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BIOSYNTHESIS OF 5-AMINOLEVULINATE FROM GLUTAMATE IN *ANABAENA VARIABILIS*

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Summary

Anabaena variabilis filaments excrete 5-aminolevulinate into the medium when incubated in the presence of levulinic acid, a competitive inhibitor of the 5-aminolevulinate utilizing enzyme, 5-aminolevulinate dehydratase (5-aminolevulinate hydro-lyase, EC 4.2.1.24). Although 5-aminolevulinate accumulation is independent of an external supply of substrate, the accumulating 5-aminolevulinate can be readily labeled by [^{14}C]glutamate or α-[^{14}C]ketoglutarate. Glycine and succinate, substrates of the classical 5-aminolevulinate synthesizing enzyme, 5-aminolevulinate synthase (succinyl-CoA:glycine *C*-succinyltransferase (decarboxylating), EC 2.3.1.37), label 5-aminolevulinate only to a very small extent.

Studies with glutamate and α-ketoglutarate labeled in specific carbon atoms show that in *A. variabilis*, as in higher plants and eukaryotic algae, all five carbon atoms of these substrates are incorporated into 5-aminolevulinate, with carbon 1 of glutamate or of α-ketoglutarate becoming carbon 5 of 5-aminolevulinate. These findings are consistent with the theory that chloroplasts evolved from cyanobacteria or from closely related organisms.

Introduction

5-Aminolevulinate is the first committed intermediate in the biosynthesis of tetrapyrroles. As such, it is the precursor of chlorophyll, heme, phycobilins and

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Abbreviations: Dimedone, 5,5-dimethyl 1,3-cyclohexanedione; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

several other molecules of central importance in cellular metabolism. In animal cells, and in previously studied bacteria it is produced from succinyl-CoA and glycine by the enzyme 5-aminolevulinate synthase [1,2]. On the hand, 5-aminolevulinate is produced in the chloroplasts of higher plants and eukaryotic algae from the intact carbon skeleton of glutamate or α-ketoglutarate [1]. The details of the latter biosynthetic pathway are largely unknown, although various reaction sequences have been proposed [1,3—6].

Cyanobacteria are typical Gram-negative bacteria in most respects, and are believed to be related to the evolutionary precursors of chloroplasts. They might, therefore, be expected to produce 5-aminolevulinate by either the chloroplast pathway, the bacterial (and animal) pathway, or both. The studies described here show clearly that the heterocyst-forming cyanobacterium (blue-green alga) *Anabaena variabilis* does, in fact, use the chloroplast pathway, producing 5-aminolevulinate from glutamate.

A brief report of this work has been presented at the eleventh International Congress of Biochemistry, July 1979, Toronto, Canada.

Materials and Methods

Materials

Levulinic acid, 5-aminolevulinate, Hepes, L-glutamate, α-ketoglutarate and dimedone (5,5-dimethyl 1,3-cyclohexanedione) were purchased from Sigma Chemical Co. [3,4-^{14}C]Glutamic acid was obtained from ICN Pharmaceuticals; all other ^{14}C-labeled organic compounds, Aquasol 2, and cellulose TLC plates (layer thickness 0.25 mm) were from New England Nuclear. Dowex 50W.X8, 200—400 mesh, was purchased from Bio-Rad Laboratories, ninhydrin spray from Pierce Chemical Company, and Silica Gel 60 TLC plates (layer thickness 0.25 mm) from EM Laboratories.

Culture conditions

Filaments of *A. variabilis* Kutz. (ATCC 29413) were grown to a concentration of 0.5—1.0 μg chlorophyll a/ml in 10 l medium at 30° C in vigorously aerated fermentors illuminated by cool, white fluorescent lamps. The medium was an 8-fold dilution of the nitrogen-free medium of Allen and Arnon [7]. The doubling time was approx. 10 h.

Incubation conditions

Filaments were harvested by filtration on Whatman No. 4 filter paper, washed with distilled water and resuspended to a concentration of 100 μg chlorophyll *a* per ml to form the incubation mixture. This incubation mixture contained in 2 ml: cyanobacterial filaments (200 μg chlorophyll *a*), 50 mM potassium levulinate, 10 mM trisodium ATP, 20 mM Hepes-KOH (pH 8.0) and either 0.1 mM sodium glutamate or other potential substrates. Reactions were started by the addition of ^{14}C-labeled substrates. In experiments lacking radioactive substrates, reactions were started by the addition of cells to the medium. Control vessels which did not contain levulinic acid and did not accumulate 5-aminolevulinate were used to estimate the amount of background label in the various samples. The amount of radioactivity in 5-aminolevulinate was obtained

in each experiment by subtracting from the counts in the sample containing levulinate the counts in a control sample (lacking levulinate).

Reaction mixtures were incubated in stoppered Warburg vessels. The central well of each vessel contained a strip of Whatman No. 1 filter paper wetted with 0.1 ml 1 M KOH. The vessels were illuminated by a 500 W incandescent lamp, and shaken at the rate of 100 rev./min for 3 h in a waterbath at 28°C. Reactions were stopped by the addition of 0.4 ml of 3 N $HClO_4$. The filter paper strips, containing KOH and absorbed CO_2, were inserted into 10 ml Aquasol 2 liquid scintillation fluid and their radioactivity determined using a Beckman LS-133 liquid scintillation system.

Purification of 5-aminolevulinate as a pyrrole

The acidified reaction mixtures were transferred into centrifuge tubes and their pH was raised to 2.5 by the addition of 1 N KOH. The tubes were kept at 4°C overnight, then centrifuged at $1000 \times g$ for 5 min and the accumulated precipitates (potassium perchlorate, proteins and cellular debris) discarded. (This and subsequent steps are summarized in Fig. 1.)

The supernatant solutions containing 5-aminolevulinate were applied to columns (12 × 31 mm) of sulfonic acid resin (Dowex 50W-X8, 200—400 mesh) equilibrated with 0.2 N (in Na^+) sodium citrate buffer (pH 3.07). The columns were eluted with a similar buffer at pH 5.10 according to the method of Beale et al. [4].

The column eluate was acidified to pH 2.5 with HCl, and extracted three times with equal volumes of diethyl ether. The aqueous fraction, containing 5-aminolevulinate, was retained and its pH was raised to 6.8 by the addition of Na_3PO_4. This fraction, supplemented with 0.5 ml ethyl acetoacetate was heated at 100°C for 15 min to form a pyrrole from the 5-aminolevulinate [8]. The solutions were cooled to 0°C, acidified to pH 2.5 with HCl and extracted three times with equal volumes of diethyl ether. The ether extracts containing the pyrrole were combined and washed once with 5 ml of 0.2 N sodium citrate buffer (pH 2.5). Residual water in the ether extract was separated by freezing. The ether extracts were evaporated nearly to dryness and were used for determinations of radioactivity by scintillation counting, of purity by chromatography, and of the concentration by colorimetry. Recovery of 5-aminolevulinate in control experiments with standard 5-amino[4-^{14}C]levulinate was 20—25%.

Chromatography of 5-aminolevulinate and 5-aminolevulinate pyrrole

5-Aminolevulinate eluted from Dowex-50 columns was chromatographed on cellulose TLC plates in *n*-butanol/acetic acid/water (25 : 4 : 10, v/v) [9]. 5-Aminolevulinate was detected on the plates by the use of ninhydrin spray. Radioactivity was assayed by the use of a Packard model 7200 radiochromatogram scanner, by autoradiography and by scintillation counting. 5-Aminolevulinate pyrrole was chromatographed on silica gel TLC plates using methyl acetate isopropanol 25% NH_4OH (45 : 35 : 20, v/v) [10]. The pyrrole was located by spraying with Ehrlich's reagent and the distribution of radioactivity determined as described above.

Samples containing 5-aminolevulinate pyrrole in aqueous solution were diluted with an equal volume of modified Ehrlich's reagent containing (in 52

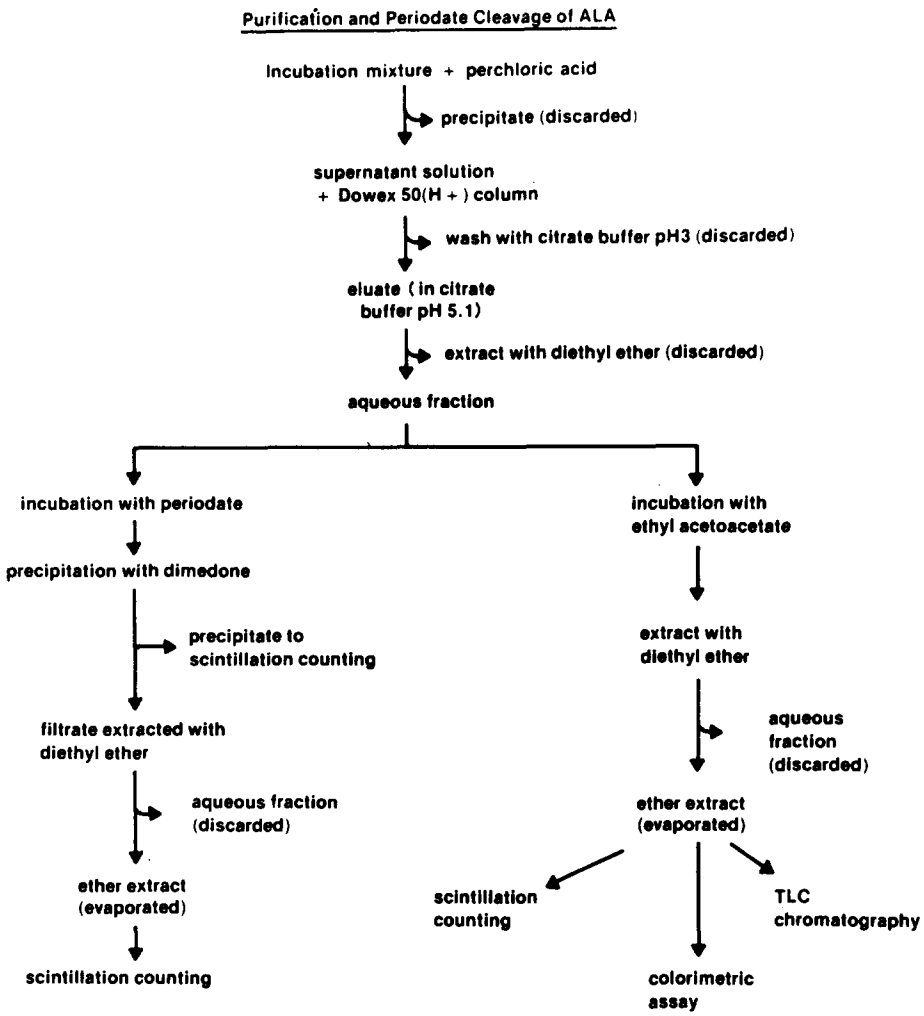

Fig. 1. Outline of the procedures for periodate cleavage of 5-aminolevulinate (ALA) and the purification of 5-aminolevulinate pyrrole.

ml) 1 g dimethylaminobenzaldehyde, 0.18 g $HgCl_2$, 42 ml glacial acetic acid and 10 ml conc. $HClO_4$. The samples were incubated at room temperature for 15 min and absorbance then measured at 553 nm [8].

Periodate cleavage of 5-aminolevulinate

5 ml samples of the ether-extracted eluate from the columns of Dowex 50 were supplemented with 50 mg 5-aminolevulinate and 100 mg $NaIO_4$, and the pH was raised to 8.5 with concentrated Na_3PO_4. Periodate cleaves 5-aminolevulinate between carbons 4 and 5, yielding succinate and formaldehyde. The succinate fragment and the formaldehyde fragment (the latter precipitated as the dimedone derivative) were separated and assayed for radioactivity using the procedure of Beale et al. [4]. The recovery of the formaldehyde fragment

(dimedone derivative) was determined from the weight of the dried precipitate (90% recovery). The recovery of the succinate fragment (50%) was estimated by assaying the recovery of standard [^{14}C]succinate from a similar, unlabeled reaction mixture.

Measurement of [^{14}C]glutamate uptake

A sample containing, in 6 ml incubation medium (see above), 5.42 μCi in 0.1 mM L-[U-^{14}C]glutamate and *A. variabilis* filaments (600 μg chlorophyll *a*) was shaken at 28°C and illuminated as described above. Portions of 0.2 ml were filtered on a 0.45 μm pore-size millipore filter, and washed with 10 ml 20 mM Hepes-KOH buffer (pH 8.0) containing 0.1 mM unlabeled L-glutamate. The filters with the washed filaments were then placed in vials containing 10 ml Aquasol 2 scintillation fluid, the filter was dissolved completely by overnight incubation and radioactivity was then measured.

Results

The requirements for 5-aminolevulinate accumulation

The optimal conditions for accumulation of 5-aminolevulinate by filaments of *Anabaena* are summarized in Table I. There was an absolute requirement for levulinate in the reaction mixture, whereas an external supply of glutamate was not required. Succinate and glycine, separately or together, α-ketoglutarate and glutamine also failed to stimulate the accumulation of 5-aminolevulinate. The stimulation by ATP has not been studied further.

The labeling of 5-aminolevulinate by glutamate

[^{14}C]Glutamate was taken up at a constant rate of 8.8 nmol/mg chlorophyll per min, for approx. 1 h. Even though 5-aminolevulinate production was not stimulated by exogenous glutamate, the 5-aminolevulinate produced was readily labeled by exogenous glutamate and α-ketoglutarate (Table II). On the other hand, [^{14}C]glycine and [1,4-^{14}C]succinate, the substrates of a typical bacterial 5-aminolevulinate synthase, label 5-aminolevulinate much less effec-

TABLE I

REQUIREMENTS FOR ACCUMULATION OF 5-AMINOLEVULINATE (ALA) BY FILAMENTS OF *ANABAENA VARIABILIS*

Complete reaction mixture contained in 2 ml: *A. variabilis* filaments (200 μg chlorophyll *a*), 50 mM potassium levulinate; 10 mM Na_3ATP, 20 mM Hepes-KOH, pH 8.0; and 0.1 mM sodium glutamate. The reaction vessels were illuminated and shaken in a water bath at 28°C for 3 h.

Incubation medium	nmol ALA accumulated	Percentage of control
Complete	18.65	100
—Levulinate	0.00	0
—Glutamate	18.58	99
—ATP	10.33	55
—Cells	0.00	0
Complete in dark	7.59	41
Complete in argon	11.19	60

TABLE II

EFFECTIVENESS OF VARIOUS SUBSTRATES IN LABELING 5-AMINOLEVULINATE (ALA) PRODUCED BY *ANABAENA VARIABILIS*

Substrate	Added radioactivity (k cpm)	Specific activity of substrate (k cpm/nmol)	Radioactivity in ALA (k cpm)	Specific activity of ALA produced (k cpm/nmol)	Radioactivity of evolved CO_2 (k cpm)
L-[U-^{14}C]Glutamate	6500	160	109.4	54.0	7.5
[1-^{14}C]Glycine	7100	25	0.5	0.3	25.9
[2-^{14}C]Glycine	4800	32	0.1	0.1	3.9
[1,4-^{14}C]Succinate	8600	41	0.2	0.1	5.0
α-[U-^{14}C]Ketoglutarate	5300	130	17.0	7.7	2.5

TABLE III

EFFECTIVENESS OF VARIOUS SPECIFICALLY LABELED GLUTAMATE MOLECULES IN LABELING 5-AMINOLEVULINATE (ALA)

Substrate	Radioactivity in L-glutamate * (k cpm)	Radioactivity in ALA pyrrole (k cpm)	Percent of added radioactivity recovered in ALA pyrrole
D,L-[1-^{14}C]Glutamate	2601	5.0	0.19
D,L-[3,4-^{14}C]Glutamate	2898	6.1	0.21
D,L-[5-^{14}C]Glutamate	2683	6.7	0.25
L-[U-^{14}C]Glutamate	5142	14.3	0.27

* Concentration of L-glutamate in the incubation mixture was 250 ± 50 μM.

tively. The fact that each of the added radioactive compounds was taken up by the cells and metabolized is shown by the accumulation of $^{14}CO_2$. These results suggest that glutamate or α-ketoglutarate rather than succinate and glycine are the substrates of the enzyme system generating 5-aminolevulinate in vivo.

Carbon atoms 1, 3 + 4 and 5 of glutamate were, within experimental error, as effective as [U-^{14}C]glutamate in labeling 5-aminolevulinate produced by *A. variabilis* (Table III). It thus appears that all five carbon atoms of glutamate are incorporated equally into 5-aminolevulinate.

In the work reported in Table II and III, purified fractions of pyrrole were used to determine the radioactivity in 5-aminolevulinate [4]. About 90% of the radioactivity in these fractions was associated with 5-aminolevulinate pyrrole as determined by TLC.

Periodate cleavage of 5-aminolevulinate was performed using fractions in which about 60% of the radioactivity was associated with 5-aminolevulinate (Fig. 1). Standard 5-amino-[4-^{14}C]levulinate and 5-amino[5-^{14}C]levulinate were used to determine the specificity of the cleavage method employed.

As shown in Table IV, carbon 1 of glutamate or of α-ketoglutarate was

TABLE IV

LOCALIZATION OF RADIOACTIVITY IN 5-AMINOLEVULINATE PRODUCED FROM SPECIFICALLY LABELED SUBSTRATES

Reactions were carried out as in Table 1. Radioactivity in the 5-aminolevulinate fraction was obtained by subtracting the counts in the control samples not containing levulinate (therefore free of 5-aminolevulinate) from the counts in the tubes containing levulinate. The subtracted counts were about 40% of the total. Dimedone derivative (C-5) and succinate fragments (C-1—C-4) were obtained and counts corrected for 90% and 50% recovery, respectively.

Substrate	Radioactivity in 5-aminolevulinate fraction (k cpm)	Percent of label recovered in C-5 (dimedone derivative)	Percent of label recovered in C-1—C-4 (succinate fragment)
[1-^{14}C]Glutamate	81.7	73.1	5.0
[5-^{14}C]Glutamate	112.2	1.2	44.5
α-[1-^{14}C]Ketoglutarate	4.7	79.7	7.1
[4-^{14}C]5-Aminolevulinate	416.2	0.4	68.6
[5-^{14}C]5-Aminolevulinate	442.9	77.6	6.4

recovered in carbon 5 of 5-aminolevulinate whereas carbon 5 of glutamate was recovered in the succinate fragment containing carbons 1—4 of 5-aminolevulinate.

Discussion

The results presented indicate that the pathway of synthesis of 5-aminolevulinate in the cyanobacterium *A. variabilis* is different from the pathway in photosynthetic and other bacteria [1,2]. On the other hand, the following observations suggest that the pathway of synthesis of 5-aminolevulinate in *A. variabilis* may be similar to the pathway in chloroplasts of higher plants and eurkaryotic algae [1,2]: (a) 5-aminolevulinate synthesis in isolated chloroplasts and in *A. variabilis* is stimulated by ATP, light and aerobic conditions (Table I and Ref. 11). Accumulation of 5-aminolevulinate is dependent on the presence of levulinate in all 5-aminolevulinate accumulating systems studied [1,2,12]. (2) 5-aminolevulinate synthesized either by chloroplasts (isolated or in vivo) or by filaments of *A. variabilis* in vivo is extensively labeled by [^{14}C]glutamate and by [^{14}C]α-ketoglutrate but labeled to a much lesser extent by [^{14}C]succinate and [^{14}C]glycine (Table II and Refs. 4, 9, 10, 13). (3) All carbon atoms of glutamate appear to be equally effective in labeling 5-aminolevulinate produced by greening barley leaves and by filaments of *A. variabilis* (Table III and Ref. 4). (4) Radioactive carbon supplied in carbon 1 of glutamate can be recovered in carbon 5 of 5-aminolevulinate produced by greening maize leaves and greening barley leaves; similar results are obtained with (1-^{14}C)-labeled glutamate or α-ketoglutarate using *A. variabilis* (Table IV and Refs. 4 and 9). This result is of particular significance because C-1 is not incorporated, but rather excreted as CO_2, when label from glutamate is incorporated into 5-aminolevulinate via the 5-aminolevulinate synthase reaction. The facts that the label from C-1 appears in 5-aminolevulinate to the same extent as other carbon atoms and that cyanobacteria lack α-ketoglutarate dehydrogenase (EC 1.2.4.2), see Ref. 14, make it improbable that the incorporation of label from glutamate proceeds via succinate and consequently via 5-aminolevulinate synthase.

It thus appears that in *A. variabilis*, as in chloroplasts of higher plants and eukaryotic algae, 5-aminolevulinate is synthesized primarily from either glutamate or α-ketoglutarate, although some slight contribution by a 5-aminolevulinate synthase is not excluded. The results are consistent with the idea that chloroplasts have evolved from cyanobacteria or from closely related organisms. Becuase no prokaryotes other than cyanobacteria have been shown to possess the 5-carbon pathway of synthesis of 5-aminolevulinate, cyanobacteria may provide a unique tool in the further elucidation of this pathway.

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