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Formation of the Chlorophyll Precursor δ-Aminolevulinic Acid in Cyanobacteria Requires Aminoacylation of a $tRNA^{Glu}$ Species

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In the chloroplasts of higher plants and algae, the biosynthesis of the chlorophyll precursor δ-aminolevulinic acid (ALA) involves at least three enzymes and a tRNA species. Here we demonstrate that in cell extracts of the unicellular cyanobacterium *Synechocystis* sp. strain PCC 6803 ALA was formed from glutamate in a series of reactions in which activation of glutamate by glutamyl-$tRNA^{Glu}$ formation was the first step. The activated glutamate was reduced by a dehydrogenase which displayed tRNA sequence specificity. Fractionation of strain 6803 tRNA by reverse-phase chromatography and polyacrylamide gel electrophoresis yielded two pure $tRNA^{Glu}$ species which stimulated ALA synthesis in vitro. These tRNAs had identical primary sequences but differed in the nucleotide modification of their anticodon. The 6803 $tRNA^{Glu}$ was similar to the sequences of $tRNA^{Glu}$ species or $tRNA^{Glu}$ genes from *Escherichia coli* and from chloroplasts of *Euglena gracilis* and higher plants. Southern blot analysis revealed at least two $tRNA^{Glu}$ gene copies in the 6803 chromosome. A glutamate-1-semialdehyde aminotransferase, the terminal enzyme in the conversion of glutamate to ALA in chloroplasts, was detected in 6803 cell extracts by the conversion of glutamate-1-semialdehyde to ALA and by the inhibition of this reaction by gabaculin.

The biosynthesis of δ-aminolevulinic acid (ALA), the universal precursor of porphyrins, occurs via at least two different biosynthetic pathways (reviewed in reference 5). In mammals, yeasts, fungi, and the purple bacteria, ALA is formed by the Shemin pathway, which involves the condensation of succinyl-coenzyme A and glycine catalyzed by ALA synthase (5). This ALA is then used in the synthesis of hemes, vitamin B_{12}, and bacteriochlorophyll. In the chloroplasts of higher plants (2), archaebacteria (9, 10), certain eubacteria (16, 33, 34), cyanobacteria (1, 7, 24, 30), algae (19, 20, 50, 51), and phytoflagellates (29, 49), ALA is formed in the C-5 pathway from the five-carbon skeleton of glutamate. Eight molecules of glutamate-derived ALA provide all of the carbon and nitrogen atoms for the formation of the porphyrin nucleus of one molecule of chlorophyll.

The transformation of glutamate to ALA occurs in the chloroplasts of higher plants and algae and requires ATP, Mg^{2+}, NADPH, and tRNA. In barley chloroplasts the required tRNA was shown to be a chloroplast-encoded $tRNA^{Glu}$ (41). Of the several proposed models of ALA synthesis by the C-5 pathway (37), the postulated mechanism shown in Fig. 1 is the best supported (22, 41). In the first step glutamate is activated by an aminoacyl-tRNA synthetase to form glutamyl-$tRNA^{Glu}$ (41). The next two postulated steps involve the reduction of the carboxyl group by an NADPH-dependent reductase to yield glutamate 1-semialdehyde (GSA) which is then transaminated by an aminotransferase to form ALA.

Synechocystis sp. strain PCC 6803 (*Synechocystis* strain 6803) is a unicellular, non-nitrogen-fixing, photosynthetic procaryote (39). This cyanobacterium is distinguished from the purple and green photosynthetic bacteria by its ability to perform plantlike oxygenic photosynthesis and by the nature of its photosynthetic apparatus, which is highly homologous to that found in chloroplasts (4, 45). Other features of *Synechocystis* strain 6803 which make it an appealing experimental system include its transformability (6, 12), its ability to grow photoheterotrophically, and the availability of techniques for genetically engineering mutants (46). We are interested in using *Synechocystis* strain 6803 as a model system in which to study the C-5 pathway of ALA formation originally described for chloroplasts (2, 20). While glutamate was shown in vivo to be the precursor of ALA in the cyanobacteria *Anabaena variabilis* (1), *Agmenellum quadruplicatum* (24), *Synechococcus* sp. strain PCC 6301 (27, 30), and *Spirulina platensis* (7), synthesis of ALA in cell extracts of cyanobacteria has not been demonstrated.

Here we report that the conversion of glutamate to ALA in crude cell extracts of *Synechocystis* strain 6803 occurs by the same $tRNA^{Glu}$-dependent pathway found in chloroplast extracts from plants and eucaryotic algae.

MATERIALS AND METHODS

General. *Chlamydomonas reinhardtii* $c137^+$ was obtained from J. Rosenbaum. *Synechocystis* strain 6803 was a gift from A. Y. Cheung. GSA was prepared and kindly given to us by C. G. Kannangara (17). *Escherichia coli* $tRNA_2^{Glu}$ was prepared by the Oak Ridge National Laboratory.

Growth of strain 6803. *Synechocystis* strain 6803 was grown photoautotrophically or photoheterotrophically (0.2% [wt/vol] glucose) at 28°C on BG-11 medium described by Rippka et al. (39) with modifications as described by Elmorjani and Herdman (8). The cells were grown under constant illumination and with constant shaking (200 rpm) to mid-log phase (A_{730} of 0.8 to 1.2) and then collected by centrifugation at 10,000 × *g* for 10 min at 4°C, washed twice with 100 mM Tricine (pH 8.0)–15 mM $MgCl_2$–1 mM dithiothereitol (DTT), and stored frozen.

Preparation of cell extracts. Frozen *Synechocystis* 6803 cells were thawed and then disrupted by passage through a French pressure cell at 16,000 to 18,000 lb/in^2. The cell homogenate was centrifuged at 100,000 × *g* for 90 min at 2°C to remove unbroken cells and membranes. After this centrifugation, glycerol was added to the clarified cell extract to give a final concentration of 10% (vol/vol). Low-molecular-weight components were removed from the cell extract by

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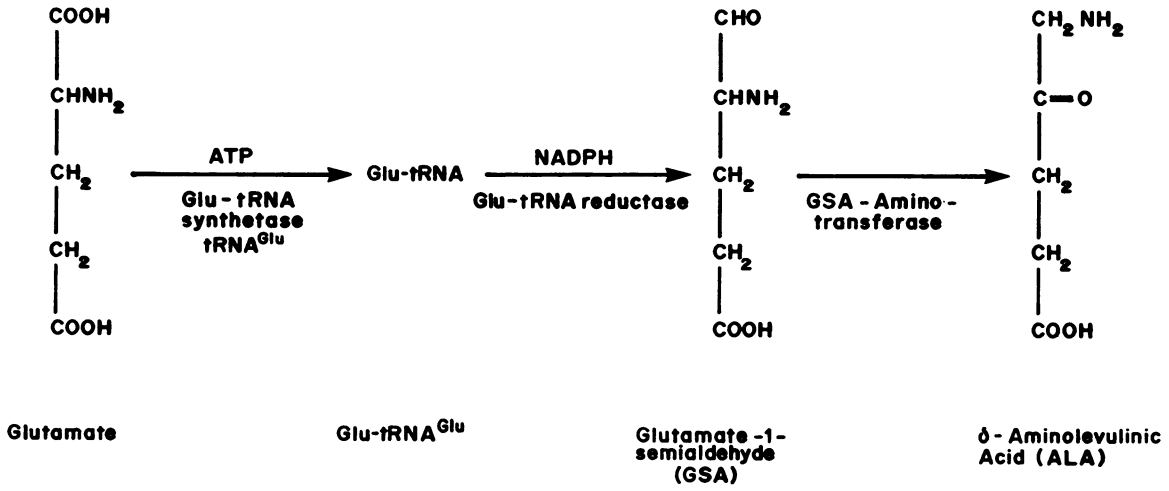

FIG. 1. Scheme of the first steps of chlorophyll synthesis. For details see text.

chromatography on a Sephadex G-25 column (2 by 50 cm) equilibrated with 100 mM Tricine (pH 8.0)–15 mM $MgCl_2$–10% glycerol–15 μM pyridoxal phosphate–1 mM DTT or by dialysis against this buffer.

For charging of tRNA, this fraction was treated with DEAE-cellulose in order to remove RNA.

Isolation and purification of tRNA. Unfractionated tRNA (420 A_{260} units) was prepared from *Synechocystis* strain 6803 cells (18 g, wet weight) by phenol extraction (31) of a cell homogenate prepared by breaking the cells in a French pressure cell at 16,000 lb/in^2 and a DEAE-cellulose step (31). The tRNA was then fractionated by RPC-5 reverse-phase chromatography (36). The resulting fractions were assayed for their ability to be aminoacylated with [^{14}C]glutamate in standard aminoacylation assays with homologous enzymes as described previously (31) and for their ability to stimulate ALA synthesis in *Synechocystis* strain 6803 cell extracts. The $tRNA^{Glu}$ species obtained were further fractionated by polyacrylamide gel electrophoresis (15%) in 8 M urea. The tRNAs were located in the gels by staining with toluidine blue O (0.4% [wt/vol] in 50% methanol–10% glacial acetic acid).

Assays for in vitro ALA formation. The formation of ALA in cell extracts was assayed by spectrophotometric measurement of the formation of the Ehrlich salt of ALA-pyrrole and by the conversion of [^{14}C]glutamate to [^{14}C]ALA. The assay for ALA formation in cell extracts (50), the purification of ALA by chromatography on Dowex 50W-X8-Na^+ (47, 50), the quantitation of ALA by reaction of ALA-pyrrole with Ehrlich reagent (50), and the identification of ALA as ALA-pyrrole by thin-layer chromatography (47) were performed as described. Briefly, the standard reaction for ALA formation (0.5 ml) contained 100 mM Tricine-NaOH, pH 8.0, 15 mM $MgCl_2$, 2 mM ATP, 1 mM NADPH, 10 mM levulinate, 0.3 M glycerol, 5 mM DTT, either 1 mM glutamate or 0.5 μCi of [^{14}C]glutamate (285 mCi/mmol), 2 to 10 mg of protein, and various tRNAs where indicated. Reaction mixtures were incubated for 2 h at 30°C with constant shaking. Reactions were terminated by the addition of 0.5 ml of 10% sodium dodecyl sulfate (SDS) and 0.05 ml of 1 M citric acid and subsequent incubation of this mixture for 3 min at 100°C. The ALA was then purified by cation-exchange chromatography on Dowex 50W-X8-Na^+ (47, 50). The Dowex-purified ALA was caused to react with ethylacetoacetate (50) to form ALA-pyrrole and then quantitated either by reaction of ALA-pyrrole with Ehrlich reagent or by determining the incorporation of ^{14}C into ALA-pyrrole when [^{14}C]glutamate was used as the substrate. In certain experiments final identification of ALA was accomplished by thin-layer chromatography of the ALA-pyrrole (47).

RNA sequencing. Enzymatic RNA sequencing of 5′- and 3′-end-labeled RNA and chemical sequencing of 3′-end-labeled RNA was performed as described by Krupp and Gross (25). Approximately 1 μg of pure tRNA was labeled at its 5′ end by using [γ-^{32}P]ATP and the phosphate exchange reaction of T4 polynucleotide kinase (25). The 3′ end of tRNA was labeled by using 5′-[^{32}P]pCp and RNA ligase (25). The 5′-terminal nucleotide was identified by complete P1 digestion of 5′-^{32}P-labeled tRNA and identification of the 5′-^{32}P-nucleotide by thin-layer chromatography (32). The sequence of the 5′ half of the anticodon stem and of the anticodon loop was confirmed by two-dimensional chromatography as described (43). The 5′-labeled oligonucleotides for homochromatography analysis were prepared by complete RNase T_1 digestion of *Synechocystis* strain 6803 $tRNA_1^{Glu}$ and $tRNA_2^{Glu}$. The position of the T_1 oligonucleotide is shown in Fig. 3.

Southern hybridizations. An oligonucleotide (5′-ACAC CUCCCUNUCACG-3′) obtained by T_1 digestion (25) of the *Synechocystis* strain 6803 $tRNA_1^{Glu}$ was used to probe Southern blots of total genomic DNA from 6803, *C. reinhardtii*, and *Hordeum vulgare* (barley). Southern blotting was performed according to Maniatis et al. (28). The hexadecameric RNase T_1 oligonucleotide was labeled at its 5′ end with T4 polynucleotide kinase and [^{32}P]ATP by employing reaction conditions described elsewhere (28). Southern blot hybridizations were performed at 42°C in a solution containing 5× SSC (1× SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0), 1× Denhardt solution (0.02% Ficoll, 0.02% bovine serum albumin, 0.02% polyvinylpyrrolidone), 50% formamide, 0.2% SDS, and 10 mM EDTA. No carrier DNA or RNA was included in the prehybridizations or hybridizations. Filters were washed four times for 30 min each in 2× SSC–0.1% SDS at 42°C, and hybridization was detected by autoradiography.

RESULTS

ALA formation in crude cell extracts. We first sought to demonstrate ALA synthesis activity in crude cell extracts of

TABLE 1. ALA formation in *Synechocystis* strain 6803 extracts[a]

Reaction mixture	Conversion of [^{14}C]glutamate into ALA (cpm)
Complete (inactivated enzyme[b]) without added tRNA[c]	620
Complete (active enzyme)	
Without added tRNA	2,940
+ tRNA (1 A_{260})	3,310
+ tRNA (4 A_{260})	7,500
+ tRNA (10 A_{260})	20,950
+ tRNA (10 A_{260}) + DNase I (10 μg)	20,580
+ tRNA (10 A_{260}) + RNase A (10 μg)	2,670
+ tRNA (10 A_{260}) treated with $NaIO_4$[d]	2,670
+ tRNA (10 A_{260}) − ATP	1,420

[a] The control reaction (0.5 ml) contained 100 mM Tricine-NaOH (pH 7.9), 0.3 M glycerol, 5 mM DTT, 15 mM $MgCl_2$, 1 mM NADPH, 2 mM ATP, 10 mM levulinate, 0.5 μCi of [^{14}C]glutamate (285 mCi/mmol), and cell extract (8 mg of protein).

[b] The enzyme extract was inactivated before the addition of substrates and cofactors by treatment with 0.5 ml of 10% SDS and 50 μl of 1 M citric acid.

[c] Synechocystis strain 6803 tRNA.

[d] 10 A_{260} 6803 tRNA in 0.1 ml of H_2O was mixed with 0.1 ml of 1 mM $NaIO_4$ in H_2O and incubated for 15 min at 23°C. The $NaIO_4$ then was inactivated by the addition of 0.1 ml of 10 mM glucose. The tRNA was mixed with 0.3 ml of 0.6 M sodium acetate and recovered by ethanol precipitation. This tRNA preparation could not be glutamylated by the homologous S-100 preparation.

Synechocystis strain 6803 following assay conditions developed for assaying ALA synthesis in extracts of plant chloroplasts. Our results (Table 1) show that ALA synthesis can be detected in the presence of ATP, Mg^{2+}, and NADPH, but without added tRNA, presumably due to the presence of endogenous tRNA. Pretreatment of the crude cell extract with RNase A (even in much greater amounts than listed in Table 1) reduced ALA formation, but did not totally abolish it. ALA synthesis was greatly stimulated when increasing amounts of *Synechocystis* strain 6803 tRNA were added. This effect could be abolished by the addition of RNase A; this demonstrates that tRNA is required for optimal ALA synthesis. In order to show that ALA synthesis requires an aminoacylated tRNA, a sample of 6803 tRNA was rendered incapable of being aminoacylated by periodate oxidation (53). *Synechocystis* strain 6803 tRNA treated with sodium periodate did not show any stimulatory effect on ALA synthesis and had little effect on the endogenous levels of ALA synthesis. The observed ATP requirement of ALA formation must be caused in the first step, since the overall reaction proceeded well with added charged Glu-tRNA^{Glu}. Treatment of cell extracts with DNase had no effect on tRNA-stimulated ALA synthesis.

RNA required for ALA synthesis is a major tRNA^{Glu} species. We next sought to identify the required RNA moiety for ALA biosynthesis in *Synechocystis* strain 6803 by fractionation of crude tRNA and sequence analysis of the active tRNA species. When tRNA from photoautotrophically grown *Synechocystis* strain 6803 was fractionated by reverse-phase (RPC-5) chromatography, at least four peaks of glutamate acceptor activity were observed (Fig. 2). However, RNA which supported in vitro ALA formation eluted in two peaks coincident with two major peaks of glutamate acceptor activity. RNA from these peaks was further purified by polyacrylamide gel electrophoresis (15% polyacrylamide, 8 M urea). After visualization of RNA by toluidine blue staining, the different RNA species were eluted from the gel and tested for glutamate acceptor activity and their ability to support ALA synthesis. Only one RNA species from each pooled fraction (termed tRNA$_1^{Glu}$ and tRNA$_2^{Glu}$, respectively) had glutamate acceptor activity. ALA-supporting activity was found associated only with tRNA$_1^{Glu}$ and tRNA$_2^{Glu}$. These results strongly suggest that the RNA species that is a cofactor for ALA synthesis is a glutamate-specific tRNA.

To confirm this conclusion, we determined the nucleotide sequence of tRNA$_1^{Glu}$ and tRNA$_2^{Glu}$ by enzymatic and chemical RNA sequencing methods (25). The primary nucleotide sequence of 76 nucleotides of these two RNAs was identical (Fig. 3). The cloverleaf model of tRNA structure possesses the NUC anticodon of glutamate-specific tRNAs. The following modified nucleotides could be deduced from the cleavage patterns obtained by chemical and enzymatic sequencing reactions (the numbering is as suggested by Sprinzl et al. [44]): position 20B, dihydrouridine (D); position 34, a highly modified, labile uridine derivative (U*); position 54, 5-methyluridine (T); position 55, pseudouridine (ψ). The modified base in the wobble position of the anticodon (N34) was further characterized by two-dimensional mobility shift

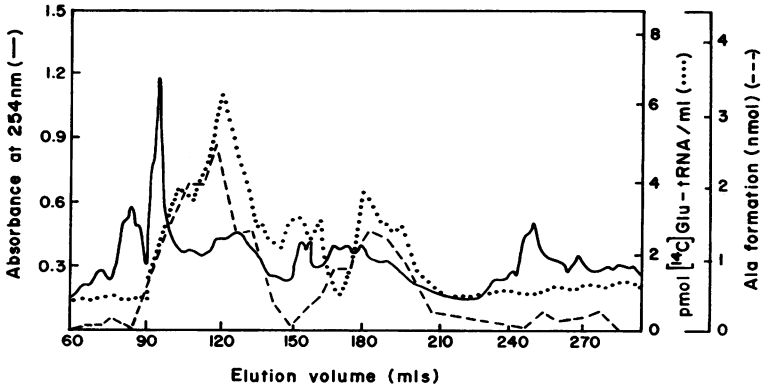

FIG. 2. RPC-5 reverse-phase chromatography of *Synechocystis* strain 6803 tRNA. Unfractionated tRNA (400 A_{260} units in 1.0 ml) were injected into a column (0.6 by 100 cm) packed with RPC-5 (36) and washed with 75 ml of buffer containing 10 mM Tris chloride (pH 7.0), 10 mM $MgCl_2$, 0.35 M NaCl, and 10 mM 2-mercaptoethanol. The RNA was eluted with a 200-ml linear gradient of 0.45 to 0.70 M NaCl in 10 mM Tris chloride (pH 7.0)–10 mM $MgCl_2$–10 mM 2-mercaptoethanol followed by a 150-ml linear gradient of 0.7 to 1.5 M NaCl in 10 mM Tris chloride (pH 7.0)–10 mM $MgCl_2$–10 mM 2-mercaptoethanol. Aliquots (0.1 ml) of fractions (1.5 ml) were used to determine glutamate acceptor activity and ALA synthesis as described elsewhere (22). Fractions eluting between elution volumes of 117 and 123 ml and 178 and 184 ml were pooled, and the RNA was precipitated with ethanol and is referred to as tRNA$_1^{Glu}$ and tRNA$_2^{Glu}$, respectively.

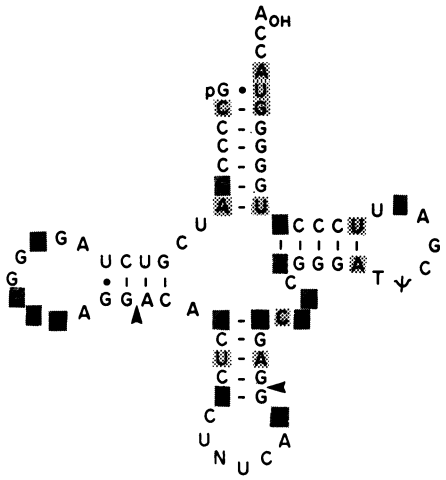

FIG. 3. Nucleotide sequence of $tRNA^{Glu}$ (EMBL/Genbank accession number M19535). The sequence was determined by base-specific enzymatic and chemical cleavage of the 5'- and 3'-end ^{32}P-labeled $tRNA^{Glu}$ from *Synechocystis* strain 6803. A fragment, indicated by the arrows, was generated by complete digestion with RNase T_1 and was used to identify modified bases of the anticodon by mobility shift analysis (43) and for use as a probe in Southern blot analysis (see Fig. 4). Shaded nucleotides designate differences in primary structure from the $tRNA^{Glu}$ genes of *E. coli* (light shading) (3) and *Hordeum vulgare* (heavy shading) (41).

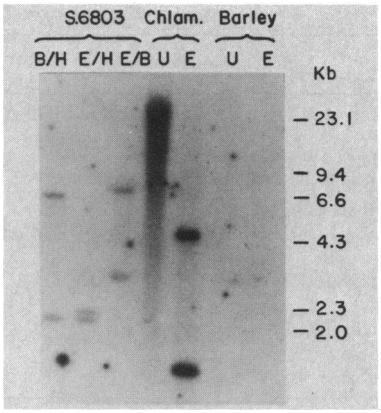

FIG. 4. Hybridization of a *Synechocystis* strain 6803 $tRNA^{Glu}$-derived [^{32}P]oligonucleotide with *Synechocystis* 6803 and *C. reinhardtii* genomic DNA. Restricted DNA (3 to 5 μg) was separated on a 0.7% agarose gel and transferred to nitrocellulose paper (28). Hybridization of a 16-mer oligonucleotide obtained by RNase T_1 digestion of *Synechocystis* strain 6803 $tRNA_1^{Glu}$ was performed in 5× SSC–1× Denhardt solution–50% formamide–0.2% SDS–10 mM EDTA for 16 h at 42°C. Nonspecifically bound probe was removed by washing the filter three times with 2× SSC–0.1% SDS at 42°C. The lanes contained *Synechocystis* 6803 (S. 6803), *C. reinhardtii* (Chlam), or barley genomic DNA, either undigested (U) or digested with the restriction endonucleases *Bam*HI (B), *Eco*RI (E), and *Hin*dIII (H) as indicated. Fragment sizes are shown in kilobases.

analysis of a nuclease T_1-resistant oligoribonucleotide spanning the anticodon loop. The nucleotide was labile under the conditions applied, giving rise to a branched pattern characteristic for sulfur-containing uridine derivatives (43) and similar to the behavior of the wobble base in $tRNA^{Glu}$ from barley chloroplasts (41). The pattern obtained was slightly different for the two isoacceptors, indicating a different degree of modification at this position. No further attempts were made to identify these nucleosides or to determine the overall pattern of modification in these two tRNAs, since no other differences in base modifications between these two tRNAs were evident.

These results prove that two of the four Glu-tRNAs of *Synechocystis* strain 6803 have a glutamate-specific anticodon. The two minor glutamate acceptor peaks observed in Fig. 2 were probably $tRNA^{Gln}$ species misaminoacylated with [^{14}C]glutamate (see Discussion).

Chromosomes of *Synechocystis* strain 6803 and *C. reinhardtii* each contain at least two copies of the $tRNA^{Glu}$ gene. There now exists extensive biochemical and genetic evidence to support the endosymbiotic origin of chloroplasts from a cyanobacteriumlike ancestor (reviewed in references 11, 26, and 52). Indeed, the *Synechocystis* strain 6803 $tRNA^{Glu}$ displays strong homology with $tRNA^{Glu}$ from chloroplasts of higher plants and algae (see Discussion). Since the complete sequences of the chloroplast genomes from *Marchantia polymorpha* (35) and *Nicotiana tabacum* (42) predict a single $tRNA^{Glu}$ gene, we are interested in determining whether a similar gene arrangement of the $tRNA^{Glu}$ gene(s) is present in *Synechocystis* strain 6803. Southern blot analysis with a ^{32}P-labeled oligonucleotide of 16 residues from $tRNA_1^{Glu}$ as a probe (arrows in Fig. 3) showed two copies of the $tRNA^{Glu}$ sequence in the chromosomes of 6803 and *C. reinhardtii* and no hybridization with barley chloroplast DNA (Fig. 4).

Effect of heterologous tRNA on ALA formation in cell extracts. We next sought to determine whether heterologous tRNAs could stimulate ALA formation in *Synechocystis* strain 6803 cell extracts. Our results (Table 2) show that tRNA from the eucaryotic photosynthetic alga *C. reinhardtii* stimulated ALA synthesis in cell extracts of *Synechocystis* strain 6803, although *C. reinhardtii* tRNA was not as stimulatory as an equal amount of *Synechocystis* 6803 tRNA. In contrast, tRNA from *E. coli*, *Bacillus subtilis*, and *Saccharomyces cerevisiae* did not stimulate ALA formation in cell extracts from *Synechocystis* 6803. The *Synechocystis* 6803 $tRNA^{Glu}$ as well as tRNA from *E. coli*, *B. subtilis*, *C. reinhardtii*, and *S. cerevisiae* could be charged with glutamate by the *Synechocystis* 6803 glutamyl-tRNA synthetase present in the cell extract (results not shown). For comparison, *Synechocystis* strain 6803 tRNA supported an approximately twofold-higher level of ALA synthesis in cell ex-

TABLE 2. Ability of various tRNAs to support ALA synthesis in cell extracts[a]

Reaction mixture	Conversion of [^{14}C]glutamate to ALA (cpm/reaction) in cell extract of:	
	Synechocystis 6803	*C. reinhardtii*
Complete (inactivated enzyme) without added tRNA	250	560
Complete		
Without added tRNA	2,360	3,270
+ *Synechocystis* 6803 tRNA (10 A_{260})	11,110	120,640
+ *C. reinhardtii* tRNA (10 A_{260})	3,400	53,980
+ *C. reinhardtii* tRNA (20 A_{260})	4,190	ND[b]
+ *E. coli* tRNA (10 A_{260})	2,290	11,330
+ *E. coli* $tRNA^{Glu}$ (1 A_{260})	1,820	15,290
+ *S. cerevisiae* tRNA (10 A_{260})	1,780	2,580
+ *B. subtilis* tRNA (10 A_{260})	1,600	ND

[a] See Table 1, footnotes *a* and *b*.
[b] ND, Not determined.

TABLE 3. GSA aminotransferase activity in cell extracts[a]

Reaction mixture	Conversion of [^{14}C]glutamate to ALA (cpm)	Unlabeled ALA formed (nmol)
Complete (inactivated enzyme) without added tRNA	340	
Complete		
Without added tRNA	2,820	
+ 10 μM gabaculin	570	
+ *Synechocystis* 6803 tRNA (10 A_{260})	15,390	
+ *Synechocystis* 6803 tRNA (10 A_{260}) + 10 μM gabaculin	1,320	
Complete (inactivated enzyme) without added GSA		0.0
Complete		
Without added GSA		0.2
+ 0.2 mM GSA		22.0
+ 0.2 mM GSA + 100 μM gabaculin		0.3

[a] See Table 1, footnotes *a* and *b*.

tracts of *C. reinhardtii* than an equal amount of *C. reinhardtii* tRNA (Table 2). As previously reported by Huang and Wang (19) and confirmed here (Table 2), *E. coli* $tRNA^{Glu}$ stimulated ALA synthesis in *C. reinhardtii* cell extracts.

ALA formation requires an aminotransferase. It has been postulated that the final step in the pathway of ALA formation in barley chloroplasts requires an aminotransferase enzyme which converts GSA to ALA (21, 23). The barley GSA aminotransferase is reported to be completely inhibited by gabaculin (3-amino-2,3-dihydrobenzoic acid) (21, 23). We could detect an enzymatic activity in cell extracts with properties analogous to those of the GSA aminotransferase from barley chloroplasts (Table 3). The tRNA-dependent conversion of glutamate to ALA by *Synechocystis* 6803 extracts was almost completely inhibited by 10 μM gabaculin. The site of action of gabaculin in *Synechocystis* 6803 extracts appears to be the GSA aminotransferase, as the conversion of synthetic GSA to ALA was completely inhibited by 100 μM gabaculin (Table 3).

DISCUSSION

ALA formation in *Synechocystis* strain 6803. Because of our interest in developing a facile system to study the biochemical, genetic, and regulatory mechanisms involved in the formation of ALA from glutamate, we have investigated this pathway in the unicellular procaryote *Synechocystis* strain 6803. The results presented here show that glutamate is converted to ALA in cell extracts of this organism. The experiments demonstrate clearly that charging of $tRNA^{Glu}$ is required to activate the glutamate for the reduction by the reductase. The formation of the *Synechocystis* 6803 Glu-tRNA can be accomplished in vitro by a homologous ligase (Fig. 2) or by *E. coli* aminoacyl-tRNA synthetase (data not shown). In analogy to the *E. coli* aminoacyl-tRNA synthetase mechanism, it is likely that the C_1 carboxyl group of glutamate is activated in this reaction. The implication of this is that dehydrogenation to GSA could occur by transacylation of the C_1 carboxyl-activated glutamyl residue to an acceptor group in the enzyme protein (e.g., a cysteine, as is the case in phosphoglyceraldehyde dehydrogenase [57]). The activation (by esterification with tRNA) of the glutamate carboxyl, which will subsequently be reduced, is a plausible mechanism that obviates the need in this pathway for an additional enzyme with hydrolytic activity required to release GSA from the tRNA (41). GSA is then transaminated to ALA by a transaminase activity. Thus, glutamyl-tRNA synthetase, a specific reductase, and a transaminase are the three enzymes involved in ALA formation.

ALA synthesis in *Synechocystis* strain 6803 can be inhibited by gabaculin. This drug is a potent inhibitor of γ-aminobutyrate aminotransferase (38) and reduces the biosynthesis of plant chlorophyll (14, 23) and of tetrapyrroles in cyanobacteria (13, 18). The target of gabaculin inhibition in barley chloroplasts is the GSA aminotransferase (23). For the cyanobacterium *Synechococcus* strain 6301, two conflicting reports place the primary site of gabaculin inhibition in a biosynthetic step either before (18) or after (13) ALA formation. Given the finding that gabaculin specifically inhibits the GSA aminotransferase of barley (23), our results indicate that in *Synechocystis* strain 6803 cell extracts, gabaculin inhibits the formation of ALA by blocking the GSA aminotransferase. A putative GSA aminotransferase protein (45 to 50 kilodaltons) has been detected on Western blots of *Synechocystis* 6803 extracts by using an antiserum prepared against the barley GSA aminotransferase (G. P. O'Neill and C. G. Kannangara, unpublished results).

tRNA specificity of the reductase. As very few chloroplast or algal $tRNA^{Glu}$ species have been sequenced and no purified reductase preparations exist, only some preliminary studies involving the ability of heterologous tRNAs to stimulate ALA synthesis have been reported. Mayer et al. (29) have reported that ALA synthesis in extracts of the phytoflagellate *Euglena gracilis* is stimulated by tRNA from organisms capable of plantlike oxygenic photosynthesis but not by *E. coli*, *S. cerevisiae*, wheat germ, archaebacterial, or bovine tRNA. In contrast, unfractionated tRNA and purified $tRNA_2^{Glu}$ from *E. coli* stimulate ALA formation in *C. reinhardtii* extracts (19). Our results show that reductase activities from different organisms vary in tRNA specificity: crude *E. coli* tRNA and purified $tRNA^{Glu}$ stimulate ALA synthesis in *C. reinhardtii* but not *Synechocystis* strain 6803 extracts. Therefore, different reductases must possess somewhat different binding sites for tRNA. Whether the discrimination is based on features of the tRNA's primary sequence or on the nature of the nucleotide modification remains to be explored.

Glutamyl-tRNA species in *Synechocystis* strain 6803. *Synechocystis* strain 6803 $tRNA^{Glu}$ is very similar to the $tRNA^{Glu}$ species from chloroplasts (44). Of the four known procaryotic $tRNA^{Glu}$ and the eight known chloroplast $tRNA^{Glu}$ sequences, the *Synechocystis* strain 6803 tRNA is more similar to chloroplast $tRNA^{Glu}$ (e.g., 95% with *Euglena gracilis* [15]; 93% with *Marchantia polymorpha* [35]; 87% with *Vicia faba* [48]) than to $tRNA^{Glu}$ species from the archaebacterium *Methanococcus vanielli* (58% [54]), the gram-negative bacterium *E. coli* (83% [3]), and the gram-

positive bacterium *Bacillus subtilis* (74% [56]). The significant homology between the $tRNA^{Glu}$ sequences of *Synechocystis* strain 6803, chloroplasts, and *E. coli* adds further evidence to the theory of the endosymbiotic origin of chloroplasts from a cyanobacterial species (11, 26, 52). It would be interesting to compare the sequence of the $tRNA^{Glu}$ from a rhodophytan algal species with the *Synechocystis* sequence, as evolutionary trees established on 16S RNA sequences place rhodophytan chloroplasts as direct descendants of cyanobacteria, whereas the euglenoids (*E. gracilis*) and the chlorophytes (*C. reinhardtii*) are more distant (55).

The four peaks of glutamate-accepting material obtained by reverse-phase chromatography of unfractionated *Synechocystis* strain 6803 tRNA probably represent four species of Glu-tRNA. As shown above by their anticodon sequence, two of these are bona fide $tRNA^{Glu}$ species (Fig. 3). These tRNAs must assume a dual role: they support ALA formation and—by deduction—also function in protein biosynthesis. The other two Glu-tRNA species are presumably $tRNA^{Gln}$ species that are mischarged with glutamate. It has recently been demonstrated that in *Synechocystis* strain 6803 as well as in chloroplasts, glutaminyl-$tRNA^{Gln}$ is formed by misaminoacylation of $tRNA^{Gln}$ with glutamate (40). The Glu-$tRNA^{Gln}$ is then converted to Gln-$tRNA^{Gln}$ by a specific amidotransferase.

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