SYNTHESIS OF δ – AMINOLEVULINIC ACID BY ISOLATED PLASTIDS

by

SIMON P. GOUGH

and

C.GAMINI KANNANGARA

Department of Physiology – Carlsberg Laboratory
Gamle Carlsberg Vej 10 – DK-2500 Copenhagen, Valby

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This paper describes a plastid preparation from spinach capable of forming δ-aminolevulinate from α-ketoglutarate. Isolated plastids from immature leaves had this ability while those from mature leaves did not. Radioactivity from α-ketoglutarate-1-^{14}C and α-ketoglutarate-U-^{14}C was incorporated into δ-aminolevulinate to an equal extent. The ability to form δ-aminolevulinate was associated with intact plastids rather than with mitochondria or microbodies. The formation of δ-aminolevulinate was stimulated by light and had a broad pH optimum from 6.5 to 8.0. The accumulation of δ-aminolevulinate was enhanced by the presence of levulinate, an inhibitor of δ-aminolevulinate dehydratase.

1. INTRODUCTION

δ-aminolevulinate (ALA) is the precursor of chlorophyll and heme in plants. It has been shown (1, 2, 13) that during greening ALA is formed from the intact carbon skeleton of glutamate or α-ketoglutarate. This is in contrast to animals, yeast and some bacteria where ALA is formed from succinyl CoA and glycine (4, 9, 15, 16). In plants ALA is the limiting metabolite for chlorophyll formation and the enzyme system forming ALA is strictly regulated. It has been proposed that this regulation operates at the level of repression and/or feedback inhibition (4, 7, 18, 19). As a first step for obtaining precise information about the nature of the genetic control of chlorophyll synthesis we

have isolated a cell-free preparation from developing leaves which catalyses the formation of ALA.

2. MATERIALS AND METHODS

2.1 Plant Material

Spinach was obtained from the local market. The apical, pale green, unexpanded leaves of spinach shoots were removed, washed and cooled at 0-4°. These are referred to as immature leaves. For comparison, fully expanded and green leaves – mature leaves – were obtained from the same shoots.

2.2 Chemicals

α-ketoglutarate-1-^{14}C (specific activity 57.4 mCi/mmole), α-ketoglutarate-U-^{14}C (specific activity 254.5 mCi/mmole) were obtained from New England Nuclear.

2.3 Preparation of chloroplasts

Precooled leaves were homogenised in ice cold grinding medium, according to HONDA et al. (6) with some modifications. 1 litre of grinding medium contained 25 g Ficoll-400, 50 g Dextran T-40,45g Tricine, 2 g $MgCl_2$. $6H_2O$ and 85.6 g sucrose; the pH of the medium was adjusted to 7.5 with KOH. The homogenate was filtered through a single layer of Miracloth and centrifuged at 1400 g for 5 minutes. The pellet was suspended in a medium containing 0.6*M* sorbitol, 0.1*M* Tricine, 0.1*M* KH_2PO_4, 0.06*M* $NaHCO_3$, 0.001*M* dithiothreitol and adjusted with KOH to pH 7.5.

2.4 Chlorophyll in the chloroplast preparation

It was measured by the method of BRUINSMA (3).

2.5 Assay for ALA-forming activity

Isolated chloroplasts were incubated in a total volume of 2 ml of the suspending medium. Chloroplasts equivalent to 200-300 μg chlorophyll were used. The incubation medium contained in addition 25 mM Na-levulinate and 2.5 μCi of radioactive substrate.

Reaction mixtures were incubated at 25°C for 15-30 minutes under a bright light (Phillips HLRG 400 W). Incubations were terminated by 100μl of 70% ice cold perchloric acid after cooling the flask in ice and adding 125 μg non-radioactive ALA.

2.6 Isolation and identification of the radioactive product as ALA

The following procedure is a modified and amplified version of that of BEALE et al. (2). The acidified reaction mixture was centrifuged and the clear supernatant adjusted to pH 2.0 with 5*M* KOH. The mixture was allowed to stand on ice to precipitate $KClO_4$. The supernatant was then subjected to ion exchange chromatography on Dowex 50W-X8 (200-400 mesh). Column dimensions: internal diameter 0.7 cm, length 4 cm (Biorad Econocolumns). 0.4 ml of wet resin bed volume was used. The resin was washed with 1*M* NaOH (1 ml) and 1ml of buffer A (Sodium hydroxide – citrate buffer, 0.2*M* in Na^+, pH adjusted to 3.1 with citric acid). The effluent pH was then 3.1. After passing the supernatant through the column, the column was washed with 2 ml of buffer A to remove glutamate. Additional glutamate was eluted with 0.5 ml of buffer B (sodium hydroxide-citrate buffer, 0.2*M* Na^+, pH adjusted to 5.1 with citric acid). Thereafter ALA was preferentially eluted with 3 ml of buffer B. The ALA in this fraction was converted into the ALA-pyrrole (2-methyl-3-carbethoxy-4(3-propionic acid)-pyrrole) according to MAUZERALL & GRANICK (12) using ethyl acetoacetate as the condensing agent. After formation of the pyrrole excess ethyl acetoacetate was removed from the solution, adjusted to pH 7.2-7.5, by three extractions with equal volumes of chloroform. The pH was then reduced to 4.5 with phosphoric acid and the ALA-pyrrole extracted by three extractions with equal volumes of diethyl ether. One drop of concentrated ammonia was added and the ether evaporated to dryness at 25° under nitrogen. The ALA-pyrrole was dissolved in a small volume of acetone and applied to cellulose (Avicel) thin layer chromatography plates. The chromatograms were developed using the butanol ammonia system of MAUZERALL & GRANICK (12). The

Abbreviations: ALA = δ-aminolevulinate

ALA-pyrrole spot was localised using 254 nm ultraviolet light. The plates were scanned for radioactivity using a Packard 7201 radiochromatogram scanner. ALA-pyrrole was eluted from the cellulose with 1 ml methanol:concentrated ammonia:water, 4:1:5,v/v/v. An aliquot was used to determine the amount of ALA-pyrrole and the radioactivity determined in the rest. To confirm the identity of the eluted ALA-pyrrole it was in some cases rechromatographed in the butanol acetic acid system (12).

2.7 Quantitive determination of ALA

ALA-pyrrole was determined spectrophotometrically at 553 nm after addition of modified Ehrlich reagent (5).

2.8 Measurement of radioactivity

Radioactivity was determined using the Beckman liquid scintillation system with Dimilume-30 as scintillation fluid.

2.9 Sucrose density gradient

Sucrose density gradients were prepared by a method modified from MIFLIN and BEEVERS (14). The density gradient was composed of w/v sucrose solutions made up in 0.1M Tricine, pH 7.5. The gradient contained from the bottom: 5 ml of 70% sucrose, 40 ml of a linear gradient from 70-45% sucrose, 5 ml of 45% sucrose, 10 ml of a linear gradient from 45 to 40% sucrose. Chloroplasts and mitochondria isolated separately were combined in 20 ml of 30% sucrose and layered on top of the gradient. The gradient was centrifuged in an MSE Mistral centrifuge for 15 minutes, 0-5° at 2000 g. The gradient was fractionated from the top with a pasteur pipette.

2.10 Catalase and cytochrome oxidase activities

These were determined as described by LÜCK (11) and WANG et al. (18).

2.11 Isolation of mitochondria

Mitochondria were obtained from the supernatant remaining after isolation of the chloroplasts as in 2.3. This supernatant was centrifuged at 30000 g for 20 minutes in a Sorval RC-2B centrifuge. Mitochondria pelleted at

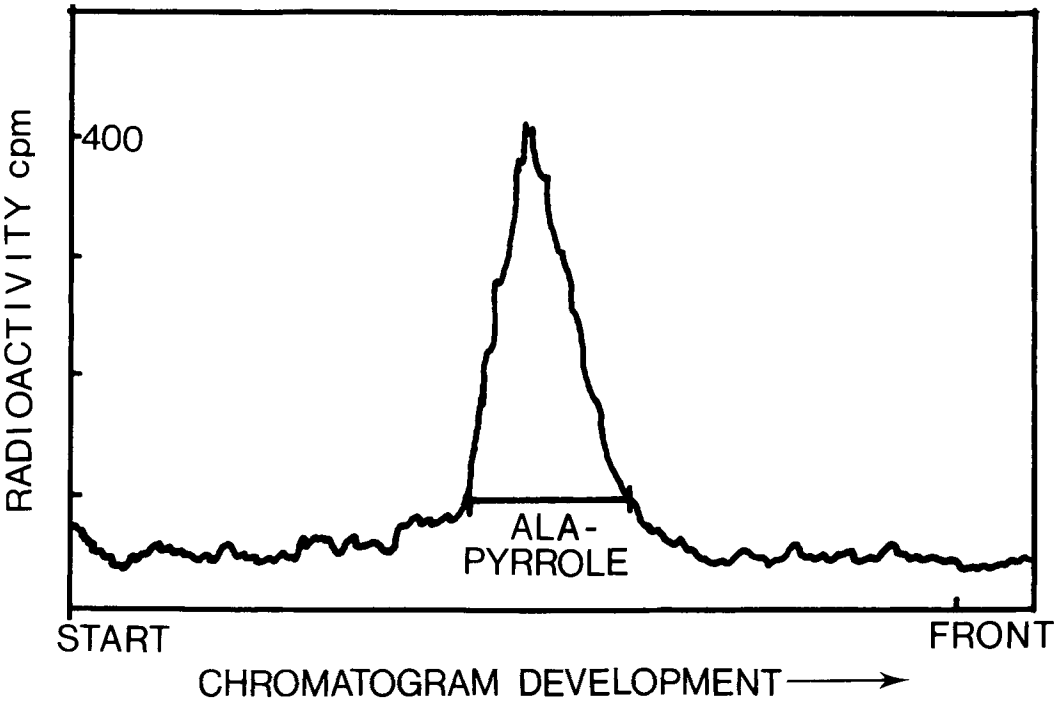

Figure 1. *Radiochromatogram scan of the ALA-pyrrole synthesized by developing chloroplasts,* Butanol: ammonia was employed as solvent system.

this speed along with chloroplast fragments and were suspended in the same medium as that used for chloroplast suspension.

2.12 Incubations under red light

In order to eliminate possible photodynamic damage, experiments to determine inhibitory effects of heme or protoporphyrin were performed under far-red light. A sharp cut-off filter (Jena RG-665) which cuts off 99.96% of light having wavelengths less than 650 nm was used together with a 150 watt tungsten light source (Tungsram 9208). Samples were placed 1 meter from the light source.

3. RESULTS

3.1 Incorporation of α-ketoglutarate-U^{14}C into ALA

Incubation of isolated plastids with α-ketoglutarate-^{14}C leads to the formation of a number of products one of which is ALA. ALA-^{14}C has been isolated and characterised, as ALA-pyrrole, by thin layer chromatography on cellulose plates in two solvent systems. Figure 1 illustrates that the final fraction obtained, contained a single radioactive substance co-chromatographing with authentic ALA-pyrrole using the butanol-ammonia solvent system. This radioactive substance was eluted from the chromatogram together with the authentic ALA-pyrrole and re-chromatographed in the second solvent system containing butanol and acetic acid. Again it cochromatographed with the authentic ALA-pyrrole near the solvent front. The addition of increasing amounts of unlabeled α-ketoglutarate diluted the incorporation of the labeled substrate (Table I).

Table I

Dilution of incorporation of α-ketoglutarate into ALA by unlabelled α-ketoglutarate.
Plastids (270 μg chlorophyll) were incubated with α-ketoglutarate-U^{14}C and various amounts of unlabelled α-ketoglutarate to give the indicated α-ketoglutarate concentrations. The atmosphere was oxygen enriched.

α-ketoglutarate μ*M*	cpm in ALA
5	4300
10	4040
25	3000
105	2425

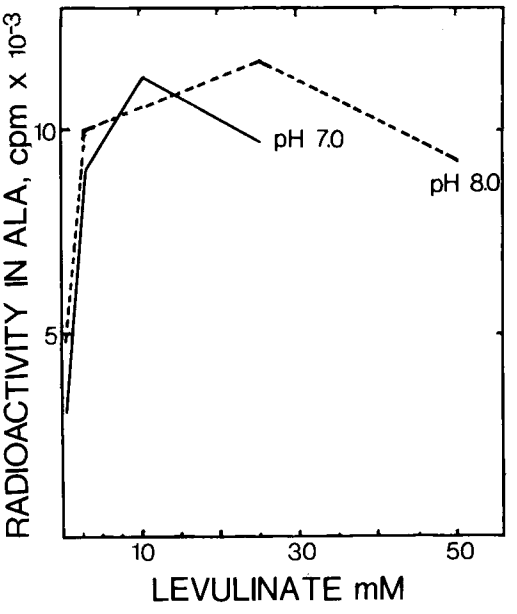

Figure 2. *Effect of different levulinate concentrations on the accumulation of ALA at pH 7.0 and 8.0.* Plastids were prepared as usual except that the plastids were suspended in medium at pH 7.0 or 8.0. Plastids (227 μg chlorophyll and 198 μg chlorophyll for pH 7.0 and 8.0 respectively) were incubated with α-ketoglutarate-U-^{14}C as described in 2.5. Counts in ALA are corrected to 250 μg chlorophyll.

3.2 Characteristics of the ALA forming system

The amount of ALA formed appeared to reach a maximum in about 15 minutes of incubation. It was stimulated by light (Table II).

Table II

Stimulation of ALA formation by light.
Plastids (270 μg chlorophyll) were incubated with α-ketoglutarate-U-^{14}C in an atmosphere enriched with oxygen.

	Cpm in ALA
Light	4300
Darkness	1000

The accumulation of ALA by suspensions of isolated plastids was increased by the presence of levulinate in the reaction mixture. Figure 2 illustrates the effect of different concentrations of levulinate on ALA accumulation. Concentrations of 10 to 25 m*M* caused optimal accumulation of ALA at pH 7.0 and 8.0.

The pH optimum for ALA synthesis in the presence of 25 m*M* levulinate was broad, ranging from 6.5 to 8.0 (Fig. 3). Only small amounts of α-ketoglutarate- ^{14}C were incorporated into ALA in plastids prepared from mature leaves of spinach when compared to those isolated from immature leaves (Table III). Highest rates of ALA synthesis obtained with these plastid preparations were in the range of 0.02 nmoles ALA produced per 15 minutes per 250 μg chlorophyll. The ability of the isolated immature plastids of spinach to synthesise ALA deteriorated even though the plastids were kept on ice. After 2 hours at 0-4° they lost 33% of their ability to make ALA.

ALA formation was not significantly effected by carrying out the incubations under partially anaerobic conditions in a nitrogen atmosphere nor by the presence of excess oxygen. We are unable to observe any stimulation or inhibition by ATP, Co-enzyme A or NADH. Both α-ketoglutarate-U-^{14}C and α-ketoglutarate-1-^{14}C were incorporated into ALA by these preparations (Table III).

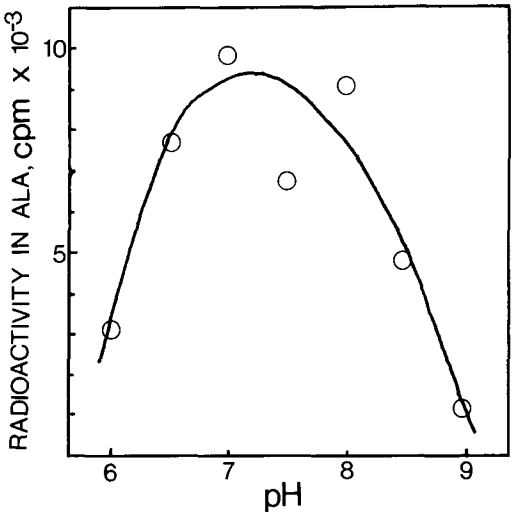

Figure 3. *The effect of pH on the formation of ALA.* Plastids were prepared as usual except that they were suspended in suspension media of the appropriate pH. Chlorophyll concentrations varied from 227 to 254 μg per sample and the counts obtained in ALA are adjusted to 250 μg chlorophyll. α-ketoglutarate-U-^{14}C was used as substrate.

Table III

ALA formation by plastids from mature and immature leaves.

Plastids from immature (198 μg chlorophyll) and mature (258 μg chlorophyll) leaves were incubated with α-ketoglutarate-U-^{14}C or α-ketoglutarate-1-^{14}C.

	Cpm incorporated into ALA/250 μg chl	
	α-ketoglutarate	
	-U-^{14}C	-1-^{14}C
Immature plastids	8791	9187
Mature plastids	423	155

3.3 Site of ALA formation within the cell

Chloroplasts and mitochondria were isolated separately, mixed together and fractionated more precisely on a sucrose gradient as described in section 2.9. The individual fractions were assayed for ALA forming ability and organelle markers. Figures 4a and b illustrate that maximal ALA synthesising ability was associated with a chlorophyll peak corresponding to intact plastids as observed by phase contrast microscopy. The major chlorophyll peak near the top of the gradient corresponded to broken chloroplasts and had only 20% of the maximal ALA synthesizing ability. The large peak of chlorophyll at the bottom of the gradient is due to a pellet containing chloroplasts. These were also associated with high ALA synthesizing ability. Mitochondria and microbodies, marked by cytochrome oxidase and catalase activities respectively were clearly separated from the region of maximal ALA synthesis.

3.4 The effect of heme and protoporphyrin on ALA formation by isolated plastids

Heme and protoporphyrin are suspected to be

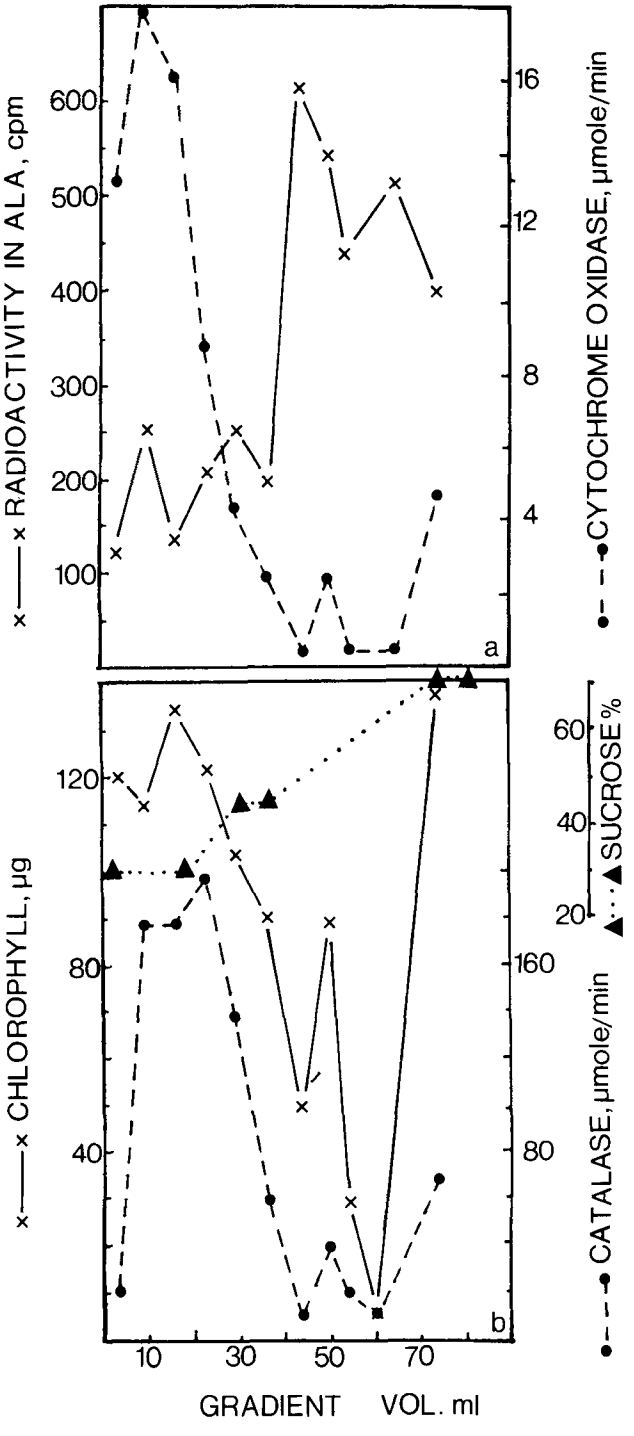

Figure 4a and b. *ALA synthesizing ability in different cell organelles.* The ability of different fractions of the sucrose gradient to incorporate α-ketoglutarate-U-^{14}C into ALA is compared to their cytochrome c oxidase activity (marker for mitochondria), catalase activity (marker for microbodies) and amount of chlorophyll (marker for chloroplast membranes).

feedback inhibitors of the enzyme system forming ALA for chlorophyll synthesis. Neither heme nor protoporphyrin at concentrations up to 200 and 6 µM respectively inhibited ALA formation significantly in the present plastid preparations (Table IV).

Table IV

The effect of heme and protoporphyrin on ALA formation.

Plastids (270 µg chlorophyll) were incubated at 25°C for 15 minutes under red light with 2.5 µCi of α-ketoglutarate-U-^{14}C. 10 µl or 1 µl of stock solutions of heme or protoporphyrin in 1:1, v/v, ethanol: 0.02*M* KOH were added to give the appropriate concentrations.

	µ*M*	Cpm incorporated into ALA
Control	0	1415
Heme	200	1473
	20	1897
Protoporphyrin	6.0	1078
	0.6	1311

4. DISCUSSION

TENG (17) reported in his doctoral thesis on a particle bound enzyme from spinach which is capable of synthesising ALA from succinyl CoA-^{14}C and glycine. This enzyme was associated with the chloroplasts. More recently BEALE and CASTELFRANCO (1) found that in greening plants glutamate and α-ketoglutarate-^{14}C were better precursors of ALA than either succinate or glycine. Experiments by BEALE et al. (2) revealed that ALA is formed from the intact carbon skeleton of glutamate.

The results presented in this paper describe an in vitro system capable of synthesising ALA from α-ketoglutarate-1-^{14}C in the presence of levulinate. The competitive inhibitor levulinate was used to prevent the ALA which was formed by the system from being further metabolised. The ALA synthesising system was associated with intact developing plastids. Synthesis of ALA in other organelles such as mitochondria or microbodies was relatively small.

Incorporation of label by the intact system approaches percentages achieved in vivo. According to BEALE et al. (2) greening barley

leaves can incorporate in 2 hours almost 1% of the fed glutamate-^{14}C into ALA. Under the best conditions reported here the isolated intact plastid preparations incorporate 0.2% of the fed radioactivity in 15 minutes.

As highest rates of ALA formation are presumably attained in actively greening leaves, it is not surprising that we found that plastids from immature spinach could form ALA. Plastid preparations from similar immature spinach leaves have been shown by KANNANGARA et al. (8) to contain plastids with developing grana and 50% of the plastid sections had prolamellar bodies. The requirement of light for ALA formation suggests either a light stimulated production of a cofactor such as ATP or NADPH or that in the dark some inhibitor prevents ALA formation. Attempts to stimulate ALA formation in the light with ATP or NADH were not successful. There may be enough ATP in our preparations to produce the quantities of ALA we observed. NADH has been shown not to enter intact plastids. Protoporphyrin or heme have been postulated to be feedback inhibitors of ALA formation in barley. In our preparations the ALA formation is not significantly inhibited by heme or protoporphyrin at the concentrations tested. However, since the enzyme system forming ALA is localised within the intact chloroplast these potential inhibitors may fail to penetrate. Further studies have been initiated to see if the ALA is synthesised in the plastids via dioxovalerate with the enzymes recently described for corn (10).

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