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Stimulation of δ-Aminolevulinic Acid Formation in Algal Extracts by Heterologous RNA[1]

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ABSTRACT

Formation of the chlorophyll and heme precursor δ-aminolevulinic acid (ALA) from glutamate in soluble extracts of *Chlorella vulgaris*, *Euglena gracilis*, and *Cyanidium caldarium* was stimulated by addition of low molecular weight RNA derived from greening algae or plant tissue. Enzyme extracts were prepared for the ALA formation assay by high-speed centrifugation, partial RNA depletion, and gel filtration through Sephadex G-25. RNA was extracted from greening barley epicotyls, greening cucumber cotyledon chloroplasts, and growing cells of *Chlorella*, *Euglena*, *Chlamydomonas reinhardtii*, and *Anacystis nidulans*, freed of protein, and fractionated on DEAE-cellulose to yield an active component corresponding to the tRNA-containing fraction. RNA from homologous and heterologous species stimulated ALA formation when added to enzyme extracts, and the degree of stimulation was proportional to the amount of RNA added. Algal enzyme extracts were stimulated by algal RNAs interchangeably, with the exception of RNA prepared from aplastidic *Euglena*, which did not stimulate ALA production. RNA from greening cucumber cotyledon chloroplasts and greening barley epicotyls stimulated ALA formation in algal enzyme incubations. In contrast, tRNA from *Escherichia coli*, both nonspecific and glutamate-specific, as well as wheat germ, bovine liver, and yeast tRNA, failed to reconstitute ALA formation. Moreover, *E. coli* tRNA inhibited ALA formation by algal extracts, both in the presence and absence of added algal RNA. *Chlorella* extracts were capable of catalyzing aminoacyl bond formation between glutamate and both the activity reconstituting and nonreconstituting RNAs, indicating that the inability of some RNAs to stimulate ALA formation was not due to their inability to serve as glutamyl acceptors. The first step in the ALA-forming reaction sequence has been proposed to be activation of glutamate via aminoacyl bond formation with a specific tRNA, analogous to the first step in peptide bond formation. Our results suggest that the RNA that is required for ALA formation may be functionally distinct from the glutamyl-tRNA species involved in protein synthesis.

The formation of ALA[3] is the first committed step in the tetrapyrrole biosynthetic pathway leading to hemes, Chls, and bilins. In plants and algae, most, and possibly all, ALA is formed via a 5-carbon pathway which utilizes the intact carbon skeleton of glutamate and is located in the chloroplast (1, 6, 18). Recent reports have indicated a requirement for RNA for this reaction in soluble enzyme extracts from barley (14), *Chlamydomonas* (12, 13), and *Chlorella* (22). The active RNA in barley chloroplasts has recently been purified and the nucleotide sequence determined (19). The ability of RNA obtained from several species of algae, higher plants, and *Escherichia coli* to stimulate ALA formation in homologous and heterologous enzyme preparations was investigated. The results suggest that the RNA requirement is universal for organisms which utilize the 5-carbon pathway of ALA biosynthesis, the reaction-specific RNA can be recognized in heterologous systems, not all acylatable $tRNA^{glu}$ species can serve this function, and the active RNA species is specific to plastids in eukaryotic cells.

MATERIALS AND METHODS

Cell Materials. Axenic cultures of *Chlorella vulgaris* Beijerinck, *Euglena gracilis* Klebs var Pringsheim, and *Cyanidium caldarium* were grown in glucose-based heterotrophic media as previously described (2, 4, 20). Cultures were grown in liquid media with rotary shaking at 25°C in complete darkness or at a light intensity of 32 $\mu E\ m^{-2}\ s^{-1}$ supplied by equal numbers of red and cool-white fluorescent tubes. *Anacystis nidulans* (*Synechococcus leopoliensis*, UTEX 625) was grown in the light in BG-11 medium (17), in an atmosphere supplemented with 1% CO_2.

Cucumber (*Cucumis sativus* L. var Beit Alpha, a gift from Moran Seeds, Inc., Modesto, CA) seeds were germinated on moist vermiculite for 5 d in the dark, then brought into the light 7 h before harvest of the cotyledons. Barley (*Hordeum vulgare* L cv Svalof's Bonus, a gift from S. P. Gough, Carlsberg Laboratory, Copenhagen) seeds were germinated in the dark on moist vermiculite for 7 d, then brought into the light 12 h before harvest of the epicotyls.

Cell Extraction for Enzyme Preparation. *Chlorella* extracts were prepared from greening cultures of strain C-10 as previously described (21). This strain forms Chl only in the light. Cells were grown in the dark, then transferred to the light to induce rapid greening. After 5 h, the culture was cooled on ice, cells were harvested by centrifugation, washed with cold water, resuspended in homogenization medium (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 23,000 p.s.i. The homogenate was treated with 500 mM NaCl for 20 min on ice, then centrifuged at 264,000*g* for 90 min. The particulate-free supernatant was passed through a column of Sephadex G-25 equilibrated with column buffer (50 mM Tricine [pH 7.9], 150 mM glycerol, 15 mM $MgCl_2$, 1.0 mM DTT, 20 μM PALP) to remove low mol wt materials and aliquots were stored frozen at −75°C.

In some cases, *Chlorella* enzyme extracts were partially depleted of endogenous RNA by subjecting the high-speed super-

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[3] Abbreviations: ALA, δ-aminolevulinic acid; ALA-pyrrole, 1-methyl-2-carboxyethyl-3-propionic acid pyrrole; DEAE, diethylaminoethyl; PALP, pyridoxal phosphate; PMSF, phenylmethylsulfonyl fluoride; RNasin, human placental RNase inhibitor.

natant to fractional $(NH_4)_2SO_4$ precipitation. Precipitations were carried out at 0°C 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 column buffer supplemented with 2.0 mM EDTA, 0.0025% PMSF, and 1.0 M NaCl, stirred at 0°C for 1 h, then precipitated with $(NH_4)_2SO_4$ at 70% of saturation. The pellet was dissolved in column buffer and passed through Sephadex G-25. Aliquots of the high mol wt fraction were stored frozen at −75°C. In other cases, the extracts were depleted of endogenous RNA by preincubation with RNase, followed by addition of RNasin to inhibit RNase action during the incubation period (22).

Initial extracts of *Euglena* were prepared from cells grown in the dark and then transferred to the light for 12 h to induce greening. Cells were broken in the French pressure cell or by grinding with mortar and pestle in liquid N_2. Later, it was found that more active preparations could be obtained by growing cells continuously in the light and homogenizing by sonication. The cell homogenates were then treated as described above for the non-RNA-depleted *Chlorella* extracts. These *Euglena* extracts appeared to be relatively free of active endogenous RNA, thus further treatment of the extract to remove RNA was unnecessary.

Cyanidium extracts were prepared from light-grown cultures of strain CPD as previously described (3).

Cell and Tissue Extraction for RNA. Low mol wt RNA was prepared as previously described for isolation of tRNA from *Euglena* (9). RNA from *Chlorella*, *Anacystis*, wild-type *Euglena*, and aplastidic *Euglena* strain W_{14}ZNalL was prepared by extraction of high-speed supernatant obtained from cells broken in RNA extraction medium (10 mM Tris-HCl [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 extraction medium), then three or four times with equal volumes of chloroform:isoamyl alcohol (24;1, v/v). Nucleic acids were precipitated by adding 2.5 volumes of absolute ethanol and cooling either overnight at −20°C or 1.5 h at −75°C. The tRNA-containing fraction was isolated by DEAE-cellulose chromatography (9). The precipitated nucleic acids were redissolved in extraction medium and applied to a column of DEAE-cellulose(Cl) previously equilibrated with extraction medium. The column was washed with wash buffer (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 eluted with elution buffer (extraction medium containing 700 mM NaCl and 1.0 mM DTT). Nucleic acids were deacylated by dissolving in deacylation buffer (500 mM Tris-HCl [pH 8.0]) and incubating at room temperature for 2 h, precipitating, and washing twice with absolute ethanol (9).

Chloroplasts were isolated from cotyledons of 7-h greening etiolated cucumber seedlings as previously described (16). Cotyledons were ground gently with buffer in a mortar and pestle, and chloroplasts were isolated by differential centrifugation. Chloroplasts were disrupted by sonication in hypotonic buffer (10 mM Tris-HCl [pH 7.5], 10 mM Mg(acetate)$_2$, 500 mM NaCl, 10 mM β-mercaptoethanol), then low mol wt RNA was isolated from a high-speed supernatant as described above for algal extracts.

Epicotyls were harvested from 12-h greening etiolated barley seedlings and homogenized in a Waring Blendor for 15 to 20 min in a volume of extraction buffer (500 mM Tris-HCl [pH 8.0], 10 mM $MgCl_2$, 1.0 mM Na_2EDTA, 100 mM NaCl, 0.5% [w/v] Na-deoxycholate, and 1.0 mM β-mercaptoethanol) equal to two-thirds the fresh weight of tissue in g, plus a volume of equilibrated phenol equal to the fresh weight of tissue in g. The homogenate was filtered through Miracloth (Calbiochem-Behring), centrifuged at 20,000*g* for 30 min, filtered through glass wool, and extracted with an equal volume of equilibrated phenol by stirring for 90 min at room temperature. Phases were separated by centrifugation, and the aqueous phase was extracted first with an equal volume of equilibrated phenol, next with an equal volume of equilibrated phenol:chloroform (1:1, v/v), then twice with equal volumes of chloroform. Na-acetate (pH 4.5) was added to a final concentration of 2%, and the nucleic acids were precipitated by adding 2.5 volumes of absolute ethanol and cooling to −20°C overnight. The tRNA-containing fraction was isolated by DEAE-cellulose chromatography as described above, with minor variations in the buffer composition (equilibration buffer was 100 mM Tris-HCl [pH 7.5], 10 mM $MgCl_2$, 1.0 mM Na_2EDTA, 100 mM NaCl, 1.0 mM β-mercaptoethanol; wash buffer was equilibration buffer with 250 mM NaCl; elution buffer was equilibration buffer with 1.0 M NaCl plus 1.0 mM DTT).

tRNA from *Chlamydomonas reinhardtii* was a generous gift from W.-Y. Wang (University of Iowa). S. D. Schwartzbach (University of Nebraska) kindly supplied whole cell tRNA and chloroplast tRNA from *Euglena gracilis* Klebs var *bacillaris* Cori and whole cell tRNA from aplastidic strain W_3BUL (derived from *bacillaris* [8]).

Assay for *in Vitro* ALA Formation. ALA formation assays with enzyme extracts of *Euglena* and *Cyanidium* were carried out as previously described for *Chlorella* (21). Incubation was for 60 min at 30°C in 1.0 ml reaction medium (50 mM Tricine, 150 mM glycerol, 15 mM $MgCl_2$, 5.0 mM ATP, 1.0 mM NADPH, 1.0 mM glutamate, 1.0 mM DTT, 20 μM PALP, and 5.0 mM levulinic acid), plus enzyme extract containing 1 to 6 mg of protein and 1 to 20 A_{260} units of the RNA being tested. Reactions were terminated by addition of 100 μl of 1.0 M citric acid and 1.0 ml 10% (v/v) SDS followed by heating for 3 min at 95°C, and ALA was isolated on Dowex-50WX8(Na). Ethylacetoacetate (60 μl) was added and the solutions were heated to 95°C for 20 min to form ALA-pyrrole (15). The product was quantitated spectrophotometrically after reaction with Ehrlich-Hg reagent (21).

Chlorella enzyme incubations were carried out as above, but in some cases the incubation time was shortened to 20 or 30 min. Also, in some cases the procedure was modified to permit a smaller incubation volume (250 μl). The low volume 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 phosphate buffer (500 mM NaOH adjusted to pH 6.8 with H_3PO_4), ALA was converted to the ALA-pyrrole with ethylacetoacetate, and measured spectrophotometrically after reaction with Ehrlich-Hg reagent, as previously described.

Assay for Glutamyl-tRNA Formation. Formation of [^{14}C]glutamate-tRNA adduct was measured by appearance of TCA-precipitable radioactivity (7, 22). Incubation was carried out for 30 min at 30°C in 250 μl reaction medium similar to the assay medium for ALA formation. NADPH and levulinic acid were omitted from this medium, and the glutamate was present at a concentration of 100 μM and a specific radioactivity of 34 cpm pmol^{-1}. Reactions were terminated by the addition of 1.0 ml 84% (v/v) acetone containing 12.5% (w/v) TCA. After standing on ice for 15 min, the mixtures were centrifuged for 2 min at 13,500*g* in the Eppendorf microcentrifuge. The pellets were washed once with 1.5 ml 67% (v/v) acetone containing 10% (w/v) TCA and 1.0 mM glutamate, then twice with 1.5 ml portions of 10% (w/v) TCA containing 1.0 mM glutamate, and finally with 1 ml 95% (v/v) ethanol. The final pellets were dissolved in 100 μl 88% (w/v) aqueous HCOOH, 1.7 ml. Tritosol (10) liquid scintillation solution was added, and radioactivity was determined by liquid scintillation spectroscopy in a Beckman LS-

100C instrument. With 8.4×10^5 cpm of added substrate, zero-time controls had fewer than 50 cpm in the final pellets, and all duplicates agreed within 15%.

Other Procedures. Protein was determined by the dye-binding method of Bradford (5) using BSA as the standard. Cell population densities were determined with a Coulter Counter (model ZBI, Coulter Electronics).

Materials. tRNAs were obtained from Sigma and Boehringer. *Escherichia coli* $tRNA^{glu}$ type II was obtained from Sigma. Cellulose DE-23 was purchased from Whatman. All other reagents were purchased from Sigma or Fisher.

RESULTS

RNA Concentration Dependence for Stimulation of ALA Formation in *Chlorella* Extracts. Upon addition of a tRNA-containing fraction from *Chlorella* to the ALA-forming enzyme system from *Chlorella*, stimulation of the ALA-forming activity was observed (Fig. 1). If the enzyme system was pretreated with RNase to degrade endogenous RNA (followed by addition of RNasin to block further RNase activity), the requirement for added RNA was nearly absolute. If the enzyme extract was instead partially depleted of endogenous RNA by differential $(NH_4)_2SO_4$ precipitation, an absolute requirement for added RNA was not observed, although the ALA-forming activity was stimulated manyfold by added RNA. Enzyme extract that was not treated to lower the endogenous RNA concentration was also stimulated by added RNA, even though in this case the activity without added RNA was higher than in the other cases. Regardless of the method employed for preparing the enzyme extract, ALA-forming activity was increasingly stimulated by addition of increasing amounts of RNA, and the stimulation was not saturated, even at 40 A_{260} units ml^{-1} of added RNA.

At all levels of added RNA, the specific activity was higher in the enzyme extract subjected to differential $(NH_4)_2SO_4$ precipitation because this treatment also resulted in a partial purification of the enzyme system. The relatively lower activity (at any given concentration of added RNA) in the enzyme extract subjected to RNase digestion is in agreement with the previously reported (21) inactivation of the enzyme system during preincubation under the conditions employed.

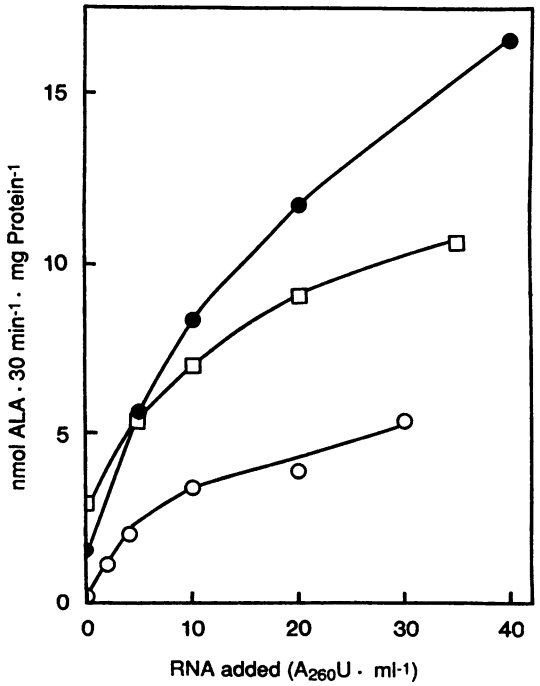

FIG. 1. Stimulation of ALA formation in enzyme extract from *Chlorella* by addition of *Chlorella* RNA. Enzyme and RNA extracts were prepared, and incubation carried out, as described in the text. Enzyme extract partially depleted of endogenous RNA by differential $(NH_4)_2SO_4$ precipitation (●); enzyme extract depleted of endogenous RNA by RNase digestion (○); enzyme extract not depleted of endogenous RNA (□).

Stimulation of ALA Formation by Added RNA in Enzyme Extracts of Various Algae. ALA formation in enzyme extracts of three different algae was stimulated by addition of RNA from either *Chlorella* or *Euglena* (Table I). Although the various enzyme extracts had different levels of ALA-forming activity, RNA from *Chlorella* and *Euglena* was approximately equally effective in stimulating ALA synthesis in all cases.

Substitution of the Required RNA with Various Heterologous RNAs. A wide range of RNAs were tested for the ability to stimulate ALA formation in enzyme extracts of *Chlorella* and *Euglena*. Stimulation was observed in all cases where the RNA was derived from green or greening cells or tissue (Table II). For example, RNA from two wild-type strains of *Euglena* was stimulatory, as was RNA derived from greening higher plant tissues (barley and cucumber) and the prokaryotic blue-green alga *Anacystis*. On the other hand, little or no stimulation was observed upon addition of RNA derived from nongreenable cells or tissue. Thus, neither of two different aplastidic *Euglena* strains provided stimulatory RNA, nor was stimulation provided by tRNA from wheat germ, yeast, bovine liver, or *E. coli*.

In two cases where chloroplast-derived RNA was tested (cucumber and *Euglena*), both were found to be stimulatory. On the other hand, RNA from the two aplastidic *Euglena* strains was not stimulatory.

In view of reported stimulation of ALA formation in *Chlamydomonas* extracts by *E. coli* $tRNA^{glu}$ (11, 12), two different forms of *E. coli* tRNA were tested in *Chlorella* and *Euglena* enzyme systems. Neither a mixture of *E. coli* tRNAs nor one glutamate-specific component, $tRNA^{glu}$ type II, was able to stimulate ALA formation in either algal enzyme system.

Inhibition of ALA Synthesis in *Chlorella* Extracts by *E. coli* tRNA. In several cases where the added RNA did not stimulate ALA formation, there appeared to be inhibition of the amount of ALA formed by *Chlorella* enzyme extract in the presence of endogenous RNA (Table II). The inhibitory effect of *E. coli* tRNA was investigated further. In the presence of 12 A_{260} units ml^{-1} of added *Chlorella* RNA, increasing inhibition of ALA-forming activity was measured with increasing amounts of added *E. coli* tRNA (Fig. 2). In the absence of added *Chlorella* RNA, the low level of ALA formation in the presence of endogenous RNA was lowered still further by the addition of *E. coli* RNA.

Table I. *Stimulation of ALA Formation by Addition of RNA Extracted from Chlorella or Euglena to Enzyme Systems Derived from Various Algae*

Enzyme and RNA extracts were prepared as described in the text. Five A_{260} units ml^{-1} of each RNA were added and incubation time was 60 min. Because of variations in the activity and degree of endogenous RNA depletion of different enzyme preparations, the activity of control incubations without added RNA is shown in all cases.

Enzyme Source	RNA Added			
	Chlorella		*Euglena*	
	− RNA	+ RNA	− RNA	+ RNA
	nmol 30 min^{-1} mg^{-1} protein			
Chlorella strain C-10[a]	0.39	7.45	0.42	6.34
Euglena strain Z (wild-type)	0.13	1.04	0.13	0.83
Cyanidium caldarium	0.11	0.43	0.11	0.38

[a] *Chlorella* enzyme preparation was depleted of endogenous RNA by differential $(NH_4)_2SO_4$ precipitation.

Table II. *Stimulation of ALA Formation by Heterologous RNA*

Various RNAs were tested for ability to stimulate ALA formation in enzyme systems derived from *Chlorella* and *Euglena*. Enzyme and RNA extracts were prepared as described in the text. Because of variations in the activity and degree of endogenous RNA depletion of different enzyme preparations, the activity of control incubations without added RNA is shown in all cases.

RNA Source	Amount Added	Enzyme System			
		Chlorella[a]		*Euglena*	
		− RNA	+ RNA	− RNA	+ RNA
	A_{260} units ml^{-1}	nmol 30 min^{-1} mg^{-1} protein			
Chlorella strain C-10					
Greening	5	1.12	6.45	0.17	1.25
	10	2.67[b]	5.72[b]		
	25	0.47[c]	4.58[c]		
Euglena strain Z (wild-type)					
Light-grown	5	0.42	6.34	0.13	0.83
Euglena strain W_{14}ZNalL (aplastidic derivative of strain Z)					
Light-grown	5	1.12	1.14	0.17	0.28
Euglena strain *bacillaris* (wild-type)					
Whole cell RNA	5			0.15	0.30
Chloroplast RNA	7.5			0.15	1.35
Euglena strain W_3BUL (aplastidic derivative of strain *bacillaris*)					
	5			0.15	0.20
Chlamydomonas reinhardtii	5	3.11	12.02	0.16	1.39
Anacystis nidulans	5	1.59	13.71	0.13	1.54
Greening etiolated cucumber cotyledon chloroplast RNA	20	1.06	2.79		
Greening etiolated barley epicotyl whole tissue RNA	20	1.06	2.12		
Wheat germ tRNA	5	1.75	1.55	0.13	0.21
	10	2.43[b]	1.88[b]		
	20	1.75	0.84		
Bakers' yeast tRNA	5	0.95	0.67	0.14	0.22
Bovine liver tRNA	5	0.95	0.94	0.14	0.21
E. coli tRNA	4	1.08	0.86		
	5			0.15	0.19
	10	4.86[b]	3.12[b]		
E. coli $tRNA^{glu}$ type II	1	0.53[c]	0.46[c]		
	2			0.16	0.16
	5	1.99	0.86		

[a] *Chlorella* enzyme preparation was depleted of endogenous RNA by differential $(NH_4)_2SO_4$ precipitation unless otherwise indicated. [b] *Chlorella* enzyme preparation was not depleted of endogenous RNA. [c] *Chlorella* enzyme preparation was depleted of endogenous RNA by RNase digestion, followed by addition of RNasin to block degradation of added RNA.

In contrast to the inhibitory effect of *E. coli* tRNA, increasing amounts of *Chlorella* RNA up to 40 A_{260} units ml^{-1} were not inhibitory, but instead increased the amount of ALA formed (Fig. 1).

Aminoacylation of Various RNAs with Glutamate by *Chlorella* Extract. *Chlorella* enzyme extract was tested for the ability to catalyze aminoacyl bond formation between glutamate and RNA from each species examined for ability to stimulate ALA formation. In all cases, aminoacyl bond formation was observed (Table III). Even those RNAs that were unable to stimulate ALA formation (aplastidic *Euglena*, wheat germ, yeast, bovine liver, and *E. coli*) served as substrates in the aminoacylation reaction. *E. coli* $tRNA^{glu}$ type II was particularly active in the aminoacylation assay, even though this RNA was inhibitory in the

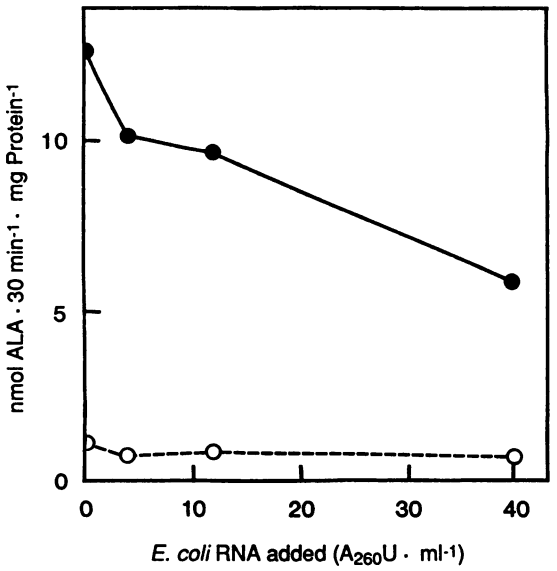

FIG. 2. Inhibition of ALA-forming activity in *Chlorella* enzyme extract by *E. coli* tRNA. Enzyme and RNA extracts were prepared, and incubation carried out, as described in the text. Enzyme extract partially depleted of endogenous RNA by differential $(NH_4)_2SO_4$ precipitation (○; similar treatment, but 12 A_{260} units ml^{-1} *Chlorella* RNA added to all samples (●).

Table III. *Aminoacylation of Various RNAs by Chlorella Extract*

Enzyme and RNA extracts were prepared as described in the text. Enzyme extract was partially depleted of endogenous RNA by differential $(NH_4)_2SO_4$ precipitation. Incubation was for 30 min in the presence of [^{14}C]glutamate (840,000 cpm, 100 μM) and 5 A_{260} units (20 A_{260} units ml^{-1}) of the indicated species of RNA. After incubation, RNA was precipitated with TCA, washed, and counted.

RNA Source	Precipitated Radioactivity
	cpm
None (endogenous level)	671
(Zero-time)	37
Greening *Chlorella* strain C-10	13,740
(Zero-time)	41
Light-grown *Euglena* strain Z (wild-type)	10,441
Light-grown *Euglena* strain W_{14}ZNalL (aplastidic derivative of strain Z)	6,761
Chlamydomonas reinhardtii	16,319
Anacystis nidulans	8,933
Greening etiolated cucumber cotyledon chloroplast RNA	5,199
Greening etiolated barley epicotyl whole tissue RNA	6,872
Wheat germ tRNA	8,140
Bakers' yeast tRNA	7,017
Bovine liver tRNA	2,336
E. coli tRNA	5,983
E. coli $tRNA^{glu}$ type II	
(1.0 A_{260} U)	17,461
(2.0 A_{260} U)	27,992
E. coli $tRNA^{phe}$	953
E. coli $tRNA^{val}$	926

ALA-forming reaction. To verify the specificity of the aminoacylation assay, *E. coli* $tRNA^{phe}$ and $tRNA^{val}$ were examined and found not to be aminoacylated with glutamate.

DISCUSSION

The essentiality of an RNA component in the reaction catalyzing formation of ALA from glutamate *in vitro* was first reported for extracts of greening barley plastids (14) and whole-cell extracts of *Chlamydomonas* (11). The general findings of these reports were confirmed for *Chlorella* extracts (22), but some differences among the three systems were observed. In particular, the range of heterologous RNAs capable of serving in the ALA-forming systems differed: in the *Chlamydomonas* system, *E. coli* $tRNA^{glu}$ could reconstitute activity in enzyme preparations depleted of RNA, whereas in the barley and *Chlorella* systems, only the RNA from the respective homologous species was capable of reconstituting activity, and tRNA from yeast, wheat germ, and *E. coli* was ineffective. For this reason it was desirable to extend the range of species examined, with the goal of determining which properties were general among glutamate-to-ALA transforming systems and which were specific to particular species.

A second goal of the present study was to determine whether the inability of some tRNAs to reconstitute ALA-forming activity might be due to the inability of the enzyme extract to utilize these RNAs as a substrate for the glutamyl-tRNA synthetase reaction. The aminoacylation reaction, in addition to being involved in peptide bond synthesis, has been proposed as the first step in ALA formation (11, 12). This proposal has been strengthened by the recent determination that the active RNA in barley chloroplasts is a tRNA containing the glutamate anticodon sequence (19). Moreover, it was shown that the 3′-CAA region, which is involved in aminoacyl bond formation with tRNAs, is also essential for the function of this tRNA in the ALA-forming process. However, it was also found that the glutamyl-tRNA that is active in ALA formation contains several base modifications in the anticodon region, and it is not yet known whether this particular tRNA also participates in peptide bond synthesis (19).

ALA formation was examined in enzyme extracts from three algae, *Chlorella*, *Euglena*, and *Cyanidium*. These species span a diverse range of groups including Chlorophyta, Euglenophyta, and Rhodophyta. In each case, activity was stimulated by the addition of RNA from either *Chlorella* or *Euglena*. Next, RNA from a diverse range of sources was tested for the ability to stimulate ALA formation in enzyme extracts of *Chlorella* and wild-type *Euglena*. One conclusion to be drawn from the results is that the ALA-forming systems investigated in this study all have similar specificities with respect to the acceptable RNAs.

Of all of the RNAs tested for the ability to stimulate ALA formation, the only ones that were effective were those derived from green or greenable cells and tissues. For example, both prokaryotic (*Anacystis*) and eukaryotic (*Chlorella*, *Chlamydomonas*, *Euglena*) algae, as well as higher plant tissue (barley, cucumber) provided effective RNA, but not *E. coli*, wheat germ, yeast, bovine liver, or aplastidic *Euglena* strains that are incapable of greening. The observations that neither of two aplastidic *Euglena* cells yielded effective RNA, and that RNA extracted from *Euglena* plastids was more effective than RNA derived from whole cells, indicates that effective RNA is localized only within the plastids in this species.

The inability of some of the tested RNAs to stimulate ALA formation was not due to inability of the enzyme extracts to use these RNAs as substrate for aminoacylation with glutamate. The specificity of the aminoacylation assay was verified by the inability of *E. coli* $tRNA^{phe}$ or $tRNA^{val}$ to be aminoacylated with glutamate. *Chlorella* enzyme extract was able to acylate all of the other tested RNAs with glutamate. The relative ability of each RNA to serve in the aminoacylation reaction was unrelated to its relative ability to stimulate ALA formation. In fact, *E. coli* $tRNA^{glu}$ type II, which was the most active RNA in the aminoacylation assay, not only did not stimulate ALA formation in either *Chlorella* or *Euglena* enzyme extracts, but inhibited ALA formation when administered to *Chlorella* enzyme extract either in the presence or absence of added *Chlorella* RNA. In earlier experiments using less pure enzyme preparations (22), we were able to detect aminoacylation of *E. coli* mixed tRNAs, but not $tRNA^{glu}$ type II, perhaps due to degradation of the RNA during the incubation. Our results with wheat germ and *E. coli* tRNA in algal enzyme extracts are in full agreement with the results previously reported for barley (14).

In summary, examination of the RNA requirement for *in vitro* formation of ALA from glutamate by enzyme extracts of several algae allows these conclusions to be drawn: (a) the algal enzyme extracts examined have similar specificities with respect to the RNAs that are effective in stimulating ALA formation; (b) effective RNA is found in cyanobacterial, algal, and higher plant cells that are green or greening, but not in cells that cannot form Chl; (c) in eukaryotic cells, the effective RNA is found within plastids, which may be the only intracellular source; (d) several of the RNAs from nongreenable cells can be aminoacylated with glutamate when incubated with *Chlorella* enzyme extract, but are nevertheless ineffective in stimulating ALA formation. Because of the differing RNA requirements observed for ALA formation and glutamyl-tRNA formation in the species examined in the present study, and considering the known acceptability of *E. coli* tRNAs for aminoacylation by chloroplast aminoacyl tRNA synthetases (23), our results suggest that there may be a structural requirement for the RNA involved in ALA formation which differentiates it from the chloroplast $tRNA^{glu}$ that is involved in protein synthesis.

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