EVIDENCE FOR A CHLOROPLAST SPECIFIC TYROSYL tRNA DEGRADING ACTIVITY

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Received June 29,1976

SUMMARY

A ribonuclease which degrades two of the four tyrosyl-tRNA isoaccepting species from green soybean cotyledons appears to be located on or in chloroplasts. Furthermore, the enzyme appears to be specific for the two chloroplastic tyrosyl-tRNAs ($tRNA^{Tyr}_{3\&4}$). The initial product of the nuclease reaction is about the size of a half-molecule. The deacylated product is not recognized by its original cognate synthetase. As estimated by fractionation on Sephadex G-150, the nuclease has a molecular weight greater than 60,000 daltons. The physiological function of the nuclease has not been determined.

Regulation of the concentrations of tRNA isoaccepting species in biological systems may serve as regulatory steps in protein synthesis (1,2). Levels of isoaccepting tRNAs are controlled by their rates of synthesis or degradation To control the rate of degradation of specific tRNA isoacceptors, ribonucleases specific for these tRNA isoaccepting species must be present in the cell. Examples of ribonucleases specific for single leucyl-tRNA isoacceptors have been demonstrated (3,4,5). Sueoka and his co-workers (3) suggested the existence of a ribonuclease which "nicks" a leucyl tRNA species from E. coli during the first two minutes after T-even phage infection. This "nicking" is believed to be associated with phage shut-down of host cell protein synthesis. In the case of plant systems investigated (4,5) no suggestion of the function of the specific ribonucleases has been made.

This report demonstrates the existence of an activity which partially degrades chloroplast tyrosyl-tRNA species, and may be localized in or associated with chloroplasts.

MATERIALS AND METHODS

RPC-5 chromatographic adsorbent was purchased from Miles Laboratories, Inc. Sephadex G-100 was purchased from Pharmacia Fine Chemical Co., and ^{3}H-L-tyrosine (8 or 40 Ci/mmole) was purchased from Schwarz-Mann, Inc. Certified Wayne Soybeans were obtained from Purdue Ag Alumni Seed Association, Remington, Indiana.

Isolation of Chloroplasts: Chloroplasts were prepared from green 6-day-old soybean cotyledons. 500 gm of cotyledons were homogenized in 1 L of grinding medium (0.5 m sucrose, 40 mM potassium phosphate, pH 7.8, 1 μM tyrosine, 0.1 mM EDTA, and 5 mM 2-mercaptoethanol) for 30 seconds at full speed in a Sorvall Omnimixer. The homogenate was filtered through cheesecloth and Miracloth, and centrifuged at 3000 xg for 10 minutes.

The 3000 xg pellet was suspended in 35% sucrose, containing 20 mM Tris-HCl, pH 7.4, 1 μM tyrosine, 0.1 mM EDTA and 0.2 mM dithiothreitol. Aliquots of the suspended pellet were layered over 25 ml 35-65% sucrose gradients followed by centrifugation at 20,000 RPM in an SW-25 Rotor for 4 hours. The OD_{650} peak on the gradient was pooled, diluted with 1 volume of cold distilled water and the chloroplasts spun down at 10,000 xg for 20 minutes.

RPC-5 Chromatography: Four tyrosine isoaccepting tRNA species were routinely observed on RPC-5 columns (6). Other minor peaks shown in elution profiles were not enzymatically recognizable after RPC-5 chromatography, or were not present in post charged profiles, and in some cases, were not consistently present in all preparations; and therefore, these minor peaks were not considered real. The four isoacceptors are referred to as $tRNA_1^{Tyr}$, $tRNA_2^{Tyr}$, $tRNA_3^{Tyr}$, and $tRNA_4^{Tyr}$ based on their elution position from RPC-5.

Isolation of tRNA and tyrosyl-tRNA Synthetases, and Assay of tyrosyl-tRNA synthetases: Total soybean soluble RNA, and BDC-ethanol fraction tRNA were prepared as previously reported (6). Cytoplasmic and chloroplastic tyrosyl-tRNA synthetases were prepared and assayed by the method previously reported (6). Cytoplasmic tyrosyl-tRNA synthetase was purified to near homogeniety and gave only a single band in gel electrophoresis. Purified chloroplast tyrosyl-tRNA synthetase was about 50% pure, showed 3 bands in gel electrophoresis, and was free of cytoplasmic tyrosyl-tRNA synthetase activity. No evidence of degradative activity for chloroplast or cytoplasmic tRNA species was observed in these purified chloroplast or cytoplasmic synthetase preparations. Cytoplasmic tyrosyl-tRNA synthetase acylated only the later two eluting tyrosyl-tRNA isoaccepting species, $tRNA_{3\&4}^{Tyr}$.

RESULTS

Acylation of tRNA by a chloroplast Lysate: A sample of the lysate obtained from gradient purified chloroplasts was incubated in a standard chloroplast enzyme reaction mixture except that the tRNA was omitted from the reaction. Under these circumstances, endogenous chloroplast tRNA will be acylated with endogenous chloroplast synthetase. Figure 1 shows an RPC-5 elution profile

Abbreviations: EDTA, ethylenediaminetetraacetic acid; BDC, benzoylated diethylaminoethyl-cellulose.

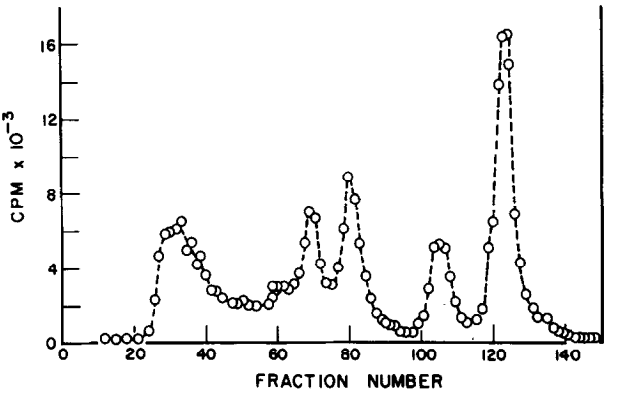

Figure 1. RPC-5 column chromatography of endogenous tRNA from a chloroplast lysate. A sample of the lysate from sucrose gradient purified chloroplasts was incubated for 10 minutes at 30°C in a standard chloroplast reaction mixture which contained 50 mM HEPES, pH 7.6, 2 mM ATP, 4 mM $MgCl_2$ and 9 μM L-^{3}H-tyrosine. The acylated tRNA was purified on DEAE cellulose (7) and chromatographed on an RPC-5 column (see Methods).

of such an acylation. The four tyrosyl-tRNA isoaccepting species of soybean cotyledons are all present in Figure 1, since no attempt was made to eliminate cytoplasmic tRNA and synthetase, nonspecifically bound to the outside of the chloroplasts. An additional pre-$tRNA_1^{Tyr}$ peak is also observed. Attempts to enzymatically deacylate the pre-$tRNA_1^{Tyr}$ peak in the presence of AMP and PPi were unsuccessful under conditions which deacylated over 90% of the four tyrosyl tRNAs. Also, the pre-$tRNA_1^{Tyr}$ peak could be deacylated by incubation at 37°C for 60 min. in 1 M Tris-HCl, pH 9.0, but not reacylated with tyrosine. These facts and the early elution position from RPC-5 of the pre-$tRNA_1^{Tyr}$ peak suggest that this peak may be a partially degraded tRNA.

G-100 Chromatography of pre-$tRNA_1^{Tyr}$: In order to estimate the size of the pre-$tRNA_1^{Tyr}$ peak, the appropriate fractions from an RPC-5 column were pooled, concentrated, combined with 10 OD_{260} units of total tRNA, and chromatographed on a 2.5 x 90 cm Sephadex G-100 column. Figure 2 shows that the radioactivity from the pre-$tRNA_1^{Tyr}$ peak elutes well behind the OD_{260} of the total tRNA, suggesting that the pre-$tRNA_1^{Tyr}$ peak is smaller than intact tRNA molecules. Each

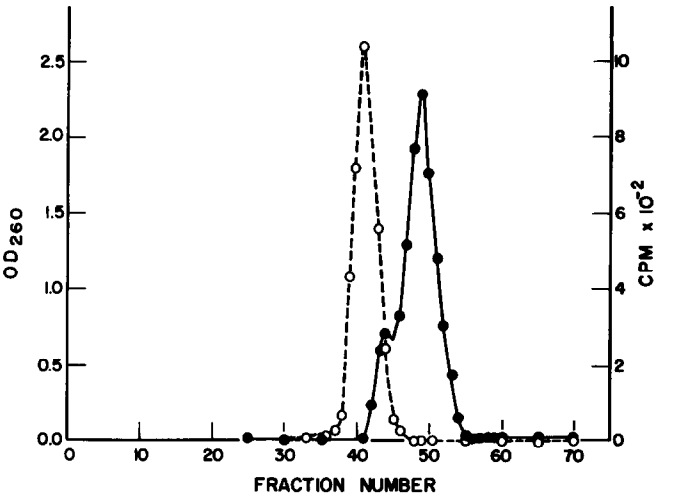

Figure 2. Sephadex G-100 column chromatography of the pre-$tRNA_1^{Tyr}$ peak from RPC-5. Fractions of radioactive pre-$tRNA^{Tyr}$ peak from an RPC-5 column were pooled, dialyzed, and concentrated by DEAE cellulose (7). After adding 10 OD_{260} units of total tRNA to the sample, the mixture was chromatographed on a Sephadex G-100 column (2.5 x 90 cm) at a flow rate of 20 ml per hr. 5 ml fractions were collected. Open circles represent OD_{260} eluted from the column and closed circles represent CPM.

of the four tyrosyl-tRNA isoacceptors is coincident with total tRNA when chromatographed on G-100 Sephadex. This evidence suggests that the chloroplast lysate contains an enzymatic activity capable of degrading one or more of the four tyrosyl-tRNAs.

tRNA Specificity of the Degrading Activity in the Chloroplast Lysate: Purified tyrosyl synthetases from soybean cotyledons (6) were employed to examine the tRNA specificity of the degrading activity. Cytoplasmic tyrosyl-tRNA synthetase acylates only $tRNA_1^{Tyr}$ and $tRNA_2^{Tyr}$, while chloroplast tyrosyl-tRNA synthetase acylates only $tRNA_3^{Tyr}$ and $tRNA_4^{Tyr}$ (6). Accordingly, samples were acylated with each of the two purified enzymes and the acylated samples were incubated either with buffer or with chloroplast lysate. Figure 3A shows the RPC-5 elution profile for a sample acylated with cytoplasmic enzyme and incubated with buffer for 15 minutes. Figure 3B shows the elution profile from an RPC-5 column of the sample acylated with cytoplasmic tyrosyl-tRNA synthetase

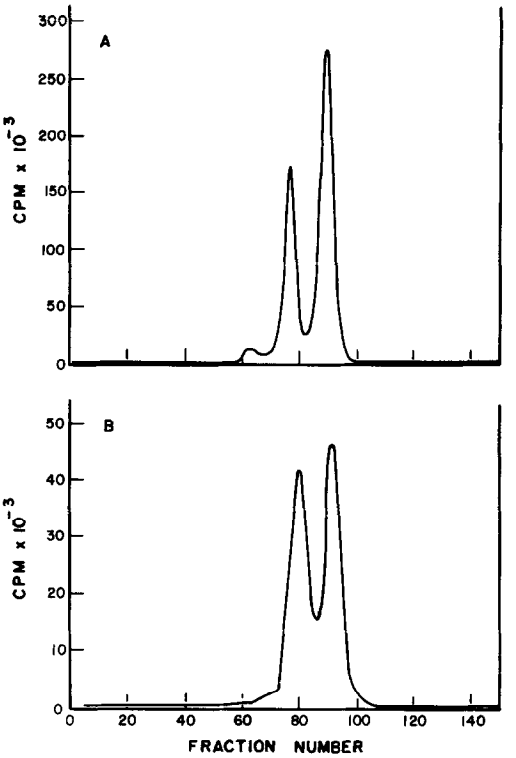

Figure 3. RPC-5 column chromatography of acylated $tRNA^{Tyr}_{1\&2}$ after incubation in the presence (A) and absence (B) of tyrosyl-tRNA degrading enzyme. Two samples of tRNA were acylated for 30 minutes at 30°C using cytoplasmic tyrosyl-tRNA synthetase and the standard cytoplasmic synthetase reaction mixture. The first sample (Figure 3A) was diluted with 20 mM potassium phosphate buffer, pH 6.7. The second sample (Figure 3B) was diluted 9 fold with buffer, and an aliquot of crude chloroplast lysate was added to make a 10-fold dilution. Both samples were then incubated for 15 minutes at 30°C prior to chromatography on an RPC-5 column (see Methods).

and incubated with chloroplast lysate for 15 minutes. $tRNA^{Tyr}_{1\&2}$ are still intact and no evidence of pre-$tRNA^{Tyr}_{1}$ peak can be found in Figure 3B. This indicates that $tRNA^{Tyr}_{1\&2}$ are not degraded by the chloroplast lysate. However, the results of the same experiment using chloroplast tyrosyl-tRNA synthetase to acylate the tRNA (see Figures 4, 4B and 4C) show that after 5 minutes incubation of $tRNA^{Tyr}_{3\&4}$ with chloroplast lysate a sizable pre-$tRNA^{Tyr}_{1}$ peak has appeared, and after 15 minutes of incubation, all of the radioactivity in the profile is

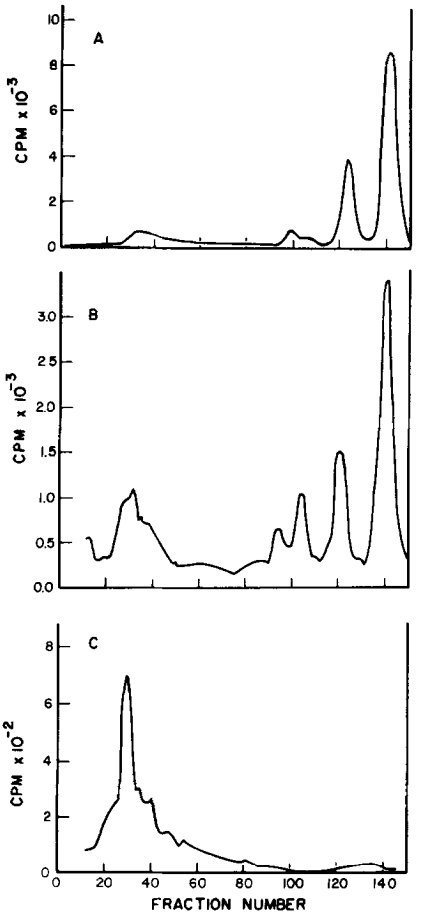

Figure 4. RPC-5 column chromatography of acylated $tRNA^{Tyr}_{3\&4}$, after incubation in the presence and absence of tyrosyl-tRNA degrading enzyme. Three samples of BDC-ethanol fraction tRNA were acylated for 30 minutes at 30°C using chloroplast tyrosyl-tRNA synthetase and the standard chloroplast synthetase reaction mixture. The first sample was diluted 10 fold with 20 mM potassium phosphate buffer, pH 6.7. The second and third samples were diluted 9 fold with buffer, and an aliquot of crude chloroplast lysate was added to make a 10-fold dilution. The first sample (Figure 4A) was incubated for 5 minutes, and the third sample (Figure 4C) was incubated for 15 minutes. RPC-5 chromatography was performed as outlined in Methods.

found in the pre-$tRNA^{Tyr}_1$ peak. Therefore, it appears that the degrading activity in the chloroplast lysate is specific for $tRNA^{Tyr}_{3\&4}$.

The specificity of the degrading activity for tRNA species charged by chloroplast tyrosyl-tRNA synthetase is also shown in Figure 5. If an enzyme

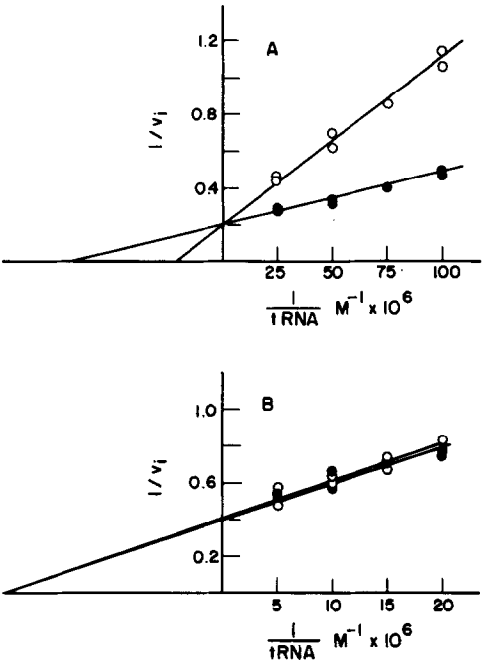

Figure 5. Inhibition of chloroplast and cytoplasmic tyrosyl-tRNA synthetases by tyrosyl-tRNA degrading enzyme. Initial velocities were determined at varying tRNA concentrations for both chloroplast and cytoplasmic tyrosyl-tRNA synthetases in the presence and absence of an aliquot of crude chloroplast lysate. Figure 5A shows a Lineweaver-Burk plot with tRNA as varied substrate for chloroplast tyrosyl-tRNA synthetase in the presence (open circles) and in the absence (closed circles) of chloroplast lysate. Figure 5B shows a Lineweaver-Burk plot with tRNA as varied substrate for cytoplasmic tyrosyl-tRNA synthetase in the presence (open circles) and in the absence (closed circles) of chloroplast lysate.

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were degrading unacylated tRNA during initial velocity assays, and acylated tRNA (whole or partly degraded) was retained by the GF/A filter, then the effect of the nuclease would be to remove substrate from the acylation assay. Utilizing tRNA as a variable substrate and under rate-limiting conditions, the Lineweaver-Burk plot would show competitive inhibition when the degrading enzyme was in the assay. Figure 5A shows that chloroplast tyrosyl-tRNA synthetase is competitively inhibited with respect to tRNA by an aliquot of chloroplast lysate. However, in Figure 5B no inhibition of cytoplasmic tyrosyl-tRNA synthetase occurs. These facts lead to the conclusion that $tRNA_{3\&4}^{Tyr}$ are being specifically degraded into the pre-$tRNA_1^{Tyr}$ peak.

DISCUSSION

While evidence exists for two other activities which partially degrade specific leucyl-tRNA species (3,4) it is very significant that a chloroplast bound activity specifically degrades chloroplast tyrosyl-tRNA. The degrading activity has been shown in two separate experiments to be specific for $tRNA^{Tyr}_{3\&4}$ and gave no evidence of degrading $tRNA^{Tyr}_{1\&2}$. Since the genetic code is assumed to be universal, the anticodon can be ruled out as the recognition site of the degrading enzyme because the same codon complement should be represented in both chloroplast and cytoplasmic tyrosyl-tRNA species. Furthermore, Williams et al (8) suggested that chloroplast and cytoplasmic tRNAs have a high degree of sequence homology, requiring the tRNA recognition mechanism of the degrading activity to be quite specific. Therefore, the possibility that the degrading activity recognizes some modified base(s) in the tRNA structure becomes highly probable. The high degree of specificity of the nuclease might also result from the initial degradation of some exposed portion of a tRNA molecule by a general nonspecific ribonuclease. While this possibility cannot be ruled out, it is highly unlikely since the degrading activity has been observed in partially purified chloroplast synthetase preparations estimated to be free of ribonucleases by standard assays. Also, tyrosyl-tRNA synthetase preparations purified on Sephadex G-150 seem to contain the nuclease which indicates that it is larger than 60,000 daltons.

At this time, the product of the nuclease reaction has not been well characterized. The pre-$tRNA^{Tyr}_1$ peak can be deacylated under mild alkaline conditions with the same kinetics as intact tRNA species, and has a distinct elution position from RPC-5 columns. It is smaller than a whole tRNA molecule by Sephadex G-100 chromatography, and it is not recognized by its original cognate synthetase. The size of the degraded tRNA has been estimated to be about one-half that of a tRNA molecule, however, this has not been rigorously demonstrated.

Two novel aspects of the nuclease are evident. It is located in or on chloroplasts and it appears to degrade chloroplast tRNAs. The physiological function of such an enzyme is at present difficult to understand. Chloroplasts isolated from both senescing cotyledons and expanding leaves contain the nuclease, which suggests that the enzyme may be operative during all stages of chloroplast development. However, it is possible that the enzyme plays a prominent role in senescence. Currently, studies are in progress to elucidate the nature of the tRNA specificity of the enzyme, the nature and size of its cleavage products, and its possible physiological mechanism.

REFERENCES

1. Sharma, O. K., Mays, L. L., and Borek, E. (1973) J. Biol. Chem., 248, 7622.
2. Ilan, J., Ilan, J., and Patel, N. (1970) J. Biol. Chem., 245, 1275.
3. Kano-Sueoka, T. and Sueoka, N. (1968) J. Mol. Biol., 37, 475.
4. Babcock, D. F., and Morris, R. F. (1973) Pl. Physiol., 52, 292.
5. Locy, R. D. and Cherry, J. H. (1972) Pl. Physiol., 50, abs.
6. Locy, R. D. (1974) Ph.D. Thesis, Purdue University, 138 p.
7. Anderson, M. B. and Cherry, J. H. (1969) Proc. Natl. Acad. Sci. U.S., 62, 202.
8. Williams, G. R., Williams, A. S. and George, S. A. (1973) Proc. Natl. Acad. Sci. U.S., 79, 3498.