nmole of phenylalanine per A_{260} unit when measured under aminoacylation conditions described in methods. Under the same conditions the combined fractions number 1 (tRNA₁^{Phe}) accepted only about 0.25 nmole Phe per A_{260} unit. This fraction, however, reaches the same aminoacylation level as fraction number 2 after an extended incubation time.

DISCUSSION

Combination of BD-cellulose and RPC-5 chromatography proved, in agreement with the suggestion of other authors (15, 16), to be effective in purification of specific tRNAs also from plant material. BD-cellulose was found to be very useful in bulk separation of tRNA^{Phe} from most of the other tRNAs. However, chromatographic patterns of tRNA^{Phe} on BD-cellulose were not reproducible. As shown by RPC-5 chromatography the tRNA^{Phe} fractions S and Et obtained from BD-cellulose contained the same components (tRNA₁^{Phe} and tRNA₂^{Phe}) though not in equal proportions. For these reasons interpretation of BD-cellulose patterns should be very cautious, especially in the high salt-organic solvent region. Both preparative procedure and analytical RPC-5 chromatography of [³H]phenylalanine-tRNA showed the presence of two tRNA fractions accepting phenylalanine (tRNA₁^{Phe} and tRNA₂^{Phe}) in barley embryos. The chromatographic behaviour of tRNA₁^{Phe} and tRNA₂^{Phe} described in the present paper indicates that tRNA₁^{Phe} derives from tRNA₂^{Phe}. Thus the two isolated tRNA^{Phe} fractions are not individual isoacceptors but different molecular forms of the same tRNA species. Recently Hiatt and Snyder (5) found in barley germs nine phenylalanine tRNAs. Results described in the present paper and similar observations of Yoshikami and Keller (18) who found three fractions of tRNA^{Phe} in wheat germ, all of which were

derived from the same primary structure, suggest that tRNA^{Phe} fractions observed by Hiatt and Synder also were not individual isoacceptors of phenylalanine.

The two isolated phenylalanine tRNAs from barley embryos (tRNA₁^{Phe} and tRNA₂^{Phe}) represented about 1% (by weight) of starting crude tRNA. In terms of phenylalanine acceptor activity the yield was about 50%. As judged by chromatography the isolated tRNA₁^{Phe} was more than 80% pure. The purity of tRNA₂^{Phe} was difficult to determine by a similar test since this fraction was not stable and was partly converted into tRNA₁^{Phe}. However tRNA₂^{Phe} did not contain any other RNA detectable by RPC-5 chromatography, except tRNA₁^{Phe}. Assays of the acceptor activity indicated about 60% purity of the two tRNA^{Phe} fractions. This is the lowest possible estimate since the two fractions contained (as shown by preliminary analysis) less than 1 mole of 3' terminal adenosine per mole of tRNA.

The two isolated fractions of tRNA^{Phe} from barley differed also by kinetics of their aminoacylation. Fraction 1 was esterified slowly, while the other one reached plateau of aminoacylation within 10 min. This difference explains why on the RPC-5 chromatograms of aminoacylated tRNA, the radioactivity of fraction 2 was much higher than that of fraction 1, while both the fractions showed about the same intensity of fluorescence. If similar differences in kinetics of aminoacylation are common, then many results describing 'major' and 'minor' isoacceptors should be reinvestigated. Recently Mushinski (17) showed, that depending on the method of aminoacyl-tRNA synthetase preparation, in normal rat liver one or two phenylalanine tRNAs can be detected. Till now it was believed that the minor, early eluting peak was an isoacceptor characteristic only for Morris hepatomas cells.

Fluorescence of tRNA^{Phe} from barley embryos closely resembles that of phenylalanine tRNAs from other eukaryotic organisms (6, 19-21). The emission maximum at 420 nm suggest that barley tRNA^{Phe} contains peroxy Y base (22) but this has to be confirmed by a separate study.

ACKNOWLEDGEMENTS

We are grateful to Professor Dr. J. Pawełkiewicz for his interest and helpful discussions of this paper. This work was supported by the Polish Academy of Sciences within the project 09.3.1.

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