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Transformation of Glutamate to δ-Aminolevulinic Acid by Soluble Extracts of *Chlorobium vibrioforme*

SIEGFRIED RIEBLE, JOHN G. ORMEROD,† AND SAMUEL I. BEALE*
Division of Biology and Medicine, Brown University, Providence, Rhode Island 02912

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Formation of the tetrapyrrole pigment precursor δ-aminolevulinic acid (ALA) from glutamate was detected and partially characterized in extracts of the strictly anaerobic green photosynthetic bacterial species *Chlorobium vibrioforme* by using assay methods derived from those developed for algae and cyanobacteria. ALA formation in *Chlorobium* extracts was saturated at 10 mM glutamate and required NADPH and ATP at optimal concentrations of 0.3 and 3 mM, respectively. Preincubation of the enzyme extract with RNase A destroyed the ALA-forming activity completely. Activity in the RNase-treated extract was restored by supplementation with *Chlorobium* RNA after addition of RNasin to block further RNase action. RNA from the cyanobacterium *Synechocystis* sp. strain PCC 6803 and *Escherichia coli* $tRNA^{Glu}$ also restored activity. Activity was inhibited 50% by 0.2 μM hemin. ALA formation was completely abolished by the addition of 5 μM 3-amino-2,3-dihydrobenzoic acid (gabaculine). These results indicate that *Chlorobium* extracts share with those of plants, eucaryotic algae, cyanobacteria, prochlorophytes, and methanogens the capacity for RNA-dependent ALA formation from glutamate.

δ-Aminolevulinic acid (ALA) is the first well-characterized, committed precursor in the biosynthesis of tetrapyrroles, including hemes, corrins, phycobilins, and chlorophylls (6). Two distinct pathways for ALA formation have been described. One route involves condensation of glycine and succinyl coenzyme A (succinyl-CoA) catalyzed by the enzyme ALA synthase (succinyl-CoA:glycine *C*-succinyltransferase [decarboxylating], EC 2.3.1.37) and occurs in animals, fungi, and some bacteria (11, 14, 26, 30, 40; Y. J. Avissar, J. G. Ormerod, and S. I. Beale, Arch. Microbiol., in press). ALA is synthesized by a second route from glutamate via the five-carbon pathway. This pathway occurs in plants (7, 36), algae (22), and many procaryotes (5, 10, 27, 33; Avissar et al., in press), including cyanobacteria (2) and methanogenic bacteria (13, 15).

Soluble cell extracts capable of ALA formation from glutamate have been obtained from phototrophic eucaryotes (16, 18, 29, 43, 44) and several procaryotic organisms, including cyanobacteria and prochlorophytes (35), *Methanobacterium thermoautotrophicum* (13), *Clostridium thermoaceticum* (33), *Escherichia coli* (5, 27), and others (Avissar et al., in press). Extracts of barley plastids (9) and *Chlorella* cells (47) have been partially purified and fractionated into three proteinaceous components and an RNA species, all of which are required for the reconstitution of ALA-forming activity. These extracts also require a reduced pyridine nucleotide, ATP, and Mg^{2+} as cofactors in vitro. A current working hypothesis for the route of ALA formation from glutamate begins with activation of the C-1 carboxyl group of glutamate by a reaction analogous or identical to the formation of glutamyl-tRNA in protein synthesis. The activated glutamate is then reduced in a pyridine nucleotide-dependent step to give an intermediate, which may be free glutamate-1-semialdehyde or a chemically equivalent compound (19, 23). This intermediate is finally transaminated to form ALA. In the case of barley, the RNA component has been identified as $tRNA^{Glu(UUC)}$ by nucleotide sequence determination (38). It has recently been shown by anticodon-based affinity chromatography that the active RNA components from spinach and from *Euglena*, *Chlorella*, *Cyanidium*, and *Synechocystis* spp. all carry the UUC anticodon (37).

Based on in vivo labeling results, several obligately anaerobic phototrophic bacterial species were recently reported to form bacteriochlorophyll from ALA derived from glutamate (1, 31, 32, 39; Avissar et al., in press). These reports are somewhat surprising in view of the fact that ALA has long been known to be formed via the ALA synthase reaction in some nonsulfur purple bacteria, including *Rhodobacter sphaeroides* (26) and *Rhodopseudomonas palustris* (42). Using techniques derived from those used for characterizing ALA formation in extracts of plants and algae, we have detected RNA-dependent ALA formation from glutamate in extracts of the green sulfur bacterium *Chlorobium vibrioforme*. The enzyme system was partially characterized and the properties were compared with those reported for other organisms.

(A preliminary account of this work has appeared in abstract form [S. Rieble, J. G. Ormerod, and S. I. Beale, Plant Physiol. **86**:S-60, 1988].)

MATERIALS AND METHODS

Growth of the organism. *Chlorobium vibrioforme* f. *thiosulfatophilum* NCIB 8327 was grown in thiosulfate-acetate-bicarbonate medium (Table 1) at 25 to 28°C in the light (incandescent lamps, 50 to 100 μE m^{-2} s^{-1}). The cell density of the cultures was monitored by measuring the apparent absorbance due to light scattering at 640 nm.

Preparation of cell extracts. All operations were carried out at 0 to 4°C. Cells were harvested during the exponential growth phase by centrifugation for 10 min at 10,000 × *g*. Cells were washed twice with cell extraction buffer (150 mM *N*-tris(hydroxymethyl)methylglycine [Tricine], 300 mM glycerol, 20 mM $MgCl_2$, 5 mM dithiothreitol [DTT], 20 μM pyridoxal phosphate, pH 7.9). Four liters of cell culture usually yielded about 3 g (fresh weight) of cells. The cells

* Corresponding author.
† Permanent address: Department of Biology, University of Oslo, Oslo 3, Norway.

TABLE 1. Composition of the growth medium

Component	Concn (g/liter)
$NaHCO_3$[a]	1.50
NaCl	1.00
$Na_2S_2O_3$	1.00
KCl	0.50
NH_4Cl	0.50
Sodium thioglycolate	0.50
$MgSO_4 \cdot 7H_2O$	0.41
$CaCl_2 \cdot 2H_2O$	0.33
KH_2PO_4	0.30
Sodium acetate $\cdot 3H_2O$	0.30
$Na_2S \cdot 9H_2O$[b]	0.10
Resazurin[c]	0.001
Microelements[d] (stock solution)	1.50 ml
HCl (1.0 N)	0.60 ml
Vitamin B_{12} (1-mg/ml stock solution)	0.20 ml

[a] Dissolved in 33 ml of H_2O, autoclaved separately, cooled to 0°C, bubbled with sterile CO_2 for 30 min, and added to cooled medium before inoculation.

[b] Added to cooled medium from a separately autoclaved 10% (wt/vol) solution.

[c] Added as an indicator of air exclusion. Resazurin is colorless in anaerobic medium and violet in the presence of air.

[d] Final concentrations of the microelements: 30 μM tetrasodium EDTA, 30 μM disodium EDTA, 30 μM $FeCl_3$, 15 μM $MnCl_2$, 15 μM $ZnSO_4$, 1.5 μM H_3BO_3, 1.5 μM $CuSO_4$, 1.5 μM Na_3VO_4, $(NH_4)_6Mo_7O_{24}$ (1.5 μM as Mo), 0.1 μM $CoCl_2$, and 0.1 μM $NiSO_4$.

were suspended in the same buffer at a ratio of 2 ml of buffer to 1 g of cells, glass powder (5-μm diameter) was added at a ratio of 3:1 (vol/vol), and the cells were broken by sonication (10 15-s bursts alternating with 45-s cooling periods). Cell debris and glass powder were removed by centrifugation for 10 min at 10,000 × *g*. The sediment was washed once with an equal volume of cell extraction buffer and centrifuged, and the supernatants were combined. This extract was centrifuged for 60 min at 285,000 × *g*. The resulting high-speed supernatant was passed through a column of Sephadex G-25 that was preequilibrated and developed with cell extraction buffer. The fraction containing the soluble proteins was stored at −75°C until use.

Assay for in vitro ALA formation. Incubations were carried out at 30°C in 0.5 ml of reaction medium consisting of cell extraction buffer supplemented with (unless otherwise noted) 5 mM levulinate, 5 mM ATP, 1 mM NADPH, and 1 mM glutamate, added together as a cofactor-substrate concentrate, and 250 μl of enzyme extract (1.2 to 1.5 mg of cell protein). The reaction was initiated by addition of the cofactor-substrate concentrate and terminated after 60 min with 200 μl of 1 M citric acid and 0.7 ml of 10% (wt/vol) sodium dodecyl sulfate. The resulting mixture was heated at 95°C for 2 min and cooled rapidly to room temperature.

Purification and quantitation of ALA. ALA was purified by ion-exchange chromatography as described previously (44). The purified ALA was pyrrolized by reaction with ethylacetoacetate (28), the 1-methyl-2-carboxyethyl-3-propionic acid pyrrole product was reacted with Ehrlich-Hg reagent (41), and the A_{553} was measured. The A_{553} of unincubated control samples was subtracted from those of incubated samples to determine net A_{553} values, and ALA concentration was calculated from a standard curve with samples containing known amounts of ALA.

Paper chromatography of 1-methyl-2-carboxyethyl-3-propionic acid pyrrole. After reaction with ethylacetoacetate, the solution containing ALA-pyrrole was adjusted to pH 3.0 with HCl and then cooled to 0°C. The solution was then extracted with three 1-volume portions of diethyl ether. The combined ether extracts were cooled to −75°C for 1 h. The ether was decanted from the ice that had formed from the water originally present in the ether solution, and the solution was then concentrated under a stream of N_2. The 1-methyl-2-carboxyethyl-3-propionic acid pyrrole was chromatographed on Whatman no. 3MM paper in a solvent of 1-butanol–1-propanol–5% (wt/vol) aqueous NH_4OH (2:1:1 [vol/vol/vol]) (7). After chromatography, either the 1-methyl-2-carboxyethyl-3-propionic acid pyrrole was visualized with Ehrlich spray reagent (200 mg of *p*-dimethylaminobenzaldehyde dissolved in 8 ml of ethanol and 2 ml of 12 N HCl) (7) or the lanes were cut into 1-cm segments and immersed in 5.5 ml of scintillation fluid (Econofluor), and radioactivity was determined by liquid scintillation spectroscopy.

***Chlorobium* small-RNA preparation.** Low-molecular-weight RNA was isolated from the soluble cell extract after the low-speed centrifugation. The supernatant was diluted with RNA extraction buffer (10 mM Tris hydrochloride, 10 mM magnesium acetate, 100 mM NaCl, 10 mM β-mercaptoethanol, pH 7.5) to a final volume of 4 ml/g of cells (12), and sodium dodecyl sulfate was added from a 10% (wt/vol) stock solution to a final concentration of 1% (wt/vol). Proteins were removed by extraction with an equal volume of phenol saturated with RNA extraction buffer. The aqueous phase was next extracted three or four times with equal volumes of chloroform-isoamyl alcohol (24:1, vol/vol) and chromatographed on DEAE-cellulose as described previously (12). The fractions containing tRNA were combined and precipitated by adding 2.5 volumes of ice-cold ethanol and cooling overnight at −20°C. Nucleic acids were deacylated by redissolving the precipitate in deacylation buffer (0.5 M Tris hydrochloride [pH 8.0]), incubating at room temperature for 2 h, precipitating with ethanol, and washing twice with ethanol (12). The precipitate was dried and dissolved in RNA storage buffer (10 mM Tris hydrochloride, 10 mM magnesium acetate, 100 mM NaCl, 1 mM DTT, pH 7.5) at a concentration of 250 A_{260} units/ml. This RNA preparation was stored at −20°C for further use.

Other procedures. Pancreatic RNase A (Sigma type I-AS) was dissolved in water (1 mg/ml), heated at 100°C for 5 min, cooled slowly, and stored at −20°C. Protein concentration was determined by the method of Bradford (8), with bovine serum albumin as the standard.

Chemicals. 1-[^{14}C]glutamate and Econofluor scintillation fluid were obtained from Du Pont-New England Nuclear. Cellulose DE-23 was from Whatman. RNasin was from Sigma Chemical Co. or Promega Biotech. All other reagents were from Sigma, Fisher Scientific, or Research Organics.

RESULTS

Characteristics of ALA formation in *Chlorobium* extracts. Soluble extracts of *Chlorobium vibrioforme* formed ALA upon incubation with glutamate and the appropriate cofactors. The incubation mixture contained 20 mM $MgCl_2$, 5 mM ATP, 1 mM NADPH, 1 mM glutamate, and 5 mM levulinic acid. Levulinic acid, a competitive inhibitor of ALA dehydratase, was included in the incubation medium to prevent further metabolism of the ALA. The absorption spectrum of the incubation product after reaction with ethylacetoacetate and Ehrlich reagent was identical to that obtained with authentic ALA. The product after reaction with ethylacetoacetate also showed chromatographic behavior identical to that of the authentic 1-methyl-2-carboxyethyl-3-propionic acid pyrrole (described below). The soluble nature of all required cell components was indicated by the presence of

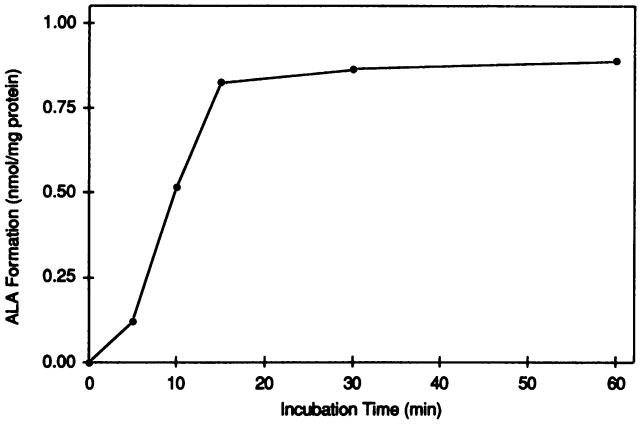

FIG. 1. Dependence of ALA formation on incubation time.

activity in the supernatant fraction after high-speed centrifugation. The time course for ALA formation during incubation indicated that under the conditions used, the reaction was completed after 20 min and no change in ALA content occurred between 20 and 60 min (Fig. 1). The amount of ALA formed during the incubation was directly dependent on the amount of protein added to the incubation mixture over the range of 0.6 to 3 mg, the highest amount tested. Heat-denatured cell extract was inactive (Table 2).

Although the cells were grown anaerobically, no precautions were taken to maintain anaerobic conditions during cell extraction and assay for ALA formation. No increase in activity was measured when the assay mixture was flushed with O_2-free N_2 and capped prior to incubation.

Substrate and cofactor requirements. Soluble cell extract showed an absolute requirement for added glutamate, NADPH, and ATP in order to form ALA (Table 2). Half-maximal activity was reached at approximately 0.4 mM glutamate, and saturation was approached at 10 mM glutamate. Direct incorporation of glutamate was determined by radioactive label incorporation. After incubation with 1-[^{14}C]glutamate, the ALA that formed was reacted with ethylacetoacetate, and the identity and specific radioactivity of the 1-methyl-2-carboxyethyl-3-propionic acid pyrrole were determined by paper chromatography. Only one Ehrlich-positive spot was detected on the paper chromatogram containing material derived from the incubation, and no Ehrlich-positive spots were detected in material derived from unincubated samples. The R_f of the pyrrole derived from the incubation product was 0.50, and this value was identical to the R_f of authentic 1-methyl-2-carboxyethyl-3-propionic acid pyrrole derived from standard ALA.

TABLE 2. Cofactor requirements for ALA formation[a]

Incubation conditions	ALA formed in nmol/mg of protein (% of control)
Complete (control)	1.56 (100)
Complete, heat-denatured enzyme	0.02 (1)
−Glutamate	0.04 (3)
−NADPH	0.02 (1)
−ATP	0.04 (3)

[a] *Chlorobium* cells were extracted and ALA-forming activity was determined as described in the text. Complete incubation mixture (0.5 ml) contained 150 mM Tricine (pH 7.8), 300 mM glycerol, 20 mM $MgCl_2$, 5 mM DTT, 20 μM pyridoxal phosphate, 5 mM levulinate, 5 mM ATP, 1 mM NADPH, 1 mM glutamate, and 1.4 mg of cell protein.

TABLE 3. Effects of RNase digestion and RNA supplementation[a]

Expt no.	Preincubation addition(s)		RNA added (A_{260} units) at 0 min	ALA formed in nmol/mg of protein (% of control)
	−15 min	−5 min		
1	—	—	— (control)	0.55 (100)
	RNase	—	—	0.01 (2)
	RNase	RNasin	*Chlorobium* (2.5)	0.54 (98)
2	—	—	— (control)	0.38 (100)
	RNase	RNasin	—	0.00 (0)
	RNase	RNasin	*Synechocystis* (2.5)	1.14 (300)
	RNase	RNasin	*E. coli* $tRNA^{Glu}$ (0.25)	0.77 (203)
	RNase	RNasin	*Chlorella* (2.5)	0.18 (47)
	RNase	RNasin	*Euglena* (wild-type) (5.0)	0.40 (105)
	RNase	RNasin	*Euglena* (aplastidic) (5.0)	0.00 (0)

[a] Incubations (0.5 ml) were carried out in complete medium containing 1.4 mg of *Chlorobium* cell protein. Incubations were started by adding substrate-cofactor mixture containing 1 mM NADPH, 5 mM ATP, and 1 mM glutamate. At the indicated times before the incubations were started, 50 ng of RNase A and/or 125 U of RNasin was added. At the beginning of the incubation, the indicated quantity and type of RNA was added. Times are minutes before the start of incubation. —, Nothing added.

ATP was optimally effective at approximately 5 mM, almost as effective at 1 mM, and somewhat inhibitory at 10 mM.

The optimum concentration of NADPH was approximately 0.3 mM, and concentrations above this value were inhibitory. NADH was a less effective cofactor than NADPH. At the highest concentration tested (10 mM), NADH was only about 10% as effective as NADPH at 0.3 mM. However, inhibition was not observed at any NADH concentration.

Effects of RNA supplementation and RNase digestion. Added RNA did not stimulate ALA formation in a cell extract that was not preincubated with RNase. The activity was presumably sustained by the presence of saturating amounts of endogenous RNA in the enzyme extract. Preincubation of the extract with RNase for 10 min prior to addition of substrates and cofactors resulted in complete loss of the activity (Table 3). Control incubations without RNase revealed that inactivation was not due to the preincubation per se. After preincubation with RNase, activity could be restored to over 95% of the control value by adding RNasin to inhibit RNase and then adding the low-molecular-weight RNA isolated from *C. vibrioforme*. *Synechocystis* RNA was even more effective than *Chlorobium* RNA (per A_{260} unit added) in restoring activity to RNase-treated extract. Other effective RNAs were those derived from *Chlorella vulgaris* and light-grown wild-type *Euglena gracilis* and commercial *E. coli* $tRNA^{Glu}$. RNA from light-grown aplastidic *Euglena* cells was ineffective.

Inhibitors. 3-Amino-2,3-dihydrobenzoic acid (gabaculine) inhibited ALA formation when added to incubation mixtures. The concentration dependence did not follow a smooth curve but rather showed a threshold relationship. The gabaculine concentration at which ALA-forming activity declined abruptly varied within the range from 0.5 to 5.0 μM from one enzyme preparation to another.

Addition of increasing amounts of hemin to the incubation mixtures resulted in progressive inhibition of ALA formation (Fig. 2). Fifty percent inhibition occurred at approximately 0.2 μM hemin.

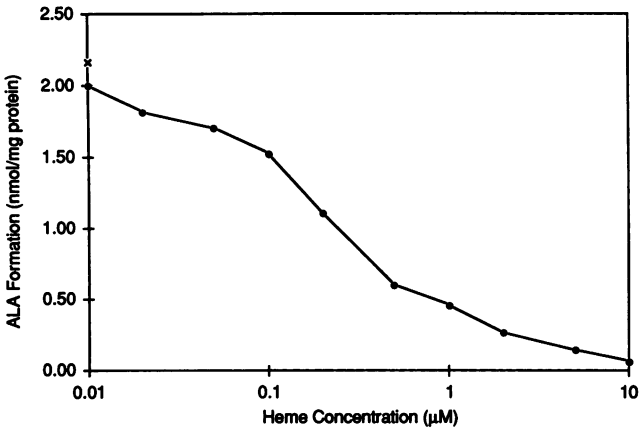

FIG. 2. Inhibition of ALA formation by heme. Activity without inhibitor is indicated by the × on the vertical axis.

DISCUSSION

Cell extracts of the obligately anaerobic photosynthetic procaryote *Chlorobium vibrioforme* catalyzed transformation of glutamate to ALA. The activity was sufficiently high to allow measurement of the ALA formed in the reaction by spectrophotometric means.

In most respects, the activity in *Chlorobium* extracts is very similar to those reported for cell or plastid extracts of other organisms (5, 13, 16, 18, 27, 29, 33, 43–45; Avissar et al., in press). In contrast to cell extracts from other phototrophic organisms thus far examined, *Chlorobium* extracts had a definite preference for NADPH over NADH as the pyridine nucleotide cofactor for ALA formation.

As with other cell extracts that can form ALA from glutamate, activity in the *Chlorobium* extract was dependent on a small RNA, which could be replaced by RNA isolated from other species that form ALA from glutamate. Interestingly, *E. coli* $tRNA^{Glu}$ was effective in supporting ALA formation in the RNase-treated *Chlorobium* extracts. *E. coli* $tRNA^{Glu}$ was previously reported to support ALA formation in *Chlamydomonas* extracts (20), but was ineffective with extracts of barley plastids (24) and *Chlorella* (46), *Euglena* (29), or *Synechocystis* (35) spp., even though, in the last three cases, it was determined that the cell extracts were capable of charging the *E. coli* $tRNA^{Glu}$ with glutamate. The mechanistic basis for the RNA discrimination and its physiological significance remain to be determined.

Addition of RNA to extracts that were not predigested with RNase did not stimulate ALA-forming activity. The lack of stimulation indicates that endogenous RNA is present at a saturating level in the *Chlorobium* extracts. This is in contrast to results reported for extracts of other organisms, in which added RNA stimulated ALA formation and saturation of the activity with respect to RNA could not be achieved.

ALA formation in the *Chlorobium* extracts was blocked by gabaculine, a potent inhibitor of the aminotransferase enzyme which catalyzes the last step in ALA formation (4, 25). The relative ineffectiveness of gabaculine at lower concentrations and the sudden onset of inhibition at a threshold concentration are understandable, because the aminotransferase is normally present in non-rate-limiting amounts in cell extracts (3, 35). Overall inhibition of ALA formation would not be observed unless the gabaculine concentration were high enough to inhibit the aminotransferase sufficiently to make that step rate-limiting. The extract-to-extract variation in the gabaculine concentration required for the onset of inhibition probably results from variation in the ratio of the aminotransferase to the other components of the ALA-forming system in the different cell extracts.

Hemin inhibited ALA formation in *Chlorobium* extracts at submicromolar concentrations. The sensitivity to hemin was somewhat lower than that reported for *Chlamydomonas* extracts (21) but higher than the sensitivity of *Chlorella* (44), *Euglena* (29), and *Synechocystis* (35) spp. and barley plastid (17) extracts. The high sensitivity to heme concentration suggests that heme is an important physiological regulator of ALA formation, acting by feedback inhibition at the enzyme level in *Chlorobium* cells.

Even though *Chlorobium vibrioforme* is a strict anaerobe, the *Chlorobium* extracts were active in ALA formation when prepared and assayed under aerobic conditions, and activity was not enhanced by incubation under N_2. This is in contrast to the results reported for another strict anaerobe, *M. thermoautotrophicum*, extracts of which were active in ALA formation only if anaerobic conditions were maintained throughout the extraction and assay (13).

In summary, the results obtained with *Chlorobium vibrioforme* show that this species produces ALA by the same pathway as oxygenic photosynthetic organisms and archaebacteria. The general characteristics, such as substrate and cofactor requirements, as well as the sensitivity to inhibition by hemin and gabaculine, are similar to those reported for the other organisms. However, ALA-forming activity in *Chlorobium* extracts is distinct in several respects, e.g., the limited ability to use NADH and the apparent saturating level of endogenous RNA present in the extracts.

It appears that the five-carbon pathway was conserved to a high degree during the course of evolution and most likely has the same essential features in all species in which it is present, independent of whether they are eucaryotes or procaryotes, phototrophic or nonphototrophic, or whether the primary tetrapyrrole end products are hemes, corrins, chlorophylls, or bacteriochlorophylls. The widespread occurrence of this pathway among phylogenetically diverse organisms suggests that it is the more ancient pathway for ALA biosynthesis. The ability of the *Chlorobium* enzyme system to accept *E. coli* $tRNA^{Glu}$, and the recent determination that *Chlorobium* is capable of natural genetic transformation (34), make this species an attractive experimental organism for molecular genetic studies of ALA formation by the five-carbon pathway.

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