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Characterization of the RNA Required for Biosynthesis of δ-Aminolevulinic Acid from Glutamate[1]

PURIFICATION BY ANTICODON-BASED AFFINITY CHROMATOGRAPHY AND DETERMINATION THAT THE UUC GLUTAMATE ANTICODON IS A GENERAL REQUIREMENT FOR FUNCTION IN ALA BIOSYNTHESIS

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ABSTRACT

The heme and chlorophyll precursor δ-aminolevulinic acid acid (ALA) is formed in plants and algae from glutamate in a process that requires at least three enzyme components plus a low molecular weight RNA which co-purifies with the tRNA fraction during DEAE-cellulose column chromatography. RNA that is effective in the *in vitro* ALA biosynthetic system was extracted from several plant and algal species that form ALA via this route. In all cases, the effective RNA contained the UUC glutamate anticodon, as determined by its specific retention on an affinity resin containing an affine ligand directed against this anticodon. Construction of the affinity resin was based on the fact that the UUC glutamate anticodon is complementary to the GAA phenylalanine anticodon. By covalently linking the 3′ terminus of yeast $tRNA^{Phe(GAA)}$ to hydrazine-activated polyacrylamide gel beads, a resin carrying an affine ligand specific for the anticodon of $tRNA^{Glu(UUC)}$ was obtained. Column chromatography of plant and algal RNA extracts over this resin yielded a fraction that was highly enriched in the ability to stimulate ALA formation from glutamate when added to enzyme extracts of the unicellular green alga *Chlorella vulgaris*. Enhancement of ALA formation per A_{260} unit added was as much as 50 times greater with the affinity-purified RNA than with the RNA before affinity purification. The affinity column selectively retained RNA which supported ALA formation upon chromatography of RNA extracts from species of the diverse algal groups Chlorophyta (*Chlorella Vulgaris*), Euglenophyta (*Euglena gracilis*), Rhodophyta (*Cyanidium caldarium*), and Cyanophyta (*Synechocystis* sp. PCC 6803), and a higher plant (spinach). Other glutamate-accepting tRNAs that were not retained by the affinity column were ineffective in supporting ALA formation. These results indicate that possession of the UUC glutamate anticodon is a general requirement for RNA to participate in the conversion of glutamate to ALA in plants and algae.

Plants and algae form the heme and Chl precursor ALA[2] from the intact carbon skeleton of glutamate (1). The cellular apparatus that catalyzes ALA formation via this five-carbon pathway has been dissected into four macromolecular components, three proteins and a small RNA which co-purifies with the tRNA fraction during DEAE-cellulose chromatography (13, 15, 23, 27). According to current models, glutamate is transformed to ALA in a sequence of three enzyme-catalyzed steps: (a) activation of glutamate by ligation to a tRNA-like molecule; (b) dehydrogenation of the activated glutamate to form glutamate-1-semialdehyde; and (c) transamination of the semialdehyde to yield ALA (Fig. 1).

The RNA component has been conclusively shown to be required for ALA production *in vitro* in several cell-free experimental systems derived from plants and algae (13, 15, 19, 25, 26). It has been hypothesized that the RNA activates the C_1 carboxyl group of glutamate for the subsequent reaction(s) in a way that is identical or analogous to the aminoacylation of tRNA during protein synthesis (5, 12, 13, 15). The participating RNA in barley chloroplast extracts has been sequenced and reported to be a tRNA-like molecule containing a highly modified UUC glutamate anticodon (21). However, commercially available *Escherichia coli* $tRNA^{Glu(UUC)}$ did not support ALA formation by the barley plastic enzyme extract (15). Although RNA extracted from several plants and algae supported ALA formation in *Chlorella* and *Euglena* enzyme extracts, *E. coli* $tRNA^{Glu(UUC)}$ was inactive, even though the enzyme extracts could charge this tRNA with glutamate (19, 26). On the other hand, *E. coli* $tRNA^{Glu(UUC)}$ did support ALA formation in *Chlamydomonas* enzyme extracts (12). From the foregoing, it is apparent that the RNA component in the ALA-forming system must be characterized from a range of sources before generalizations can be drawn regarding anticodon or other structural requirements.

To determine the generality of the occurrence of a UUC glutamate anticodon in RNA capable of supporting ALA formation, we have adapted a chromatographic procedure for fractionating tRNA based on affinity interaction between complementary anticodons of mobile and matrix-bound tRNA (11), and were able to isolate relatively large quantities of $tRNA^{Glu(UUC)}$ by filtration through a resin containing bound $tRNA^{Phe(GAA)}$, the anticodon

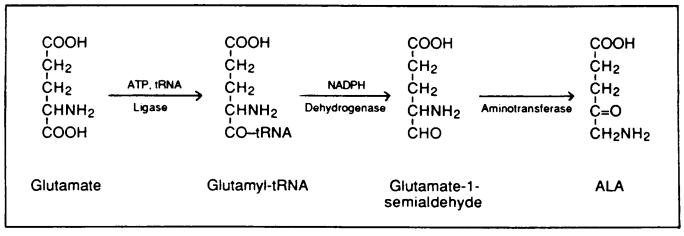

FIG. 1. Hypothetical biosynthetic sequence of ALA formation from glutamate via the RNA-dependent five-carbon pathway.

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[2] Abbreviations: ALA, δ-aminolevulinic acid; ALA-pyrrole, 2-methyl-3-carbethoxy-4-(3-propionic acid)-pyrrole; PALP, pyridoxal phosphate; PMSF, phenylmethylsufonyl fluoride; Tricine, *N*-(2-hydroxy-1,1-bis-[hydroxymethyl]ethyl)glycine.

of which is complementary to that of $tRNA^{Glu(UUC)}$. RNA was isolated from several algal and plant species which have been demonstrated to form ALA via the five-carbon pathway. In all cases, the component that is capable of participating in the ALA-forming process was specifically retained on the column, whereas nonretained glutamate-accepting RNAs were ineffective in supporting ALA formation. These results indicate that possession of the UUC glutamate anticodon is a general requirement for RNA to function in this capacity. A preliminary account of this work has appeared in abstract form (22).

MATERIALS AND METHODS

Cell Materials. Axenic cultures of *Chlorella vulgaris* Beijerinck, *Synechocystis* sp. PCC 6803, *Cyanidium caldarium*, and *Euglena gracilis* Klebs var Pringsheim were maintained on glucose-based heterotrophic media as previously described (2, 3, 19, 24, 26). Cultures were grown in liquid media with rotary shaking at 25°C in complete darkness or at a light intensity of 32 μE $m^{-2}s^{-1}$ supplied by equal numbers of red and cool-white fluorescent tubes. Fresh spinach leaves were purchased locally.

Cell Extraction for Enzyme Preparation. These procedures were carried out as previously described (24). Cultures were cooled on ice, and cells were harvested by centrifugation, resuspended in algal cell homogenization buffer (100 mM Tricine [pH 7.9], 300 mM glycerol, 15 mM $MgCl_2$, 1.0 mM DTT, 20 μM PALP) and broken by passage through a French pressure cell at 20,000 p.s.i. Unbroken cells and debris were removed by centrifugation for 15 min at 10,000*g*. The clarified homogenate was stirred with 500 mM NaCl for 20 min on ice, then centrifuged at 264,000*g* for 90 min. The particulate-free, clear yellow supernatant was partially depleted of endogenous RNA by fractional $(NH_4)_2SO_4$ precipitation and NaCl treatment. Precipitations were carried out on ice by stirring the high-speed supernatant supplemented with 2.0 mM EDTA, 0.0025% PMSF, and $(NH_4)_2SO_4$ at 35 or 60% of saturating concentration. The fraction precipitating between 35 and 60% of saturation was redissolved in enzyme fractionation buffer (50 mM Tricine [pH 7.9], 1.0 M glycerol, 15 mM $MgCl_2$, 1.0 mM DTT, 20 μM PALP), supplemented with 2.0 mM EDTA, 0.0025% (w/v) PMSF, and 1.0 M NaCl, stirred on ice for 30 min, then precipitated with $(NH_4)_2SO_4$ at 70% of saturation. The 1.0 M NaCl treatment and 70% $(NH_4)_2SO_4$ precipitation steps were repeated once, and the final precipitate was dissolved in enzyme fractionation buffer and desalted by chromatography through a column of Sephadex G-25 equilibrated with enzyme fractionation buffer. Aliquots of the high mol wt fraction were stored frozen at −75°C. The fraction precipitating between 60 and 100% of saturation in the initial $(NH_4)_2SO_4$ fractionation was stored at −75°C for later isolation of RNA.

Cell and Tissue Extraction for Low Molecular Weight RNA. The procedure employed for the algae was similar to that previously described for isolation of tRNA from *Euglena* (7). RNA was prepared by extraction of high-speed supernatant obtained from cells broken (by sonication for *Euglena* and French Press for other algae) in RNA extraction medium (10 mM Tris-Cl [pH 7.5], 10 mM $Mg(acetate)_2$, 100 mM NaCl, 10 mM β-mercaptoethanol). Supernatants were adjusted to 1% (w/v) with SDS and then extracted once with an equal volume of phenol (previously saturated with RNA extraction medium). The phenol phase was back-extracted with an equal volume of RNA extraction medium. In some cases, the procedure began with the fraction precipitating between 60 and 100% of saturation with $(NH_4)_2SO_4$ obtained during the enzyme preparation (see above). The precipitate was dissolved in RNA extraction medium, passed through Sephadex G-25, and then extracted with an equal volume of phenol (previously saturated with RNA extraction medium). In some cases, to reduce the volume before chloroform extraction, nucleic acids were precipitated from the pooled aqueous phases after phenol extraction by adding 2.5 volumes of absolute ethanol and cooling either overnight at −20°C in an ethanol bath or for 1.5 h at −75°C, followed by centrifugation to collect the nucleic acids. The pellet was dissolved in RNA extraction medium and extracted three or more times with equal volumes of chloroform:isoamyl alcohol (24:1, v/v). The tRNA-containing fraction was isolated by DEAE-cellulose chromatography (7). Nucleic acids (20 A_{260} units/ml) were dissolved in RNA extraction medium and applied to a column of DEAE-cellulose(Cl) previously equilibrated with RNA extraction medium. The column was washed with RNA extraction medium containing 250 mM NaCl until the A_{260} of the effluent was below 0.05. The low mol wt nucleic acid fraction was then eluted with DEAE-cellulose column elution buffer (RNA extraction medium containing 700 mM NaCl and 1.0 mM DTT instead of β-mercaptoethanol) and precipitated with 2.5 volumes of absolute ethanol. RNA was dissolved in aminoacyl-tRNA deacylation buffer (500 mM Tris-Cl [pH 8.0]) at a concentration of 30 A_{260} units ml^{-1} and deacylated by incubating at room temperature for 2 h, precipitated with 2.5 volumes of absolute ethanol, and washed with absolute ethanol (7). RNA was dried by vacuum desiccation and redissolved in RNA extraction medium containing 1 mM DTT instead of 10 mM β-mercaptoethanol.

Fresh prewashed spinach leaves were deveined and cleaned of damaged regions, and homogenized in a Waring Blendor for 15 min at low speed with a mixture of plant tissue homogenization buffer (500 mM Tris-Cl [pH 8.0], 10 mM $MgCl_2$, 1.0 mM Na_2EDTA, 100 mM NaCl, 1 mM β-mercaptoethanol, and 0.5% [w/v] Na-[7-deoxy]-cholate) and phenol (previously saturated with RNA extraction medium) at a ratio of 2:2:3 (w/v/v) tissue:buffer:phenol. The slurry was squeezed through four layers of Miracloth wrapped in two layers of cheesecloth, and then centrifuged at 10,000*g* for 10 min. The aqueous phase was reextracted with an equal volume of fresh phenol (previously saturated with RNA extraction medium), then twice with chloroform:isoamyl alcohol (24:1 v/v), and finally precipitated with 2.5 volumes absolute ethanol after addition of one-tenth volume 20% (w/v) Na-acetate (pH 4.5). This material was then chromatographed on DEAE-cellulose and deacylated as described above.

Preparation of the Affinity Column. The methods used here are adapted from those previously described by Grosjean *et al.* (11). In this procedure, the RNA is first reacted with periodate to open the 3′ ribosyl moiety and form aldehyde groups from the vicinal hydroxyl groups. Hydrazine-activated polyacrylamide (14) then reacts with the aldehydes to covalently link the RNA to the gel matrix.

To prepare the activated gel matrix, dry Bio-Gel P-200 polyacrylamide gel beads (hydrated bead diameter 80–150 microns) were swollen in methanol, soaked in excess deionized water for 2 d, and then suspended in polyacrylamide gel storage buffer (200 mM NaCl, 2.0 mM Na_2EDTA, 100 mM H_3BO_3 [pH 7.3]; and 0.02% [w/v] NaN_3). Before use, a portion was thoroughly washed with deionized water by vacuum filtration through Whatman No. 1 filter paper on a Büchner funnel. Approximately 25 ml of wet gel and a freshly prepared 60% (w/v) solution of hydrazine hydrate were warmed separately by immersion in a 47°C constant temperature bath for 45 min. Warm hydrazine solution (25 ml) was added to the warm gel and the slurry stirred for 8 h in a glass-stoppered flask, using a submersible magnetic stirrer in the 47°C bath. The derivatized gel was washed by vacuum filtration with 200-ml portions of 100 mM NaCl. After each wash, the filtrate was tested for residual free hydrazine by reaction with 2,4,6-trinitrobenzoic acid sulfate, until the indicator color was a pale violet, indicating that the filtrate was free of unreacted hydrazine (14).

The affine ligand was prepared from commercial $tRNA^{Phe(GAA)}$. The RNA (5 mg containing at least 100 A_{260} units) was dissolved in 5 ml periodate reaction buffer (100 mM Na-acetate [pH 5.0],

10 mM $Mg[acetate]_2$. Freshly prepared periodate solution (5 ml of 20 mM periodic acid, 100 mM Na-acetate [pH5.0]) was added to the dissolved tRNA and the mixture was incubated in the dark for 30 min at room temperature. The reaction was terminated by the addition of 1.75 ml 2.0 M KCl and incubation on ice for 10 min. Insoluble potassium periodate was sedimented by centrifugation at 10,000*g* for 10 min. The supernatant was removed and the RNA was precipitated at −75°C for at least 1 h by the addition of one-tenth volume 20% (w/v) Na-acetate (pH 5.0) and 2.5 volumes of absolute ethanol. The RNA was sedimented by centrifugation at 20,000*g* for 30 min, washed with 6 ml cold absolute ethanol and again sedimented by centrifugation at 20,000*g* for 10 min. The final RNA precipitate was dried *in vacuo* for 1 h.

Coupling of the periodate-cleaved $tRNA^{Phe}$ to the hydrazine-activated polyacrylamide beads was accomplished by dissolving the RNA in 5 ml RNA-polyacrylamide coupling buffer (200 mM Na-acetate [pH 4.8]) and mixing with approximately 2.5 ml of wet hydrazine-activated gel (equilibrated in the same buffer). The coupling reaction was allowed to proceed for 15 h at 4°C with gentle shaking. The derivatized gel was transferred to a water-jacketed column (Pharmacia, 10 mm diameter by 20 cm long), where it was held between two support-plungers and washed extensively with 200 mM Na-acetate (pH 4.8), then with affinity column elution buffer (10 mM Na-acetate [pH 4.8], 1.0 M NaCl, 10 mM EDTA), and finally with affinity column storage buffer (200 mM NaCl, 2.0 mM Na_2EDTA, 100 mM H_3BO_3 [pH 7.3], and 5.0 μM pentachlorophenol). Measurement of the A_{260} of the initial eluate indicated that over 90% of the RNA became bound to the gel matrix, and extensive washing did not liberate additional RNA. The column was stored at 2°C in affinity column storage buffer.

Chromatography of RNA on the $tRNA^{Phe}$-Polyacrylamide Column. The column was washed extensively with affinity column wash buffer (10 mM Na-acetate [pH 4.8], 1.0 M NaCl, 10 mM $MgSO_4$) and equilibrated to a jacket temperature of 1°C with a circulating water-ethanol mixture. The RNA sample was applied in a small volume of RNA extraction medium, allowed to enter the gel bed, and then the flow was stopped for 15 to 20 min to facilitate interaction with the $tRNA^{Phe}$ affine ligand. The column was next washed at 1°C with 20 to 25 ml of affinity column wash buffer at a flow rate of about 30 ml h^{-1}, and 1-ml fractions were collected. The flow was then stopped and the column equilibrated to a jacket temperature of 32°C. Elution was continued with affinity column elution buffer, again collecting 1-ml fractions. After elution was completed, the column was regenerated by washing extensively with affinity column elution buffer and then with affinity column storage buffer. The RNA content of each fraction was determined by measuring A_{260} using a Cary 219 spectrophotometer.

Assay for *in Vitro* ALA Formation. ALA formation assays were carried out as previously described (24). Incubation was for 30 min at 30°C in a 250 μl reaction volume containing enzyme extract, the RNA being tested, and reaction medium (50 mM Tricine [pH 7.9], 1.0 M glycerol, 15 mM $MgCl_2$, 5.0 mM ATP, 1.0 mM NADPH, 1.0 mM glutamate, 20 μM PALP, and 5.0 mM levulinic acid). Incubations were terminated by addition of 12.5 μl 100% (w/v) TCA, cooled for 10 min on ice, then the precipitate removed by 2 min centrifugation at 13,500*g* in an Eppendorf microcentrifuge. Supernatant aliquots (200 μl) were neutralized with 66 μl 500 mM Na_3PO_4 plus 300 μl ALA assay buffer (500 mM NaOH adjusted to pH 6.8 with H_3PO_4) then 25 μl ethylacetoacetate was added and the solutions were heated to 95°C for 15 min to form ALA-pyrrole. The product was quantitated spectrophotometrically after reaction with an equal volume of Ehrlich-Hg reagent. RNA was prepared from the affinity column fractions by precipitation with 2.5 volumes absolute ethanol for 16 h at −20°C, centrifugation for 30 min in a microcentrifuge at 4°C, washing with cold absolute ethanol, and drying *in vacuo* for 1 h.

Assay for Aminoacyl-tRNA Formation. A tritium-based filter-binding assay adapted from a previously described procedure (16) was used. Incubation mixtures contained RNA, enzyme extract, and acylation medium (50 mM Tricine [pH 7.9], 1.0 M glycerol, 15 mM $MgCl_2$, 5.0 mM ATP, 20 μM PALP) in a total volume of 100 μl. Reactions were initiated by the addition of substrate (10 nmol [^{3}H]amino acid containing 2.4×10^6 to 3.00×10^6 cpm radioactivity, 100 μM final amino acid concentration) and allowed to proceed for 30 min at 30°C. To terminate the reactions, 75 μl aliquots were applied to glass microfiber filters (each consisting of one-half of a 24 mm diameter Schleicher & Schuell No. 32 glass microfiber filter disk), the filters were placed vertically in 20-ml glass vials containing 1.0 ml cold 10% (w/v) TCA, and the TCA solution was allowed to wick up into the filters. The filters became saturated with TCA solution within less than 1 min. The vials were then filled with 10% TCA and incubated for 20 min at 5°C, after which the solution was decanted. The vials were next filled with cold 5% (w/v) TCA, incubated for 20 min at 5°C, and the solution decanted. A total of three 5% TCA washes were performed, followed by a cold ethanol:diethyl ether (1:1, v/v) wash, and finally a diethyl ether wash. The filters were allowed to air dry before transfer to plastic scintillation vials, and then 5.0 ml Econofluor scintillation solution were added. The vials were shaken and stored for 16 h in darkness to dissipate chemiluminescence before the radioactivity was determined by liquid scintillation spectroscopy in a Beckman LS-100C instrument. Tritium counting efficiency was approximately 25%. Counting durations were sufficient to achieve a counting error of not more than 2% (except backgrounds, which were counted to 5% error). With 2.4×10^6 to 3.0×10^6 cpm of added substrate, background controls had approximately 500 cpm, and all duplicates agreed within 15%.

Other Methods. Protein was determined by the dye-binding method of Bradford (4) using BSA as the standard. Algal cell population densities were determined with a Coulter Counter (model ZBI, Coulter Electronics).

Materials. L-[3, 4-^{3}H]Glutamic acid was purchased from ICN Radiochemicals, L-[4,5-^{3}H]leucine and Econofluor premixed scintillation solution were from New England Nuclear. *E. coli* $tRNA^{Glu}$ type II and brewers' yeast $tRNA^{Phe}$ type V were from Sigma. Cellulose DE-23 was from Whatman. Bio-Gel P-200 polyacrylamide beads were from Bio-Rad. Miracloth was from Calbiochem-Behring. All other reagents were from Fisher, Sigma, or Research Organics.

RESULTS

Specificity of the Affinity Column. To test the effectiveness of the affinity resin, 2.0 A_{260} units of commercially available *E.coli* $tRNA^{Glu}$ were applied to the column at 1°C. After the $tRNA^{Glu}$ was applied, the column was washed at 1°C with the Mg-containing affinity column wash buffer. Bound material was eluted at 32°C with the Mg-free, EDTA-containing affinity column elution buffer. Total recovery of applied material was 97% (based on A_{260}). The major portion (60%) of the applied material remained bound to the column and was eluted in fractions 22 through 32 after the buffer was changed and the temperature raised, although a significant minor portion (37%) of the applied material was not retained by the column, and appeared in fractions 1 through 21. However, 92% of the recovered glutamate-accepting ability was eluted with the retained material, and only 8% eluted with the nonretained material (Fig. 2). Based on glutamate-accepting ability per A_{260} unit, a 1.5-fold purification of the commercial $tRNA^{Glu}$ was achieved by this affinity chromatography procedure.

To test the column specificity, extracted *Chlorella* RNA was applied to the column, and effluent fractions were tested for both

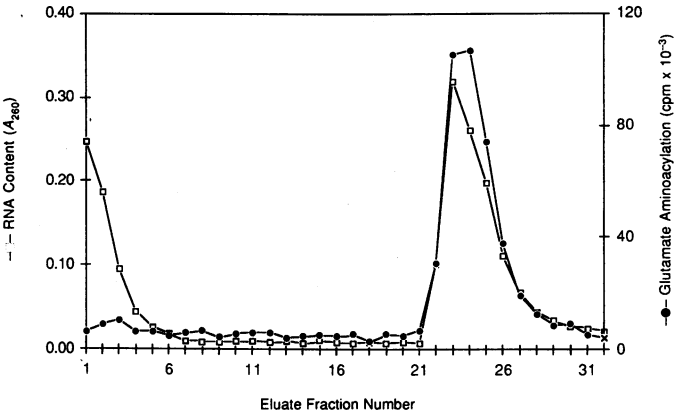

FIG. 2. Elution profile of *E. coli* $tRNA^{Glu}$. Two A_{260} units were applied to the affinity column prepared by covalently attaching brewers' yeast $tRNA^{Phe}$ to polyacrylamide gel beads as described in the text. Application of the $tRNA^{Glu}$ and initial elution with high-Mg affinity column wash buffer were carried out at 1°C. After 21 1-ml fractions were collected, the column temperature was raised to 32°C and elution was begun with low-Mg affinity column elution buffer. Each column fraction was assayed for A_{260} and glutamate-accepting ability as described in the text. The endogenous level of glutamate-accepting material is indicated by an × on the ordinate axis.

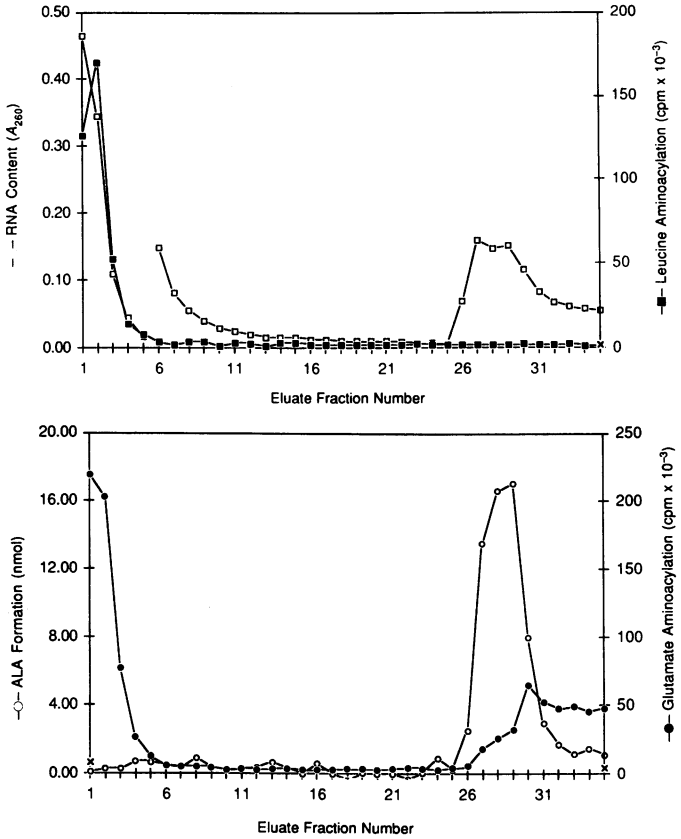

FIG. 3. Elution profile of *Chlorella* RNA. A DEAE-cellulose purified tRNA-containing fraction of *Chlorella* RNA (25 A_{260} units) was applied to the affinity column. Initial elution with high-Mg affinity column wash buffer was carried out at 1°C. After 25 1-ml fractions were collected, the column temperature was raised to 32°C and elution was begun with low-Mg affinity column elution buffer. Each column fraction was assayed for A_{260}-absorbing material, glutamate- and leucine-accepting abilities, and the ability to stimulate ALA formation when incubated with a *Chlorella* enzyme extract, as described in the text. The A_{260} values of the first five fractions have been divided by 20. Endogenous levels of leucine and glutamate-accepting material, and ALA-forming activity, are indicated by ×s on the ordinate axes.

glutamate- and leucine-accepting ability. Whereas all of the leucine-accepting material eluted before the buffer and temperature change, a portion of the glutamate-accepting material was retained and did not elute until the buffer was changed and the temperature raised (Fig. 3)

Column effluent fractions from the *Chlorella* RNA fractionation were also tested for the ability to stimulate ALA formation when added to a *Chlorella* enzyme extract containing the complete cofactor and substrate mixture required for the transformation of glutamate to ALA. The fractions eluting after the buffer and temperature change were capable of stimulating ALA formation to a much greater extent than the early-eluting fractions (Fig. 3).

Possible causes for the discrepancy between the elution profiles of material capable of accepting glutamate and supporting ALA formation were assessed. First, it was possible that the nonretained material contained an inhibitor of the ALA-forming reactions, and might therefore mask the presence of RNA capable of stimulating ALA formation. This possibility was tested by supplementing a complete ALA formation assay system (containing unfractionated *Chlorella* RNA) with nonretained and retained RNA. Some inhibition was observed with added nonretained material, and stimulation with added retained material (Table I). Greater inhibition occurred when increasing concentrations of nonretained material were added to incubations containing retained RNA. However, no more than 50% inhibition was reached even with the highest amounts of added nonretained material. The highest amounts added (4.0 A_{260} units) correspond to about 1.5 times the amount present in the nonretained fractions measured for the ability to stimulate ALA formation without additional unfractionated RNA (Fig. 3).

Stimulation of ALA Formation by Retained and Nonretained RNA. The concentration dependence for stimulation of ALA formation by the early- and late-eluting column fractions was determined. For the late-eluting material, stimulation of ALA formation was detected even at the lowest level of added RNA (0.0025 A_{260} unit), and increasing amounts of ALA were formed with increasing amounts of added RNA, up to the maximum amount added (0.06 A_{260} unit) (Fig. 4). With the nonretained RNA fraction, some degree of stimulation of ALA formation was measured at high RNA concentrations, and inhibition was observed at the highest RNA concentrations added. Per A_{260} unit added, the retained RNA was approximately 200 times more effective than the nonretained RNA in stimulating ALA formation (Fig. 4). In another experiment, starting with a different RNA extract, the retained RNA was 15.8-fold more active, and the nonretained RNA was 23 fold less active than the unfractionated material, in stimulating ALA formation (Table I).

Affinity Purification of $tRNA^{Glu}$ from Various Organisms. RNA was isolated from a number of different organisms and applied to the $tRNA^{Phe}$-polyacrylamide column. Column fractions were assayed for RNA concentration and the abilities to accept glutamate and support ALA formation, with an enzyme system derived from *Chlorella*. RNA from spinach, *Cyanidium*, *Euglena* and *Synechocystis* had elution profiles for both activities that were similar to those of *Chlorella* RNA (*cf.* Figs. 3 and 5). Total recovery of applied A_{260} units ranged from 77 to 92%, and total recovery of material capable of stimulating ALA formation ranged from 61 to 104%. Based on the degree of stimulation of ALA formation per A_{260} unit of RNA added to the incubation mixtures, the enrichment of activity of retained RNA, compared to unfractionated material, ranged from 12- to 50-fold (Table II).

Table I. *Effect of Retained and Nonretained Affinity Column Eluate on ALA Formation in Chlorella Enzyme Extracts*

Standard ALA formation assays were carried out as described in the text, in *Chlorella* enzyme extracts containing 0.58 mg protein and supplemented with unfractionated *Chlorella* RNA, nonretained affinity column effluent material, and retained material, or combinations of these. Incubations were for 30 min at 30°C

Added RNA Identity and Quantity	ALA Formation
(A_{260} units)	*nmol*
None (endogenous level)	0.58
Unfractionated (1.25)	5.18
Unfractionated (1.25) + nonretained (2.00)	3.86
Unfractionated (1.25) + retained (0.04)	6.01
Nonretained (2.00)	0.90
Retained (0.04)	2.87
Retained (0.04) + nonretained (0.25)	2.86
Retained (0.04) + nonretained (0.50)	2.85
Retained (0.04) + nonretained (1.00)	2.75
Retained (0.04) + nonretained (2.00)	2.21
Retained (0.04) + nonretained (3.00)	2.00
Retained (0.04) + nonretained (4.00)	1.84

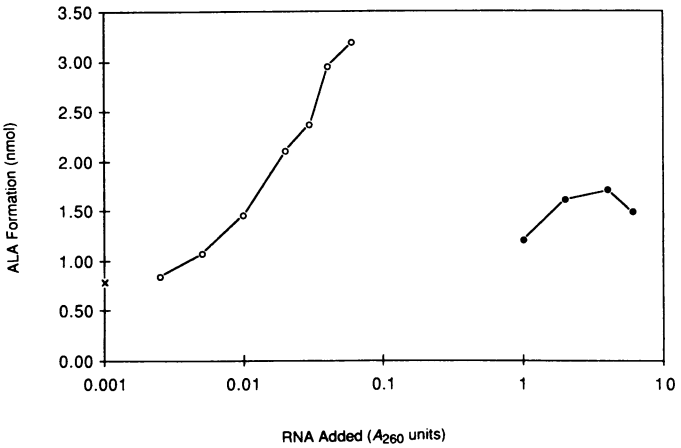

FIG. 4. Concentration dependence for stimulation of ALA formation in *Chlorella* enzyme extract by affinity-separated fractions of *Chlorella* RNA. (●), Nonretained fraction; (○), retained fraction. The endogenous level of ALA-forming activity, without added RNA, is indicated by an × on the ordinate axis.

With some RNA extracts, material in several of the column fractions eluting before the buffer and temperature change was capable of stimulating ALA formation to varying degrees. This material did not co-elute with the major portion of the nonretained RNA, but appeared in column fractions eluting somewhat later. One possible cause of the incomplete retention of material capable of supporting ALA formation is the presence, in the unfractionated RNA mixture, of sufficient $tRNA^{Phe}$ to compete with the matrix-bound $tRNA^{Phe}$ for complexing with the mobile $tRNA^{Glu}$. This possibility was tested by adding 5 A_{260} units of brewers' yeast $tRNA^{Phe}$ to 25 A_{260} units of *Chlorella* RNA before applying the mixture to the affinity column. The presence of the added $tRNA^{Phe}$ caused incomplete retention of the material capable of supporting ALA formation (Fig. 6), in contrast to its complete retention from unsupplemented *Chlorella* RNA (Fig. 3). As was observed in the cases of cellular RNA extracts exhibiting this behavior, the early-eluting material capable of supporting ALA formation did not co-elute with the major portion of the nonretained RNA, but appeared in later column fractions.

With all of the algal and plant RNAs examined, it was observed that the elution profiles of retained RNA capable of supporting ALA did not coincide with those of glutamate-accepting material. The differences are most apparent in the elution profiles from *Cyanidium* and *Synechocystis* (Fig. 5). In some cases, the profiles suggest the existence of two or more peaks of retained glutamate-accepting material. If the bound RNA was eluted by raising the temperature from 1°C to 32°C in two steps instead of one, it was also possible to partially resolve the bound *Chlorella* RNA into two distinct peaks of A_{260} and glutamate-accepting material (data not shown). In all cases, the material capable of supporting ALA formation eluted with the earliest-eluting portion of the retained glutamate-accepting RNA.

DISCUSSION

The RNA that participates in ALA formation from glutamate was affinity-purified by a column chromatography procedure based on anticodon-anticodon interaction, taking advantage of the fact that the UUC glutamate anticodon is complementary to the GAA phenylalanine anticodon. The anticodon-anticodon coupling between tRNAs has been shown to be highly sensitive to temperature, total salt concentration, and the presence of Mg^{2+} (11). Binding is enhanced by low temperature, high salt concentration, and the presence of Mg^{2+}. This procedure has the capacity for relatively large yields of purified RNA, in contrast to the gel electrophoretic methods previously used (21). In addition to providing a facile isolation of the RNA required for ALA biosynthesis via the five-carbon pathway, this method has provided a means of rapidly screening RNA from a number of species. Our results indicate that possession of a UUC glutamate anticodon is a universal requirement of RNA that is capable of supporting ALA formation from glutamate, and that other glutamate-accepting RNAs, which are not retained by the affinity resin, are incapable of supporting ALA formation.

Under the conditions employed, glutamate-accepting activity in commercial *E. coli* $tRNA^{Glu(UUC)}$, was selectively retained, whereas leucine-accepting activity in extracted *Chlorella* RNA was not retained. Complete separation of a portion of the glutamate-accepting tRNA from other amino acid accepting tRNAs (as assessed by leucine acceptance) in a mixed tRNA fraction obtained from *Chlorella* was achieved during a single chromatographic step. Almost all of the material capable of stimulating ALA formation, when added to a *Chlorella* enzyme extract, was retained by the column, indicating that the only component in the RNA extract capable of supporting ALA formation contains a UUC glutamate anticodon. However, a considerable fraction of the total glutamate-accepting material was not retained by the affinity column.

Possible causes for the discrepancy between the ability of the nonretained material to accept glutamate but not stimulate ALA formation were assessed. One possibility is that the nonretained column eluate fractions contained RNA capable of supporting ALA formation, but this activity was masked by inhibitory material also present in these fractions. Although nonretained material inhibited ALA formation to some degree, the amount of inhibition was relatively small and was insufficient to mask the presence of RNA capable of supporting ALA synthesis, if it were present in the nonretained fractions. Therefore, most of the nonretained glutamate-accepting material must represent RNA that can be charged with glutamate, but which does not contain a UUC anticodon that is complementary to the GAA anticodon of the bound $tRNA^{Phe}$ ligand. One component of this material might be $tRNA^{Glu}$ bearing the other glutamate anticodon, CUC. Because the CUC anticodon is not complementary to the GAA anticodon of the bound $tRNA^{Phe}$, $tRNA^{Glu(CUC)}$, would not be expected to be retained on the affinity column.

Another component of the nonretained glutamate-accepting

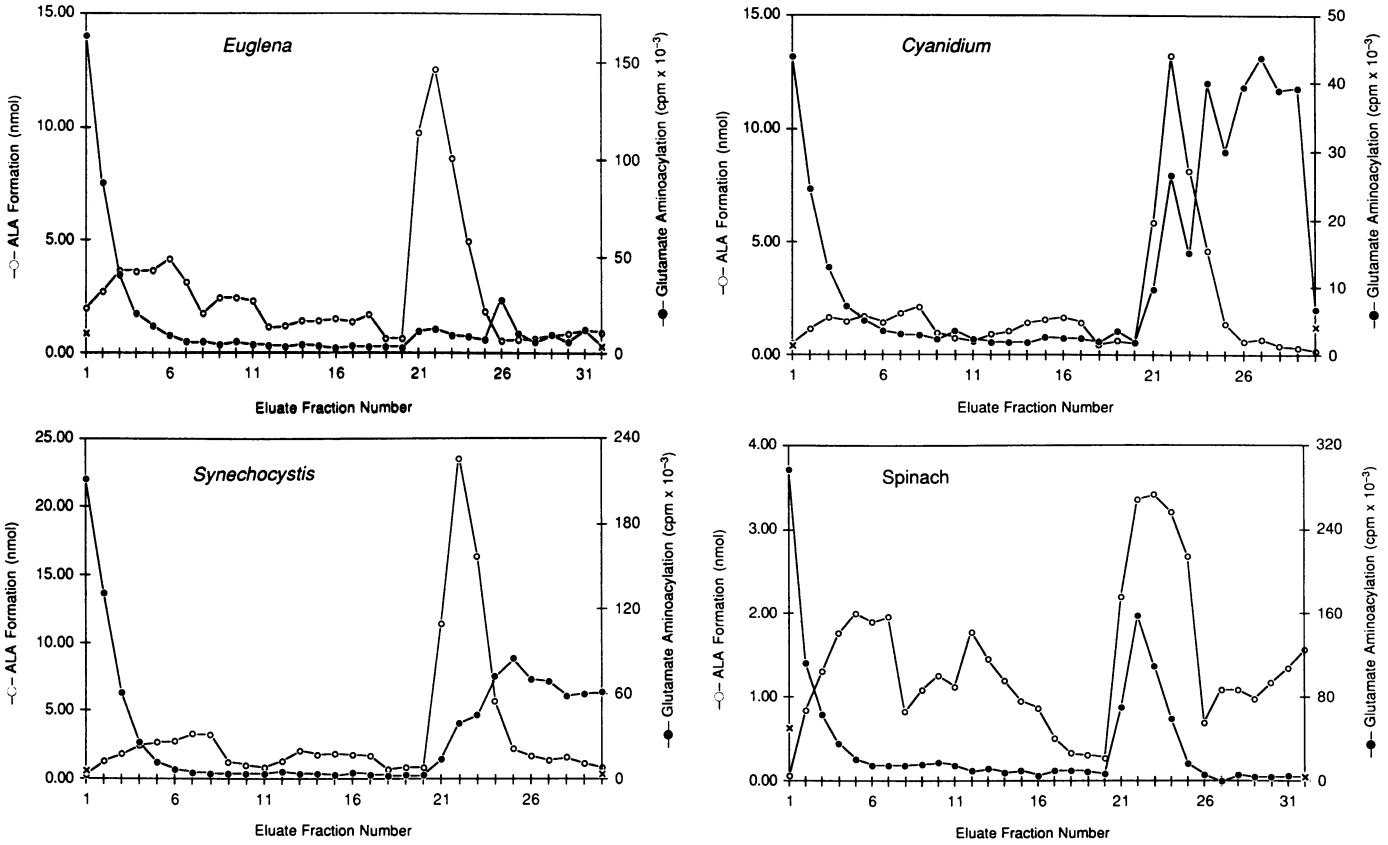

FIG. 5. Elution profiles of *Euglena*, *Cyanidium*, *Synechocystis*, and spinach RNA. DEAE-cellulose purified tRNA-containing fractions were applied to the affinity column. The amounts applied were (in A_{260} units) 37.5 (*Euglena*), 50 (*Cyanidium*), 20 (*Synechocystis*), and 80 (spinach). Initial elution with high-Mg affinity column wash buffer was carried out at 1°C. After 20 1-ml fractions were collected, the column temperature was raised to 32°C and elution was begun with low-Mg affinity column elution buffer. Each column fraction was assayed for glutamate-accepting ability and the ability to stimulate ALA formation when incubated with *Chlorella* enzyme extract. Endogenous levels of glutamate-accepting material and ALA-forming activity are indicated by ×s on the oridinate axes.

Table II. *Stimulation of ALA Formation in Chlorella Enzyme Extracts by Unfractionated and Affinity-Purified RNA*

Standard ALA formation assays were carried out as described in the text, in *Chlorella* enzyme extracts containing 0.58 mg protein and supplemented with unfractionated RNA or affinity-purified RNA obtained from the indicated sources. Incubations were for 30 min at 30°C.

Added RNA	Amount Added	ALA Formation	Stimulation
	A_{260} *units*	*nmol*	Δ *nmol* A_{260} *unit*$^{-1}$
Endogenous level	0.00	0.65	
Chlorella unfractionated	1.25	4.98	3.43
Chlorella affinity-purified	0.04	4.79	108.12
Euglena unfractionated	2.50	5.29	1.76
Euglena affinity-purified	0.05	3.79	57.17
Euglena affinity-purified[a]	0.03	2.83	74.18
Cyanidium unfractionated	5.00	4.99	0.92
Cyanidium affinity-purified	0.07	3.60	44.69
Synechocystis unfractionated	1.00	7.10	6.47
Synechocystis affinity-purified	0.07	6.35	76.04
Synechocystis affinity-purified[a]	0.04	4.57	94.64
Spinach unfractionated	2.50	1.88	0.50
Spinach affinity-purified	0.09	1.32	7.92
Spinach affinity-purified[a]	0.02	1.14	28.44

[a] Data from retained column fraction having highest ability to stimulate ALA formation per A_{260} unit. All other data are from the retained column fraction having highest total ability to stimulate ALA formation.

material might be tRNA bearing the glutamine anticodons UUG and CUG. In some bacterial species, $tRNA^{Gln}$ is first charged with glutamate by glutamyl-tRNA synthetase, and then the glutamyl-$tRNA^{Gln}$ is converted to glutaminyl-$tRNA^{Gln}$ by a specific amidotransferase that utilizes glutamyl-$tRNA^{Gln}$ as a substrate (17, 28). $tRNA^{Gln}$ species present in the RNA extracts might therefore be capable of being charged with glutamate by the enzyme extracts, but because they do not bear anticodons that are complementary to the bound ligand, they would not be retained by the affinity column. In any case, our results indicate

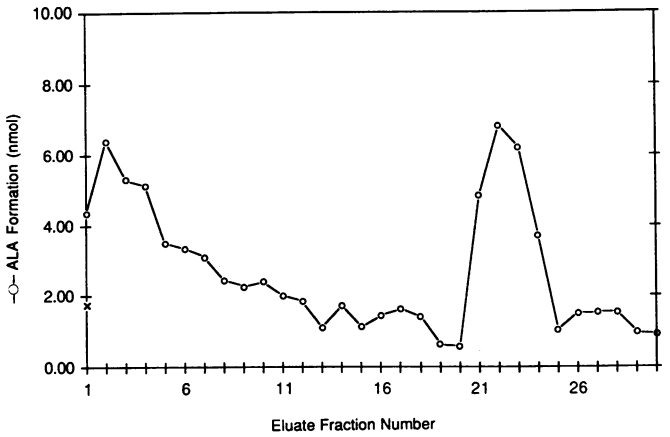

FIG. 6. Effect of added $tRNA^{Phe}$ on elution profile of *Chlorella* RNA. Brewers' yeast $tRNA^{Phe}$ (5 A_{260} units) was added to 25 A_{260} units of DEAE-purified *Chlorella* RNA, and the mixture applied to the affinity column. Elution was carried out as described for the other samples. Each column fraction was assayed for the ability to stimulate ALA formation when incubated with *Chlorella* enzyme extract. The endogenous level of ALA-forming activity is indicated by an × on the ordinate axis.

that UUC anticodon-bearing RNAs are the only species present in the cell extracts that are capable of participating in the ALA-forming reactions when added to *Chlorella* enzyme extracts, even though other RNA species are present which can be charged with glutamate by the enzyme extracts.

It was recently shown that RNA extracted from plant and algal species representing a diverse phylogenetic range are capable of supporting ALA formation when added to enzyme extracts of *Chlorella* and *Euglena* (19, 26). Several of these RNAs were fractionated by affinity chromatography. In all cases, material capable of supporting ALA formation when added to *Chlorella* enzyme extracts was retained by the column. Also, in all cases, as with *Chlorella* RNA, additional material was present which accepted glutamate, but was not retained by the column and did not support ALA formation.

Although possession of a UUC glutamate anticodon therefore appears to be necessary for participation in the ALA-forming system, it should be stressed that possession of a UUC anticodon together with the capacity to accept glutamate is insufficient to confer the ability to participate in the ALA-forming system. Even though *E. coli* $tRNA^{Glu(UUC)}$ was retained by the affinity column and was charged with glutamate when incubated with *Chlorella* enzyme extract, this tRNA did not stimulate ALA formation in extracts of *Chlorella* (26), *Euglena* (19), or barley plastids (15). However, it should be noted that *E. coli* $tRNA^{Glu(UUC)}$ was reported to support ALA formation in *Chlamydomonas* extracts (12).

In some cases, when RNA extracts were affinity-chromatographed, a portion of the material capable of supporting ALA formation eluted after the major portion of the nonretained material, but before the major fraction of retained material. This behavior could be duplicated with the *Chlorella* RNA extract by adding $tRNA^{Phe}$ to the RNA before affinity chromatography. Presumably, the added free $tRNA^{Phe}$ competed with the matrix-bound $tRNA^{Phe}$ for complexing with the applied $tRNA^{Glu}$. This competition had the effect of reducing the apparent efficiency of the affinity column.

It is apparent that the elution profiles of the bound material capable of accepting glutamate do not coincide precisely with the elution profiles of material that stimulates ALA formation. This difference suggests the existence in the RNA extracts of more than one $tRNA^{Glu(UUC)}$-like species, not all of which participate in ALA formation. In the case of RNA derived from eukaryotic species, which contain three separately compartmented pools of tRNA, this phenomenon could be predicted for the following reason: It was previoously noted that only plastid-containing wild-type *Euglena* cells yielded RNA capable of supporting ALA formation, whereas aplastidic mutant cells did not, even though RNA from aplastidic cells accepted glutamate (19). It therefore appears that RNA that is effective in ALA formation exists only within the plastids of eukaryotic cells, even though other cellular compartments may contain $tRNA^{Glu(UUC)}$ species that elute from the affinity column slightly differently from the RNA that participates in ALA formation. However, the affinity column elution profiles provide evidence for more than one $tRNA^{Glu(UUC)}$-like species even in the RNA derived from the prokaryote *Synechocystis*. In this case, there is no possibility of separately compartmented tRNA pools.

The observation that the material capable of supporting ALA formation eluted with the earliest-eluting portion of the glutamate-accepting bound RNA suggests that the anticodon-anticodon interaction of this RNA species with the affine ligand is weaker than that of the other bound RNAs. In this regard, it should be noted that the barley plastid RNA that functions in ALA formation has a highly modified anticodon region, with two of the three anticodon bases modified (21). This barley RNA contains a novel pseudouridine in the second position of the anticodon, as well as a 5-methylaminomethyl-2-thiouridine at the wobble position, as do other $tRNA^{Glu}$ species (9). Replacement of the uridine with a pseudouridine at the second position in the anticodon would be predicted to significantly weaken the stability of the anticodon-anticodon couple with the GAA phenylalanine anticodon used as the affine ligand (10).

Our results suggest that the RNA that participates in ALA formation may be structurally different from the tRNA that takes part in protein synthesis. In the cases of plastids and prokaryotes, which probably contain only one gene for $tRNA^{Glu(UUC)}$, the differentiation of the tRNA with respect to the two functions could arise by post-transcriptional modification of the tRNA precursor molecules. tRNA is known to participate in several biosynthetic reactions other than protein synthesis (6, 8, 18, 20). In some cases, the tRNA that is involved in the nonprotein synthesizing reaction is inactive in protein synthesis and its aminoacyl-tRNA adduct cannot bind ribosomes (6). It is not yet known whether the RNA that supports ALA formation is capable of participating in protein synthesis. The affinity chromatography method described here, coupled with further purification steps, can provide sufficient quantities of individual RNA species to permit this question to be answered experimentally.

A final question concerns the possible benefit in using a $tRNA^{Glu}$-like molecule, that is distinct from the $tRNA^{Glu}$ that participates in protein synthesis, as a cofactor in ALA biosynthesis. An attractive hypothesis is that overall coordinate regulation of tetrapyrrole and protein biosynthesis could be economically achieved by control over total $tRNA^{Glu}$ synthesis, and fine regulation of the relative rates of synthesis of protein and tetrapyrroles could be exerted by control over the enzyme(s) that modify the RNA to enable it to support ALA formation.

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