Transformation of Glutamate to δ-Aminolevulinic Acid by Soluble Extracts of Synechocystis sp. PCC 6803 and Other Oxygenic Prokaryotes*

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δ-Aminolevulinic acid is the first committed precursor in the biosynthesis of hemes, phycobilins, and chlorophylls. Plants and algae synthesize δ-aminolevulinic acid from glutamate via an RNA-dependent 5-carbon pathway. Previous reports demonstrated that cyanobacteria form δ-aminolevulinic acid from glutamate in vivo. We now report the direct measurement of this activity in vitro. Three oxygenic prokaryotes were examined, the unicellular cyanobacteria Synechocystis sp. PCC 6803 and Synechococcus sp. PCC 7002 (Agmenellum quadruplicatum PR-6) and the chlorophyll a- and b-containing filamentous prochlorophyte Prochlorothrix hollandica. δ-Aminolevulinic acid-forming activity was detected in soluble extracts of all three species. δ-Aminolevulinic acid formation by Synechocystis extracts was further characterized. Activity depended upon addition of reduced pyridine nucleotide, ATP, and Mg²⁺ to the incubation mixture. NADPH was a more effective pyridine nucleotide than NADH at low concentrations, but NADPH inhibited δ-aminolevulinic acid formation above 1 mM, whereas NADH did not. The pH optimum was about 7.6, and the ATP concentration optimum was 0.1 mM. Activity was stimulated by addition of RNA derived from Synechocystis sp. PCC 6803 or Chlorella, and abolished by preincubation with RNase A. After RNase inactivation, activity was restored by addition of RNasin to block further RNase action, followed by supplementation with Synechocystis RNA. Activity was inhibited by micromolar concentrations of hemin, as was previously found with plant and algal extracts. Complete dependence on added glutamate could not be achieved. Radioactivity was incorporated into δ-aminolevulinic acid when the incubation mixture contained 1[14C]glutamate. Activity in the Synechocystis enzyme extract was stimulated by the addition of a partially purified enzyme fraction from Chlorella. It thus appears that prokaryotic oxygenic organisms share with chloroplasts the capacity for biosynthesis of photosynthetic pigments from glutamate via the RNA-dependent 5-carbon pathway.

δ-Aminolevulinic acid is the first committed precursor in the biosynthesis of tetrapyroles including hemes, phycobilins, and chlorophylls (1). Two distinct pathways for δ-aminolevulinic acid formation have been described. One route involves condensation of glycine and succinyl-CoA, catalyzed by the enzyme δ-aminolevulinic acid synthase (succinyl-CoA:glycine C-succinyltransferase (decarboxylating), EC 2.3.1.37), and occurs in animals, yeast, fungi, and some bacteria (2, 3). δ-Aminolevulinic acid is synthesized by a second route from glutamate via the 5-carbon pathway. This pathway occurs in plants (4), algae (5), and some prokaryotes including obligately anaerobic photosynthetic bacteria (6-8), methanogenic bacteria (9), and possibly in other strict anaerobes such as Bacteroides and Clostridium (10-12).

Soluble cell extracts capable of δ-aminolevulinic acid formation from glutamate have been obtained from barley and maize plastids (13, 14) and extracts of the unicellular eukaryotic algae Chlamydomonas reinhardtii (15), Chlorella vulgaris (16), Euglena gracilis (17), and Cyanidium caldarium (18). Active extracts have also been obtained from the methanogen Methanobacterium thermoautotrophicum (19). Soluble extracts of barley plastids (20) and Chlorella cells (21) have been partially purified and fractionated into three proteinaceous components, all of which are required, in addition to an RNA component, for the reconstitution of δ-aminolevulinic acid-forming activity. These extracts require a reduced pyridine nucleotide, ATP, Mg²⁺, and a low molecular weight RNA as cofactors in vitro. A current working hypothesis for the route of δ-aminolevulinic acid formation from glutamate begins with activation of the C₁ carboxyl group of glutamate by a reaction analogous or identical to the formation of glutamyl-tRNA for protein synthesis (Fig. 1). The activated glutamate is then reduced in a pyridine nucleotide-dependent step, and the reduced product, which may be free glutamate l-semialdehyde, is finally transaminated, perhaps internally, to form δ-aminolevulinic acid. In the case of barley, the RNA component has been identified as tRNA^Glu(UUC) by nucleotide sequence determination (22). It has recently been shown by anticodon-based affinity chromatography that the active RNA components from spinach, Euglena, Chlorella, and Cyanidium all carry the UUC glutamate anticodon (23). A UUC anticodon-containing RNA that was capable of supporting δ-aminolevulinic acid formation by Chlorella preparations was also extracted from the cyanobacterium Synechocystis (23).

Cyanoacteria are potentially attractive experimental materials for isolation of the genes coding for the macromolecular components of the δ-aminolevulinic acid-forming system. Until now, the only direct evidence supporting the operation of the 5-carbon δ-aminolevulinic acid-forming pathway in cyanobacteria has been based on in vivo label incorporation (24-27). We report here the formation of δ-aminolevulinic acid from glutamate in soluble extracts of two cyanobacteria, Synechocystis sp. PCC 6803 and Synechococcus sp. PCC 7002, and of another oxygenic prokaryote, the filamentous prochloro-

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mixture had the composition of assay buffer but contained in addition (unless otherwise noted) 5 mM levulinic acid, 5 mM ATP, 5 mM NADH, and 1 mM glutamate added together from a concentrated cofactor-substrate mixture, and 1–3 mg of soluble protein. The reaction was started by adding the cofactor-substrate mixture, and stopped after 60 min of incubation by the addition of 100 µl of 1 M citric acid and 1 ml of 10% (w/v) sodium dodecyl sulfate.

**Purification and Quantitation of δ-Aminolevulinic Acid—δ-Aminolevulinic acid was purified by ion exchange chromatography as previously described (16). The purified δ-aminolevulinic acid was pyrolized by reaction with ethylacetocetate (33), the 1-methyl-2-carboxyethyl-2-propionic acid methyl ester that was reacted with Ehrlich’s reagent (34), and the Aα50 was measured. The Aα50 of unincubated control samples was subtracted from those of incubated samples to determine net Aα50 values, and δ-aminolevulinic acid was calculated from a standard curve with samples containing known amounts of δ-aminolevulinic acid.

**Paper Chromatography of 1-Methyl-2-carboxyethyl-3-propionic Acid Pyrrole—After reaction with ethylacetocetate, the solution containing 1-methyl-2-carboxyethyl-3-propionic acid 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 diethyl ether extract was then evaporated to near dryness. The dried extract was then dissolved in 90% (v/v) aqueous NH₄OH (2:1) (4). The 1-methyl-2-carboxyethyl-3-propionic acid pyrrole was visualized after chromatography by Ehrlich’s spray reagent (200 mg of p-diethylaminobenzaldehyde dissolved in 8 ml of ethanol and 2 ml of 12 N HCl) (4) or the lanes were cut into 1-cm segments, immersed in 5.5 ml of scintillation fluid (Econofluor), and radioactivity was determined by liquid scintillation spectroscopy.

**Synechocystis Small RNA Preparation—Low molecular weight RNA was isolated from the soluble cell extract after the high-speed centrifugation. The supernatant was diluted with RNA extraction buffer (10 mM Tris-HCl (pH 7.5), 10 mM Mgacetate), 100 mM NaCl, 20 mM mercaptoethanol, and the final volumes were adjusted to 9 ml with ice-cold ethanol and cooling overnight at -25°C. The precipitated nucleic acids were redissolved in RNA extraction buffer and chromatographed on DEAE-cellulose as previously described (35). The fractions containing RNA were combined and precipitated by adding 2.5 volumes of ice-cold absolute ethanol and cooling overnight at -25°C. Nucleic acids were decayed by redissolving the precipitate in deacylation buffer (0.5 M Tris-HCl (pH 8.0)), incubating at room temperature for 2 h, precipitating with ethanol, and washing twice with ethanol (35). The pellet was dried and dissolved in RNA storage buffer (10 mM Tris-HCl (pH 7.5), 0.1 mM Mgacetaetate), 100 mM NaCl, 1 mM DTT) at a concentration of 200 A260 units ml⁻¹. This RNA preparation was stored at -25°C for further use.

**Extraction and Assay Procedures for δ-Aminolevulinic Acid Synthase—Cells of Synechocystis and Euglena were harvested as described above, but the procedures changed to one of two δ-aminolevulinic acid synthase incubation buffers. δ-Aminolevulinic acid synthase incubation buffer A was 75 mM Hepes (pH 7.8), 50 mM glycine, 25 mM succinic acid, 20 mM MgCl₂, 5 mM EDTA, and 0.1 mM pyridoxal-P (31). δ-Aminolevulinic acid synthase incubation buffer B was 150 mM Tricine (pH 7.8), 500 mM glycine, 50 mM glycine, 20 mM succinic acid, 20 mM MgCl₂, 1 mM DTT, and 20 μM pyridoxal-P. The supernatant derived from the first centrifugation step after cell disruption was used in the assays. Cell extracts were incubated for 30 min at 37°C in δ-aminolevulinic acid synthase incubation buffer. The incubations were started by the addition of succinyl-CoA, which was generated by the method of Simon and Shemin (36). The total volume of the incubation mixture was 1 ml. Incubations were terminated by addition of 50 µl of 100% (w/v) aqueous trichloroacetic acid solution. Precipitate was removed by centrifugation for 1 min at 13,000 x g in the microcentrifuge. One-half milliliter of supernatant was adjusted to 6.8 with approximately 170 µl of 0.5 M NaPO₄, then 25 µl of ethylacetocetate was added, and the solution was heated to 95°C for

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1 The abbreviations used are: Tricine, N-tris(hydroxymethyl)methylglycine; DTT, dithiothreitol; Hepes, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid.
15 min to form 1-methyl-2-carboxethyl-3-propanoyl acid pyrrole, which was quantitated as described above.

Other Procedures—RNase A (Sigma Type I-AS) was dissolved in water (1 mg ml⁻¹), heated to 100 °C for 5 min, cooled slowly, and stored at −25 °C. Cell population densities were determined with a Coulter Counter (Model ZBI, Coulter Electronics). Protein concentration was determined by the method of Bradford (57), using bovine serum albumin as a standard.

Chemicals—[14C]Glutamate and Ecosfluor scintillation fluid were obtained from DuPont-New England Nuclear. Cellulose DE-23 was from Whatman. RNA and agaroze-linked bacteria's yeast hexokinase were from Sigma. All other reagents were from Sigma, Fisher, or Research Organics.

RESULTS

Characterization of the δ-Aminolevulinic Acid-forming Reaction in Extracts of Synechocystis—Soluble extracts of Synechocystis formed δ-aminolevulinic acid upon incubation with substrate and cofactors appropriate for δ-aminolevulinic acid formation from glutamate. The incubation mixture contained 40 mM MgCl₂, 5 mM ATP, 5 mM NADH, 1 mM glutamate, and 5 mM levulinic acid. Levulinic acid, a competitive inhibitor of δ-aminolevulinic acid dehydratase, was included in the incubation medium to prevent further metabolism of the δ-aminolevulinic acid. The absorption spectrum of the incubation product after reaction with ethylacetoacetate and Ehrlich reagent was identical to that of authentic δ-aminolevulinic acid. The product after reaction with ethylacetoacetate also had identical chromatographic behavior to that of the authentic 1-methyl-2-carboxethyl-3-propanoyl acid pyrrole (see below). The soluble nature of all required cell components was indicated by the presence of activity in the supernatant fraction after high-speed centrifugation. This was true even when an alternate extraction procedure was used, in which cells were disrupted in high-phosphate buffer, which allowed the removal of a substantial fraction of cell protein by sedimenting intact membrane-bound phycobilisomes in the high-speed centrifugation step. The time course for δ-aminolevulinic acid formation during incubation indicated that under the conditions used, the reaction was completed after 30 min, and that no change in δ-aminolevulinic acid content occurred between 30 and 60 min. The amount of δ-aminolevulinic acid formed during the incubation was directly dependent on the amount of protein added to the incubation mixture over the range of 1.5 to 5 mg, the highest amount tested. Maximum δ-aminolevulinic acid formation occurred at approximately pH 7.6, and the temperature optimum was approximately 30 °C.

Synechocystis extract prepared in high-phosphate extraction buffer had a higher specific δ-aminolevulinic acid-forming activity than extract prepared in standard extraction buffer (Table I). The high-phosphate concentration preserves the integrity of the phycobilisomes and facilitates their removal at the high-speed centrifugation step. Per mg of Synechocystis protein, the preparation was about 7.5-fold more active than Synechocystis-Soluble extracts of Synechocystis-Soluble extracts of Synechocystis extract prepared in high-phosphate extraction buffer. Incubation mixtures (see below).

TABLE I

| Incubation conditions | δ-Aminolevulinic acid formation |
|----------------------|---------------------------------|
|                      | nmol mg⁻¹ protein | % control |
| Complete             | 0.52              | 100       |
| Complete, heat-denatured enzyme | 0.00              | 0         |
| −RNA                 | 0.27              | 52        |
| −NADH                | 0.00              | 0         |
| −ATP                 | 0.00              | 0         |
| −Mg²⁺                | 0.01              | 2         |
| Complete             | 3.98              | 100       |
| −RNA                 | 2.67              | 67        |
| −RNA, + 5 A₅₉₀ units Chlorella | 3.43              | 86        |

FIG. 2. Dependence of δ-aminolevulinic acid (ALA) accumulation on pyridine nucleotide concentration. Cells were disrupted in high-phosphate extraction buffer. Incubations (0.5 ml) contained 1.25 mg of Synechocystis cell protein, 2.5 A₅₉₀ units of Synechocystis RNA, and the indicated concentration of added NADH (○) or NADPH (●). Note the logarithmic concentration scale and the zero concentration value indicated by × on the left axis.

δ-aminolevulinic acid was for NADH as the pyridine nucleotide than with NADPH.

With cell extract prepared in standard extraction buffer, added ATP was required (Table I). However, with cell extract prepared in high-phosphate extraction buffer, sufficient residual ATP was carried through the gel filtration step to eliminate the need for added ATP. To demonstrate an ATP requirement in extract prepared in high-phosphate extraction buffer, the extract was preincubated for 10 min at 30 °C with assay buffer plus agarose-linked hexokinase (10 units ml⁻¹) and 25 mM dextrose. The agarose-linked hexokinase was then removed by filtration and the reaction was initiated by adding glutamate, NADPH, levulinic acid, and ATP. The optimal ATP concentration was 0.1 mM, and incubations without added ATP had about 20% of the maximal activity (Fig. 3).

Effects of RNA Supplementation and RNase Digestion—Added RNA was not absolutely required, but it stimulated δ-aminolevulinic acid formation (Table I). Chlorella RNA stimulated δ-aminolevulinic acid formation by Synechocystis ex-
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**Fig. 3.** Dependence of \(\delta\)-aminolevulinic acid (ALA) accumulation on ATP concentration. Incubations (1 ml) contained 2.8 mg of Synechocystis cell protein and the indicated amount of added Synechocystis RNA.

The activity without added RNA is presumably due to the presence 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 nearly complete loss of the activity (Table II). Preincubation of the extract alone without added RNase for 15 min before starting the reaction by the addition of substrate and cofactors had no effect on the resulting activity. The degree of inactivation was dependent on the amount of RNase added, with nearly complete inactivation occurring at 50 ng of added RNase (Fig. 5). After preincubation with RNase, activity could be restored to over 70% of the control value by adding RNasin to inhibit RNase, and then adding the low molecular weight RNA isolated from Synechocystis (Table II). If RNasin was added before RNase, the amount of \(\delta\)-aminolevulinic acid formed was 89% of the control value, indicating that neither reagent had other effects on the enzyme activity.

**Identification of Glutamate as the \(\delta\)-Aminolevulinic Acid Precursor—Absolute dependence of the reaction on added glutamate could not be demonstrated. In normally prepared cell extracts, omission of glutamate from the incubation mixture resulted in only slightly less \(\delta\)-aminolevulinic acid accumulation than in complete incubation medium (Table III). Even after passing the cell extract twice through Sephadex G-25, \(\delta\)-aminolevulinic acid-forming activity in the absence of added glutamate was still about 80% of the value with complete incubation medium. Addition of 1 \(\mu\)l of a saturated solution of phenylmethylsulfonyl fluoride in isopropyl alcohol (approximately 0.002% (w/v) final concentration) to the 1-ml incubation mixture had no effect on \(\delta\)-aminolevulinic acid formation or glutamate dependence.

An experiment was carried out to assess the possibility that the observed glutamate independence was due to the presence of glutamyl-tRNA in the extract and its co-elution with the protein-containing fraction during gel filtration. Cell extract was preincubated with RNase, then passed once or twice through Sephadex G-25, and finally incubated with RNasin plus deacylated tRNA. Under these conditions, activity in the absence of added glutamate was reduced to about 50% of the value with 1 mM glutamate added (Table III).

Glutamate was identified as the substrate for the reaction by radioactive label incorporation. The specific activity of the 1-\([^{14}C]\)glutamate added to the incubation mixture was 55.3 mCi mmol\(^{-1}\). After the incubation with 1-\([^{14}C]\)glutamate, \(\delta\)-aminolevulinic acid that formed was reacted with ethylace-
TABLE III

Effects of various treatments on glutamate dependence

| Cell extract treatment schedule | δ-Aminolevulinic acid formation | Glutamate dependence |
|---------------------------------|-------------------------------|---------------------|
|                                 | nmol mg⁻¹ protein             | % Glu of +Glu       |
| Gel-filtered once               | 0.58                          | 79                  |
| Gel-filtered twice              | 0.48                          | 83                  |
| Gel-filtered, RNase-treated, RNasin added | 0.43 0.50 86 | |
| RNase-treated, gel-filtered once | 0.32 0.57 56                  | |
| RNase-treated, gel-filtered twice | 0.26 0.54 48                 | |

δ-Aminolevulinic acid formation via δ-aminolevulinic acid synthase-catalyzed condensation of glycine and succinyl-CoA in extracts of Synechocystis. The low-speed supernatant after cell disruption was used to avoid possible loss of the enzyme activity. Extracts prepared from Euglena were used as positive controls (31). Incubations were carried out in two different δ-aminolevulinic acid synthase incubation buffers, one (buffer A) corresponding to that used for measurement of δ-aminolevulinic acid synthase in Euglena extracts (31), and the other (buffer B) closer in composition to the buffer used for measuring δ-aminolevulinic acid formation from glutamate, but with glycine and succinyl-CoA added. δ-Aminolevulinic acid formation was not detected in Synechocystis extracts with either assay condition, whereas under the same conditions δ-aminolevulinic acid formation (2.4–2.7 nmol of δ-aminolevulinic acid) via the synthase reaction was readily detected in the Euglena extracts. The possibility was examined that the presence of δ-aminolevulinic acid synthase in the Synechocystis
Table IV

Stimulation of δ-aminolevulinic acid formation in Synechocystis extracts by affinity-purified Chlorella δ-aminolevulinic acid-forming enzyme fractions

| Added Chlorella enzyme fraction | δ-Aminolevulinic acid formation |
|--------------------------------|-------------------------------|
|                                | nmol | % stimulation |
| None                           | 0.54 | 0             |
| Blue-Sepharose-unbound (0.26 mg) | 0.65 | 20            |
| ADP-agarose-unbound (0.41 mg)   | 0.82 | 52            |
| ADP-agarose-bound (0.10 mg)     | 2.98 | 452           |
| Blue-Sepharose-unbound (0.74 mg) + ADP-agarose-unbound (0.58 mg) | 0.00 |            |
| Blue-Sepharose-unbound (0.74 mg) + ADP-agarose-bound (0.15 mg) | 0.46 |            |
| ADP-agarose-unbound (0.58 mg) + ADP-agarose-bound (0.15 mg) | 0.24 |            |

Table V

Comparative δ-aminolevulinic acid formation by cell extracts of three species

| Species                   | Cell extract | Added RNA | δ-Aminolevulinic acid formation |
|---------------------------|--------------|-----------|--------------------------------|
|                           | mg protein   | A_{260} | nmol mg^{-1} protein |
| Synechocystis sp. PCC 6803 | 3.0          | 0       | 0.35                |
| Synechococcus sp. PCC 7002 | 3.0          | 5       | 0.71                |
|                           | 6.5          | 0       | 0.19                |
|                           | 6.5          | 5       | 0.21                |
| P. hollandica             | 2.0          | 0       | 1.39                |

The activity in extracts of all three species was sufficiently high to allow measurement of the δ-aminolevulinic acid formed in the reaction by spectrophotometric means. δ-Aminolevulinic acid was identified as the incubation product by comparison of the absorption spectrum after reaction with ethylacetocetate and Ehrlich reagent to that of authentic δ-aminolevulinic acid reaction product. Additional evidence was identical chromatographic behavior of the ethylacetocetate reaction product with that of authentic 1-methyl-2-carboxyethyl-3-propionic acid pyrrole (see below).

δ-Aminolevulinic acid-forming activity was further characterized in extracts of the unicellular cyanobacterium *Synechocystis* sp. PCC 6803. In most respects, the δ-aminolevulinic acid biosynthetic activity in *Synechocystis* extracts is very similar to those reported from eukaryotic cell or plastid extracts (13-18). Activity required ATP, reduced pyridine nucleotide, Mg^{2+}, and a small tRNA-like molecule. The addition of an amino donor, other than glutamate, to the reaction mixture was not required. Heat-denatured extract was inactive.

The requirements for ATP and Mg^{2+} are consistent with the proposed mechanism of glutamate activation by aminocyclation of a tRNA (21, 22, 38, 39). As with the eukaryotic cell extracts thus far examined, *Synechocystis* extracts had greater δ-aminolevulinic acid-forming activity with NADPH as the pyridine nucleotide cofactor than with NADH. The optimal concentration of NADPH was approximately 0.5 mM, and higher concentrations inhibited δ-aminolevulinic acid formation. With NADH, inhibition at higher concentrations was not observed. The physiological reason for the preference of the *Synechocystis* extracts for NADPH is unknown, but the preference of the plastid-derived extracts for NADPH is consistent with the generally greater availability of this reduced pyridine nucleotide, compared to NADH, in plastids (40).

Absolute dependence of δ-aminolevulinic acid formation on added glutamate could not be achieved in *Synechocystis* extracts. Cell extracts were passed through a Sephadex G-25 column to remove low molecular weight components before δ-aminolevulinic acid-forming activity was assayed. This procedure would be expected to deplete the extracts of endogenous glutamate and cause the reaction to become dependent on the addition of exogenous glutamate. However, the *Synechocystis* extracts still formed δ-aminolevulinic acid without added glutamate, and addition of glutamate stimulated the activity only by about 25%. The possibility was considered that the true carbon substrate was not glutamate, but α-ketoglutarate, which might be generated during the incubation. However, α-ketoglutarate inhibited δ-aminolevulinic acid formation by about 20%. Another possible explanation for the lack of glutamate dependence is that substrate quantities of glutamyl-tRNA might be present in the cell extract and co-elute with the protein fraction during gel filtration. This possibility was tested by digesting the cell extract with RNase and then gel filtering to remove the RNA fragments. After this treatment, δ-aminolevulinic acid-forming activity of the final extract was absolutely dependent on added RNA. The added RNA had been pretreated to ensure complete deacylation. Nevertheless, the dependence on glutamate was still not complete, and δ-aminolevulinic acid formation was stimulated only two fold by added glutamate.

One possible cause for the independence on added glutamate is that small, but sufficient, amounts of glutamate might be generated during the incubation by the action of proteolytic enzymes. Dependence on added glutamate was not increased by addition of the protease inhibitor phenylalkylsulfonyl...
fluoride. However, the responsible protease might not be inhibited by this reagent. Another possibility is that some glutamate might remain tightly bound to proteins through the gel filtration steps, and be released only during the incubation.

Although absolute dependence on added glutamate could not be achieved, glutamate was determined to be the carbon substrate for \( \delta \)-aminolevulinic acid formation by measuring specific transfer of label from \( \text{[1-}^{14}\text{C]} \) glutamate to \( \delta \)-aminolevulinic acid during the incubation. Glutamate labeled at C\(_1\) can transfer label to \( \delta \)-aminolevulinic acid only via the 5-carbon pathway, and this carbon atom is lost upon indirect incorporation of glutamate into \( \delta \)-aminolevulinic acid via the \( \delta \)-aminolevulinic acid synthase-catalyzed reaction (4, 41). Therefore, the high degree of label transfer from \( \text{[1-}^{14}\text{C]} \) glutamate to \( \delta \)-aminolevulinic acid measured in the Synechocystis cell extract is interpreted to indicate a direct precursor role. Co-migration of the only radioactive spot with the 1-methyl-2-carboxyethyl-3-propionic acid pyrrole upon paper chromatography indicates the high radiopurity of the isolated 1-methyl-2-carboxyethyl-3-propionic acid pyrrole that was quantitated for specific radioactivity determination. The decrease in the specific radioactivity of the \( \delta \)-aminolevulinic acid product to about one-third of the specific radioactivity of the glutamate added to the incubation mixture is consistent with the existence, or generation, of endogenous glutamate in the incubation mixture. The calculated 3-fold dilution of glutamate specific radioactivity is also consistent with the measured 50–100% stimulation of \( \delta \)-aminolevulinic acid formation by added glutamate, with the unstimulated rate attributed to endogenous glutamate.

Because of earlier doubts raised by the apparent glutamate independence, an attempt was made to detect \( \delta \)-aminolevulinic acid formation from glycine and succinyl-CoA in the Synechocystis extracts. As a positive control, \( \delta \)-aminolevulinic acid synthase activity was measured in Euglena extracts, which were previously demonstrated to contain the enzyme (31). No \( \delta \)-aminolevulinic acid synthase activity was detected in the Synechocystis extracts. Moreover, when Euglena extract was added to Synechocystis extract, all of the measured \( \delta \)-aminolevulinic acid synthase activity could be attributed to the added Euglena extract, with neither inhibition nor stimulation caused by the Synechocystis extract.

The \( \delta \)-aminolevulinic acid formation in the Synechocystis extract is dependent on a small RNA. Activity with endogenous RNA was destroyed by RNase digestion of the extract prior to incubation. The extract was largely reactivated by addition of a fraction of Synechocystis RNA that was isolated by phenol-chloroform extraction and DEAE-cellulose chromatography. Addition of this RNA fraction to extracts that were not RNase-predigested stimulated \( \delta \)-aminolevulinic acid-forming activity. The degree of stimulation was proportional to the amount of added RNA. RNA isolated from Chlorella by the same procedure also stimulated \( \delta \)-aminolevulinic acid formation, but not as effectively as Synechocystis RNA. Synechocystis RNA was previously found to stimulate \( \delta \)-aminolevulinic acid formation in extracts of Euglena (17) and Chlorella (23). The cross-species acceptability of the RNA fractions from Synechocystis, Euglena, and Chlorella indicates that the RNAs are similar to each other, but different in some way from glutamyl-tRNAs from other species, which did not support \( \delta \)-aminolevulinic acid formation in these cell extracts, even though these RNAs could be acetylated with glutamate (17, 42).

Hemin inhibited \( \delta \)-aminolevulinic acid formation in Synechocystis extracts at micromolar concentrations. The sensitivity to hemin was somewhat lower than in Chlorella (16) or Chlamydomonas (43) extracts, but about the same as reported for barley plastid extracts (44), and suggests that heme may be an important physiological regulator of \( \delta \)-aminolevulinic acid formation, acting by feedback inhibition at the enzyme level, in Synechocystis cells.

\( \delta \)-Aminolevulinic acid formation in Synechocystis extracts was highly stimulated by the addition of one of three affinity-purified enzyme fractions of the \( \delta \)-aminolevulinic acid-forming system isolated from Chlorella (21). Preliminary results suggest that this fraction is also rate limiting in Chlorella extracts. Based on the known affinity properties of the resins used in the separation, it was concluded that this component corresponds to the dehydrogenase enzyme (21). The stimulation caused by the Chlorella protein fraction was more than additive, since the Chlorella fractions, either singly or in any combination of two, formed only small amounts of \( \delta \)-aminolevulinic acid in the absence of Synechocystis protein (21). The ability of a Chlorella enzyme fraction to stimulate \( \delta \)-aminolevulinic acid formation in Synechocystis extracts suggests that the reaction intermediates are the same in both species, and that species-specific interaction of enzyme components is not required for \( \delta \)-aminolevulinic acid formation.

The procedure for detection of \( \delta \)-aminolevulinic acid formation in Synechocystis extracts was also used to measure \( \delta \)-aminolevulinic acid formation in extracts of another cyanobacterial species, Synechococcus sp. PCC 7002. Synechococcus extracts had somewhat lower activity than the Synechocystis extracts, and activity was stimulated by the addition of Synechocystis RNA. The lower activity of Synechococcus can be attributed to the facts that neither the growth conditions nor the extraction and incubation conditions were optimized for this species.

\( \delta \)-Aminolevulinic acid-forming activity was also detected in extracts of the filamentous prochlorophyte \( P. \) hollandica. Prochlororhizis is the first member of the Prochlorophyta to be cultured in the laboratory (30), and the first in which the route of \( \delta \)-aminolevulinic acid formation has been determined. We previously found that RNA isolated from Prochlororhizis supports \( \delta \)-aminolevulinic acid formation in Euglena enzyme extracts (17). The observation that Prochlororhizis forms \( \delta \)-aminolevulinic acid via the 5-carbon pathway is consistent with the proposed candidacy of the chlorophyll a- and b-containing Prochlororhizis as precursors to green algal and plant chloroplasts.

Substantial progress has recently been made in several laboratories toward understanding the biochemistry of \( \delta \)-aminolevulinic acid formation and its pivotal role in the regulation of tetrapyrrole biosynthesis. Progress with cell-free systems to date has been made almost exclusively with material derived from plants and eukaryotic algae. Oxygenic prokaryotes are especially attractive experimental materials for the next logical step in the investigation of mechanism of \( \delta \)-aminolevulinic acid formation and its regulation, isolation of the genes coding for the macromolecular components of the \( \delta \)-aminolevulinic acid-forming system. The detection and partial characterization of \( \delta \)-aminolevulinic acid formation in extracts of two genetically transformable cyanobacteria that have been used for molecular genetic studies (45, 46) is an important first step in the preparation for genetic studies.

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