BIOSYNTHESIS OF δ-AMINOLEVULINIC ACID IN THE UNICELLULAR RHODOPHYTE, CYANIDIUM CALDARIUM

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ABSTRACT: Levulinic acid, a competitive inhibitor of δ-aminolevulinic acid dehydrase, inhibited chlorophyll-a and phycocyanobilin synthesis by 50% in vivo in the unicellular rhodophyte, Cyanidium caldarium. Inhibition of tetrapyrrole synthesis was accompanied by accumulation of δ-aminolevulinic acid in amounts equivalent to the difference between chlorophyll-a and phycocyanobilin synthesis in control and levulinic acid treated cells. δ-Aminolevulinic acid produced by levulinic acid treated cells incubated with specifically labeled glycine, α-ketoglutarate and glutamate was degraded in alkaline periodate to the succinic acid (C_1-C_4) and formaldehyde (C_5) derivatives. The distribution of radioactivity in these fragments suggested that the carbon skeleton of δ-aminolevulinic acid is derived directly from α-ketoglutarate and glutamate.

The first step in the porphyrin pathway in animals and bacteria is catalyzed by δ-aminolevulinic acid synthetase (succinyl-CoA;glycine C-succinyltransferase[decarboxylating], EC 2.3.1.37) which condenses glycine and succinyl CoA to form δ-aminolevulinic acid (ALA) (1). ALA synthetase activity has been demonstrated in vitro in photosynthetic bacteria (2), non-photosynthetic bacteria (3), yeast (4), avian erythrocytes (5) and mammalian tissues (6,7) but not in plants. Failure to observe ALA synthetase activity in extracts of algae or higher plants is surprising because these organisms produce porphyrin derivatives such as chlorophyll, heme and bile pigment in amounts and at rates in vivo well within the limits of detection in ALA synthetase assays (8-10).

Recent work has demonstrated that administered α-ketoglutarate and glutamate are more efficiently incorporated into ALA than are glycine and succinate in barley leaves, bean leaves and cucumber cotyledons (11). Furthermore, the carbon skeleton of glutamate is apparently converted to ALA intact in bean leaves in a manner incompatable with ALA synthesis from succinyl CoA and glycine (12). This indicates that ALA in higher plants may arise by a different metabolic route than occurs in animals and bacteria and suggests that repeated failure to detect ALA synthetase activity in green plant extracts may have resulted from the use of compounds in ALA synthetase assays which are not substrates for the enzyme in plants that makes ALA.

The present investigation was undertaken to examine the possibility than an alternate mode of ALA biosynthesis occurs in algae.

MATERIALS AND METHODS

Materials. Levulinic acid, ALA hydrochloride, p-dimethylamino-benzaldehyde, periodic acid and 5,5-dimethyl-1,3-cyclohexanedione (dimedone) were obtained from Sigma Chemical Company. Dowex 50W-X8 was purchased from BioRad Laboratories. DL-[1-^{14}C] glutamic acid, DL-[5-^{14}C] glutamic acid, [1-^{14}C] glycine and [2-^{14}C] glycine were obtained from New England Nuclear. [1-^{14}C] α-Ketoglutaric acid and [5-^{14}C] α-ketoglutaric acid were purchased from Amersham-Searle.

The Organism. Cyanidium caldarium is a unicellular rhodophyte (13) which produces chlorophyll-a and phycocyanin when grown in the light. Dark-grown cells are unable to make these photosynthetic pigments but when placed in the light they synthesize chlorophyll-a and phycocyanin. Phycocyanobilin, the prosthetic group of phycocyanin, is a linear tetrapyrrole structurally related to mammalian bile pigment (14).

Culture Conditions. Dark-grown cells were placed in the light in medium to which levulinic acid (4 mM) was added. Levulinic acid is a competitive inhibitor of ALA dehydrase (porphobilinogen synthase; δ-aminolevulinate hydro-lyase [adding δ-aminolevulinate and cyclyzing); EC 4.2.1.24), the second enzyme in the porphyrin pathway (15). Maximum ALA accumulation in algal cells was observed in medium containing 4 mM levulinic acid. Levulinic acid concentrations from 1-100 mM were tested. In each experiment, 25 ml cell suspensions (10^8 cells/ml) were incubated at 38° under fluorescent lights (ca. 2000 lux) on a rotary shaker for 12 hours. Radiolabeled compounds were added to duplicate control and levulinic acid treated cells and incubation was continued for another 4 hours in the light.

Measurement of ALA as a Pyrrole. After incubation, cell suspensions were made 5% with respect of $HClO_4$, frozen overnight, thawed and centrifuged for 10 minutes at 10,000 x g at 4°. An aliquot of the supernatant (ca. one fiftieth of the total volume) was adjusted to pH 6.8 with 0.5 M Na_3PO_4, ALA was condensed with

ethylacetoacetate, and ALA was estimated as the 2-methyl-3-carbethoxy-4-(3-propionic acid)-pyrrole derivative with Ehrlich reagent according to the method of Mauzerall and Granick (16). Another aliquot of the supernatant (one third of the total volume) was adjusted to pH 2.5, extracted three times with diethyl ether, the pH was adjusted to 6.8, and ALA was condensed with ethylacetoacetate as above. The resulting ALA-pyrrole was extracted into diethyl ether from the solution at pH 2.5, the diethyl ether was evaporated to dryness, and the residue was assayed for radioactivity in 14 ml of Aquasol in a Beckman liquid scintillation spectrometer at 86% efficiency.

Isolation of ALA. An aliquot (one third of the total) of the supernatant from the frozen and thawed cells was adjusted to pH 6.8 and applied to Dowex 50W-X8 columns (3 ml bed volume in 10 ml plastic syringes) which had been washed with 5 ml of 1 N NaOH and 20 ml of sodium citrate buffer, pH 3.1 (0.2 N in Na^+, pH adjusted with citric acid). The columns were washed with 20 ml of the same buffer and ALA was eluted with 20 ml of pH 5.1 sodium citrate buffer (0.2 N in Na^+, pH adjusted with citric acid).

Periodate Cleavage of ALA. ALA in an aliquot of the Dowex 50W-X8 column was cleaved in alkaline periodate to formaldehyde (C_5) and succinic acid (C_1-C_4) as described (Figure 1) (12). The formaldehyde fragment was precipitated as the dimedone derivative, collected on 0.22 μ GSWP Millipore filters by vacuum filtration, dried, weighed and assayed for radioactivity. The resulting filtrate was adjusted to pH 3.0 with 1 N HCl, the succinic acid fragment was extracted into diethyl ether, the diethyl ether was dried over anhydrous sodium sulfate, evaporated to dryness and the residue was weighed and assayed for radioactivity.

Respiration of Radiolabeled Compounds. One μCi of each specifically labeled compound (2 μCi/umole) was added to duplicate suspensions of dark-grown cells which had been incubated in the light for 12 hours in medium containing 4 mM levulinic acid. Respired CO_2 was collected on glass fiber filter discs moistened with 1 N KOH. The filter discs were suspended from the bottom of rubber stoppers used to seal the 50 ml Erlenmeyer flasks containg 25 ml cell suspensions. At 30 minute intervals over a 4 hour incubation period in the light, the filter discs were changed and assayed for radioactivity.

RESULTS

Effect of Levulinic Acid. Chlorophyll-a and phycocyanobilin synthesis was reduced by approximately 50% in dark-grown cells incubated in medium containing 4 mM levulinic acid in the light for 16 hours (Table I). The nmoles of ALA accumulated under these conditions was nearly exactly equivalent to that needed to account for the difference between tetrapyrrole synthesis in control and levulinic acid treated cells. Cells which had been incubated with 4 mM levulinic acid for 16 hours in the light produced as much chlorophyll-a and phycocyanobilin as control cells when placed in fresh medium lacking levulinic acid and incubated for an additional 16 hours. Control cells in medium lacking levulinic acid did not contain detectable quantities of ALA.

Table I. Effect of levulinic acid on tetrapyrrole synthesis in Cyanidium caldarium. Dark-grown cells were incubated in medium with levulinic acid (4 mM) for 16 hours in the light. Control cells were similarly incubated in medium lacking levulinic acid.

COMPOUND	CONTROL		LEVULINIC ACID (4mM)	
	ALA Equivalents* (nmoles)	Inhibition (%)	ALA Equivalents* (nmoles)	Inhibition (%)
Chlorophyll-a**	481 ± 17	0	229 ± 8	52
Phycocyanobilin**	312 ± 19	0	174 ± 4	44
ALA	0	-	437 + 44	-
Total	793 ± 36		840 ± 56	

* ALA equivalents are equal to 8 times the molar amounts of phycocyanobilin and chlorophyll-a produced in algal cells, reflecting their formation from 8 moles of ALA.

** Chlorophyll-a was estimated from the absorbance at 675 nm using a specific absorption coefficient of 84.0 l/gm cm; phycocyanin was estimated from the absorbance at 620 nm using a specific absorption coefficient ($E_1^{1\%}_{cm}$) of 7.7; both pigments were estimated in vivo as described previously (10). Spectral overlap of the absorption bands of each pigment was corrected for (14); the nmoles of phycocyanobilin were determined by assuming that this phycobiliprotein is 4% phycocyanobilin chromophore by weight (10) and that the molecular weight of phycocyanobilin is 584 (13).

Labeling Experiments. The incorporation of radiolabeled compounds into ALA in C. caldarium is shown in Table II. These results show that C_1 and C_5 of α-ketoglutarate and glutamate were at least 2-3 times more effective than C_2 of glycine as precursors of ALA. In addition, C_1 of glycine was poorly and inefficiently incorporated into ALA under the conditions employed.

The distribution of radioactivity in the periodate cleavage products of ALA (Figure 1) produced in cells incubated with specifically labeled compounds and levulinic acid is shown in Table III. Several features of ALA synthesis in C. caldarium are evident from these results. First, the data showing the percent of applied label in the respective periodate cleavage products reveals

Table II. Incorporation of radiolabeled compounds into ALA. Dark-grown C. caldarium cells were incubated in the light in medium containing levulinic acid (4 mM) for 12 hours, radiolabeled compounds were added, and incubation was continued for an additional 4 hours in the light.

Compound	ALA Recovered* From Cells (nmoles)	dpm Recovered** in ALA-Pyrrole x 10^3	dpm Added to Cells x 10^{-6}	% of Applied Label in ALA-Pyrrole
[1-^{14}C] glycine	451	5	20	0.03
[2-^{14}C] glycine	458	70	21	0.33
[1-^{14}C] α-ketoglutarate	420	128	13	1.00
[5-^{14}C] α-ketoglutarate	483	189	18	1.05
DL-[1-^{14}C] glutamate	472	94	13	0.72
DL-[5-^{14}C] glutamate	455	164	22	0.74

* ALA in the supernatant of frozen and thawed cells was purified on Dowex 50W-X8 columns, converted to the pyrrole derivative, extracted into diethyl ether, estimated spectrophotometrically, and assayed for radioactivity.

** Recovered radioactivity represents the net dpm in experimental samples (average of 2) after subtraction of background values (average of 2) in control samples from a 25 ml cell suspension (10^8 cells/ml) incubated with radiolabeled compounds but not administered levulinic acid.

that (a) C_1 of α-ketoglutarate and glutamate was preferentially incorporated into C_5 of ALA and (2) C_5 of glutamate was preferentially incorporated into the succinic acid fragment of ALA. Second, C_1 of α-ketoglutarate and glutamate was incorporated at least 10 times more efficiently into C_5 of ALA than was C_2 of glycine. Thirdly, C_2 of glycine was about equally distributed between the formaldehyde and succinic acid fragments of ALA in a manner inconsistent with ALA synthesis via ALA synthetase (ie., C_2 of glycine becomes C_5 of ALA in the reaction catalyzed by ALA synthetase).

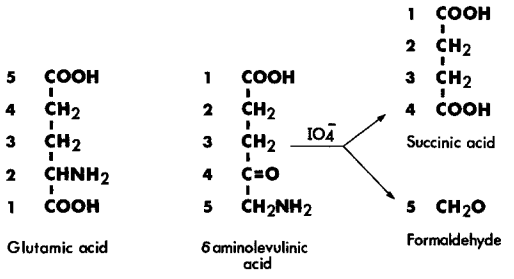

Figure 1. Diagram of the carbon numbering system for glutamic acid, δ-aminolevulinic acid, and the periodate cleavage products of δ-aminolevulinic acid.

Table III. Distribution of radioactivity in ALA. The experimental protocol employed is described in Table II.

Compound	dpm Added x 10^6	dpm in C_5* x 10^3	% Applied Label in C_5	dpm in C_1-C_4** x 10^3	% Applied Label in C_1-C_4
[1-^{14}C] glycine	20	4	0.02	0.2	0.001
[2-^{14}C] glycine	21	9	0.05	7	0.03
[1-^{14}C] α-ketoglutarate	13	105	0.80	7	0.06
[5-^{14}C] α-ketoglutarate	22	56	0.26	39	0.18
DL-[1-^{14}C] glutamate	13	174	1.34	9	0.07
DL-[5-^{14}C] glutamate	18	39	0.22	90	0.50

* The dpm in C_5 have been corrected to 100% yield of C_5 as the dimedone derivative. Actual yields of C_5 as the dimedone derivative were 51% or greater.

** The dpm in C_1-C_4 have been corrected to 100% yield. The actual yield of the succinic acid derivative in each treatment was 20% or greater.

Respiration Experiments. The respiration of specifically labeled compounds in C. caldarium cells is shown in Figure 2. Each of these compounds was rapidly taken up by the algal cells and respired at variable rates except [2-^{14}C] glycine which was largely taken up but not converted to $^{14}CO_2$.

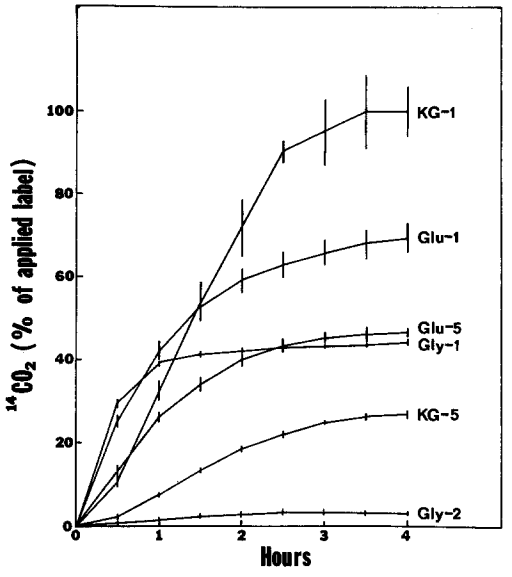

Figure 2. Respiration of radiolabeled compounds in cells of C. caldarium.

DISCUSSION

The present results have shown that levulinic acid reversibly inhibits tetrapyrrole biosynthesis in the unicellular rhodophyte, C. caldarium. The stoichiometry between inhibition of tetrapyrrole synthesis and ALA accumulation suggests that (a) the ALA accumulated was that destined for chlorophyll-a and phycocyanobilin synthesis and (b) levulinic acid specifically effects the porphyrin pathway in C. caldarium by acting as an in vivo inhibitor of ALA dehydrase.

The labeling experiments described show that α-ketoglutarate and glutamate are better precursors of ALA in C. caldarium than is glycine. The distribution of radioactivity in the periodate cleavage products of ALA is consistent with the premise that the carbon skeleton of ALA in C. caldarium is derived directly from the carbon skeleton of α-ketoglutarate and glutamate. However, some radioactivity from C_5 of α-ketoglutarate and glutamate was recovered in C_5 of ALA. This could be explained by the fact that [5-^{14}C] α-ketoglutarate (or that derived from glutamate by transamination) becomes labeled at C_1 (average of 50%) after one turn around the Krebs cycle. Radioactivity in the periodate cleavage

products of ALA would thus tend to be evenly distributed to the extent that the label position was randomized. The appearance of label from C_1 of α-ketoglutarate and glutamate in the succinic acid fragment of ALA is more difficult to explain. Metabolism of α-ketoglutarate via the Krebs cycle would result in loss of C_1 by decarboxylation. Refixation of $^{14}CO_2$ would probably result in random labeling of numerous cell constituents over a 4 hour incubation period. However, the respiration data suggest that radioactivity appearing in ALA cannot be explained by refixation of respired CO_2 because C_1 of glycine and C_5 of α-keto glutarate was respired to nearly the same extent yet C_1 of glycine was not incorporated significantly into ALA.

Finally, the present results with C. caldarium suggest that ALA in algae may arise from the carbon skeleton of α-ketoglutarate or glutamate by the same pathway which appears to be present in barley leaves, bean leaves and cucumber cotyledons (11). Beale et al (12) have discussed in detail the possible mechanisms and intermediates which might be involved in synthesis of the carbon skeleton of ALA from glutamate. If this alternate pathway is responsible for ALA synthesis in all plants, then the first committed intermediate in the porphyrin pathway (ie., ALA), is produced by a rate limiting, regulatable enzyme which must have been completely different in the evolutionalry antecedents of modern day plants and animals.

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