BIOSYNTHESIS OF δ -AMINOLEVULINATE IN CYANIDIUM CALDARIUM: CHARACTERIZATION OF tRNA^{Glu}, LIGASE, DEHYDROGENASE AND GLUTAMATE 1-SEMIALDEHYDE AMINOTRANSFERASE

by

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The unicellular red algae *Cyanidium caldarium* converts the intact carbon skeleton of glutamate to δ -aminolevulinate. The components required for this conversion are: tRNA^{Glu}, ligase, dehydrogenase, glutamate 1-semialdehyde aminotransferase, ATP, NADPH and Mg²⁺. The enzymes and the tRNA involved in δ -aminolevulinate synthesis were isolated from *Cyanidium caldarium* and characterised. Soluble proteins from greening cells were separated by gel filtration on Sephacryl S-300 and passed sequentially through Blue Sepharose, Matrex gel Red A and chlorophyllin Sepharose. The ligase and the dehydrogenase activities were bound to Blue Sepharose as well as Matrex gel red A. Chlorophyllin Sepharose retained the tRNA^{Glu}, while glutamate 1-semialdehyde aminotransferase passed through the affinity columns and was collected in the run-off protein fraction. The run-off fraction also contained δ -aminolevulinate dehydratase and porphobilinogen deaminase. Glutamate was converted to glutamate 1-semialdehyde in the presence of tRNA^{Glu}, ATP, NADPH and Mg²⁺, either by the Blue Sepharose-bound protein fraction or the Matrex gel Red A-bound protein fraction. In the presence of the aminotransferase-containing fraction, the glutamate 1-semialdehyde produced was converted into δ -aminolevulinate. Two compounds in addition to glutamate 1-semialdehyde and glutamyl tRNA^{Glu} were produced from glutamate when the Blue Sepharose-bound protein fraction was incubated with tRNA^{Glu}, ATP, NADPH and Mg²⁺. One of these was converted into δ -aminolevulinate upon incubation with the run-off protein fraction and was assumed to be an additional intermediate in the δ -aminolevulinate biosynthesis pathway. The tRNA preparation from *Cyanidium caldarium* gave a minor and a major peak of glutamate acceptor activity when analysed by High Pressure Liquid Chromatography on a C₁₈ column containing trioctyl methylammonium chloride. The ability to convert glutamate to glutamate 1-semialdehyde was associated with the major peak of glutamate acceptor activity. Glutamate 1-semialdehyde aminotransferase of *Cyanidium caldarium* was stimulated by low concentrations (up to 100 μ M) of pyridoxal 5' phosphate and pyridoxamine 5' phosphate. At higher concentrations (0.5 to 5 mM), pyridoxamine 5' phosphate was further stimulatory while pyridoxal 5' phosphate was inhibitory. Gabaculine inhibited glutamate 1-semialdehyde aminotransferase and was more effective than the gabaculine-pyridoxal 5' phosphate adduct, m-carboxyphenyl pyridoxamine 5' phosphate.

Abbreviations: IM_{Blue} = protein that binds to Blue-Sepharose; IM_{Red} = protein that binds to Matrex gel Red A agarose; IM_{Green} = material that binds to the chlorophyllin-Sepharose; RO_{Green} = proteins that do not bind to Blue-Sepharose, Matrex red gel Red A, chlorophyllin-Sepharose and pass through these affinity columns; CPP_p = m-carboxyphenyl pyridoxamine 5' phosphate.

1. INTRODUCTION

Cyanidium caldarium is a unicellular algae which accumulates chlorophyll *a*, phycocyanin and allophycocyanin as light harvesting pigments. It is a eukaryote and is classified as a red algae although it does not produce phycoerythrin in contrast to most other red algae. *Cyanidium caldarium* grows in light at 42 °C in inorganic culture media at pHs around 2. It is also able to grow in the dark in the presence of glucose. Under these conditions it does not accumulate chlorophyll or phycocyanins and the cells are yellow. Transfer of yellow cells to light induces pigment synthesis (2). Both light-grown and dark-grown cells produce a large amount of cytochrome *c*-553 (4). The linear tetrapyrrole, phycocyanobilin in *Cyanidium* is formed from heme by the action of the enzyme heme oxygenase (2). The δ -aminolevulinic acid required for the synthesis of all these tetrapyrroles is made from glutamate (11, 15, 17). In vitro synthesis of δ -aminolevulinic acid from glutamate has been demonstrated in extracts from *Cyanidium caldarium* and it has been shown that this synthesis is RNA-dependent (18). In this paper we present the isolation and partial characterization of the components required for the conversion of glutamate to δ -aminolevulinic acid in *Cyanidium caldarium*.

2. MATERIALS AND METHODS

2.1. Materials

Cyanidium caldarium strain III D2 was a gift from Professor ROBERT F. TROXLER, Boston University School of Medicine, Boston MA, USA. 3-Amino-2,3-dihydrobenzoic acid hydrochloride (gabaculine) was from Fluka AG, Switzerland. $^{14}\text{C}(\text{U})$ -glutamic acid (specific activity 292 mCi per mmole) was from New England Nuclear, Boston MA, USA. L-(2,3- ^3H)-glutamic acid (25 Ci per mmole) was from Amersham, UK.

2.2. Growth conditions of *Cyanidium caldarium* III D 2

The cells were grown heterotrophically in the dark at 42 °C according to (2). The growth medium contained 1% glucose, 2 mM KH_2PO_4 ,

Figure 1. Sephacryl S-300 gel filtration of a soluble protein extract from *Cyanidium caldarium* cells.

Soluble proteins from cells (120 g wet weight) grown in the dark for five days and greened for 72 h were analysed.

10 mM NH_4Cl , 1 mM MgSO_4 , 0.5 mM CaCl_2 and 0.1 mM FeCl_3 /ethylenediamine tetraacetic acid. Micronutrients were also added according to (2). After five days of growth, cells were centrifuged down and one volume of packed cells was suspended in 20 volumes of the above medium without glucose. They were incubated with shaking in the light ($5 \times 20\text{W}$ fluorescent tubes) at 30 °C for 24 hours except for the experiments presented in Figures 1 and 9 where enzymes and RNA were prepared from 72 hour-greened cells.

2.3. Preparation of soluble protein extracts

Cells were harvested by centrifugation and washed with cold (4 °C) extraction buffer containing 0.1 M Tricine-NaOH pH 9.0, 0.3 M glycerol, 25 mM MgCl_2 and 1 mM dithiothreitol. Cell pellets were suspended in fresh buffer and passed six times through an ice cold French pressure cell at 8000 p.s.i.g. The suspension of disrupted cells was centrifuged at 300000 g for 30 minutes at 4 °C. The supernatant is referred to as the soluble protein extract.

2.4. Protein fractionation

2.4.1. Gel filtration and serial affinity chromatography

The soluble protein extract from *Cyanidium caldarium* was fractionated by gel filtration and serial affinity chromatography using columns of Sephacryl S-300, Blue Sepharose, Matrex gel Red A and chlorophyllin Sepharose (12, 16).

The columns used had the following dimensions: Sephacryl S-300 5×45 cm, Blue Sepharose 2.5×12 cm, Matrex gel Red A 2.5×8 cm, chlorophyllin Sepharose 2.5×12 cm. Gel filtration and affinity chromatography were performed in a cold room at 2 °C using the extraction buffer given in section 2.3. The elution profile from a Sephacryl S-300 column is shown in Figure 1. The early eluting peak contained membrane vesicles, ribosomes and high molecular weight proteins and was discarded. The eluate following the first peak was applied to the Blue Sepharose, Matrex gel Red A, and chlorophyllin Sepharose columns connected in series. The trailing side of the second ultra-violet absorbing peak from Sephacryl S-300 was pink due to the presence of cytochrome c-553. Once this region of the eluate had entered the Blue Sepharose, the gel filtration column was disconnected from the affinity columns and buffer was applied directly to the Blue Sepharose column.

2.4.2. Elution of affinity bound proteins with NaCl, desalting and protein concentration

The proteins not adsorbed by the three affinity columns were collected and are referred to as the RO_{Green} fraction. The proteins bound to the Blue Sepharose and the Matrex gel Red A were eluted with 1 M NaCl dissolved in buffer containing 0.1 M Tricine-NaOH pH 9.0, 0.3 M glycerol, 25 mM MgCl₂ and 1 mM dithiothreitol and desalted by passing through a Sephadex G-50 column (2.5×45 cm). These protein fractions were then concentrated using an Amicon pressure dialysis cell fitted with a PM 30 filter and were referred to as 1M_{Blue} and 1M_{Red} respectively. The protein content of the 1M_{Blue} and 1M_{Red} preparations was approximately 10 mg per ml and that of RO_{Green} was about 20 mg per ml. These protein solutions were aliquoted as 0.8 to 1 ml portions in cryotubes, quickly frozen in liquid nitrogen and stored at -70 °C.

2.4.3. Isolation of tRNA from the chlorophyllin Sepharose-bound material

The tRNA of *Cyanidium caldarium* bind to the chlorophyllin Sepharose column and were eluted with the 1 M NaCl containing buffer given

in section 2.4.2. This eluate was concentrated by pressure dialysis using a PM 10 filter without first removing the salt. Thereafter, the concentrate was washed twice with buffer-saturated phenol to remove the proteins and the aqueous (upper) layer was further washed with chloroform:isoamyl alcohol (24:1), three times to remove the phenol. The aqueous (upper) layer was collected and one-tenth volume of 2.7 M Na-acetate pH 5.5 and 2.5 volumes of ethanol were added before leaving overnight at -18 °C. The precipitated tRNAs were collected by centrifugation and dissolved in 500 µl of sterile water and stored at -18 °C.

2.5. Enzyme Assays

2.5.1. Glutamic acid tRNA ligase

1 ml incubation mixtures of 20 mM HEPES pH 6.8, 3 mM MgCl₂, 1 mM dithiothreitol, 0.5 mM ATP, 10 µCi of ³H-Glutamate, 5 to 10 A₂₆₀ units RNA, and 0.5 mg enzyme protein. Incubations were carried out at 28 °C for 20 minutes and stopped by placing the incubation tubes in ice and adding 20 µl of ice cold trichloroacetic acid (1 g dissolved in 1 ml of water). The precipitate was collected on a filter paper disc by vacuum filtration, and washed thoroughly with ice cold 5% (w/v) trichloroacetic acid followed by 96% ethanol. The filter was then dried under an infra red lamp, and the activity of the tRNA ligase determined by counting the precipitated radioactivity. The other amino acid tRNA ligase activities were assayed in the same way using 10 µCi of the appropriate ³H-amino acid.

2.5.2. Dehydrogenase

The dehydrogenase involved in the conversion of glutamate to glutamate 1-semialdehyde was assayed in a coupled assay with glutamic acid tRNA ligase. In a total volume of 200 µl in Eppendorf tubes, the assay mixture contained the following: 2 mM ATP, 2 mM NADPH, 1 µCi of ¹⁴C(U)-L-glutamate, 10A₂₆₀ units of RNA (unless otherwise described in the text), 0.5 mg protein 1M_{Blue} and/or 0.5 mg protein 1M_{Red}, and the components of the extraction buffer. The pH of the reaction mixture was adjusted to 7.9 by adding a volume of extraction buffer pH 5.2 (pH

unadjusted), equal to the volume of enzyme solution added. Incubations were at 28 or 37 °C for 20 or 40 minutes as indicated in the figure legends. The reactions were stopped by placing the tubes in ice and adding 8 μ l of ice cold 7% perchloric acid. Precipitated proteins were removed by centrifugation in an Eppendorf centrifuge at 4 °C, and 70 μ l of the clear supernatant was analysed by a High Pressure Liquid Chromatography system coupled to the radioactivity detector described in section 2.6.

In experiments to determine the pH optimum of the dehydrogenase activity, the enzyme fraction was passed through a Sephadex G-25 desalting column using a buffer containing 10 mM Tricine-NaOH pH 9.0, 0.3 M glycerol, 25 mM MgCl_2 and 1 mM dithiothreitol. Aliquots (80 μ l) of buffers containing 0.1 Tricine-NaOH adjusted to various, appropriate pHs, 0.3 M glycerol, 25 mM MgCl_2 and 1 mM dithiothreitol were added to 80 μ l of this enzyme solution to obtain the desired pHs.

2.5.3. Glutamate 1-semialdehyde aminotransferase

Glutamate 1-semialdehyde aminotransferase activity was measured by one of two methods. A. Utilizing ^{14}C -labelled glutamate 1-semialdehyde

^{14}C -Labelled glutamate 1-semialdehyde was generated by the ligase and dehydrogenase coupled enzyme reactions described in 2.5.2. The ^{14}C -labelled glutamate 1-semialdehyde was converted to ^{14}C - δ -aminolevulinate by incubating with 0.5 mg protein from the RO_{Green} fraction, 1 mM Na-levulinate, and 0.1 mM pyridoxamine phosphate for 30 minutes at 28 °C. The assays were performed in a total volume of 300 μ l in Eppendorf tubes. The ^{14}C -labelled substrate was generated either in a preincubation or during the incubation with RO_{Green} fraction. The formation of ^{14}C -labelled product was monitored by a High Pressure Liquid Chromatography system coupled to a radioactivity detector described in section 2.6.

B. Utilizing chemically synthesised glutamate 1-semialdehyde

One ml assays were carried out in pH 7.9 extraction buffer containing 0.5 mg RO_{Green} protein, 1.0 mM levulinate, approximately 25

μM glutamate 1-semialdehyde, and pyridoxal 5' phosphate or pyridoxamine 5' phosphate as indicated in the text. Incubations were performed at 28 °C for 40 minutes and stopped by the addition of 20 μ l of 70% perchloric acid. Precipitated proteins were removed by centrifugation. The supernatants were transferred to 15 ml graduated tubes and pHs adjusted to 7.0 with 0.5 M Na_3PO_4 . Ethyl acetoacetate (100 μ l) was added and the mixtures placed in a boiling water bath for 20 minutes. Thereafter, the tubes were cooled to room temperature and the volume adjusted to 1.5 ml with water. Modified Ehrlich's solution (1.5 ml) was then added and the absorbance at 553 nm and 525 nm was recorded after 20 minutes (8).

2.5.4. δ -Aminolevulinate synthesis from glutamate

Conversion of ^{14}C -(U)-L-glutamate to δ -aminolevulinate was studied as given in section 2.5.3. A.

2.5.5. Detection of δ -aminolevulinate dehydratase and porphobilinogen deaminase

These enzymes were detected by incubating the protein (0.5 mg) preparations with 10 mM δ -aminolevulinate or 35 μM porphobilinogen for 20 minutes at 28 °C. In a total volume of 200 μ l, the incubation mixtures contained 0.1 M Tricine-NaOH (pH 8), 0.3 M glycerol, 25 mM MgCl_2 and 1 mM dithiothreitol. At the end of the incubation, 20 μ l of 70% perchloric acid was added and placed in ultra violet light for about 5 minutes. A red fluorescence indicated the presence of uroporphyrin. In the samples incubated with 10 mM δ -aminolevulinate the appearance of pink colour upon addition of 200 μ l of modified Ehrlich's reagent indicated the formation of porphobilinogen.

2.6. High Pressure Liquid Chromatographic separation of ^{14}C -labelled products formed in the enzyme assays

Radioactive glutamate 1-semialdehyde and δ -aminolevulinic acid and other compounds formed in the enzyme assays were separated on

a reverse phase C_{18} column (Waters, Novopac 5μ) using a Waters 6000A solvent delivery system. The mobile phase was slightly modified from that described in (16) and contained 50 mM NaH_2PO_4 , adjusted with phosphoric acid to pH 3.0, 1.74 g per litre sodium dodecyl sulphate (Pierce, SequenalTM grade) and 1% t-amyl alcohol. The column was equilibrated for one to two hours at a flow rate of 2 ml per minute and the samples were fractionated with the same flow rate. The radioactivity in the eluate from the reverse phase column was monitored continuously, using a Berthold HPLC Radioactivity Monitor LB505.

The identity of the peaks representing glutamate and δ -aminolevulinate was determined by injection of the authentic ^{14}C -labelled compounds. Glutamate 1-semialdehyde was identified by co-injection of unlabelled carrier and by analysing the eluate for glutamate 1-semialdehyde by ninhydrin staining or enzyme assays as described in (8).

2.7. Fractionation of tRNAs by High Pressure Liquid Chromatography

Approximately 100 A_{260} units of RNA from the $1M_{\text{Green}}$ fraction was injected onto a C_{18} ODS-Hypersil column containing trioctyl methyl ammonium chloride as previously described (12). The tRNAs were separated using a four hour linear gradient of 0.5 M ammonium acetate, pH 5.0, to 2.3 M ammonium acetate, pH 5.5, both containing 0.02% sodium azide. The

Figure 2. Fractionation of *Cyanidium caldarium* tRNAs by High Pressure Liquid Chromatography.

The RNAs (80 A_{260} units) obtained from the chlorophyllin Sepharose-bound material of 72 h greened cells were separated on a C_{18} ODS-Hypersil column containing trioctyl methyl ammonium chloride as described in section 2.7 and analysed for their abilities to ligate with glutamate as given in section 2.5.1 and for glutamate 1-semialdehyde synthesis as given in section 2.5.2.

flow rate was 0.5 ml per minute. The eluate from this column was collected in 1 ml fractions in sterile plastic tubes. The RNA was precipitated by the addition of 2.5 ml cold 95% ethanol and placed overnight in a freezer at -18°C . Precipitated RNA was collected by centrifugation for 30 minutes at $2500\times g$, dried, and dissolved in 100 μl sterile distilled water.

Table I. Distribution of tRNA and the enzymes involved in δ -aminolevulinate synthesis in the four fractions obtained after serial affinity chromatography.

Fraction obtained after serial affinity chromatography eluting from:	tRNA	Glutamic acid tRNA ligase	Dehydrogenase	Glutamate 1-semialdehyde aminotransferase
Blue Sepharose ($1M_{\text{Blue}}$)	nd	2.1×10^4	1.4×10^4	nd
Matrex gel Red A ($1M_{\text{Red}}$)	nd	0.8×10^4	2.2×10^4	nd
Chlorophyllin Sepharose ($1M_{\text{Green}}$)	420 A_{260} units	nd	nd	nd
Run-off protein (RO_{Green})	nd	nd	nd	4.7×10^4

The enzyme activities are given as cpm per mg protein per minute. These values represent amounts of: ^3H -(U)-L-glutamate ligated to tRNA for ligase, ^{14}C -glutamate 1-semialdehyde produced from ^{14}C -(U)-L-glutamate for dehydrogenase and ^{14}C - δ -aminolevulinate produced from ^{14}C -glutamate 1-semialdehyde for the aminotransferase. nd = not detectable.

2.8. Purification of glutamate 1-semialdehyde aminotransferase by isoelectric focussing

An aqueous solution (45 ml) containing 20% glycerol, 2 mM dithiothreitol, 200 μ M pyridoxamine 5' phosphate, 3% octyl glucoside and 2.5% ampholytes (pH 3-10) was applied to a preparative isoelectric focussing apparatus (RotoforTM, Bio-Rad). After a pre-run for one hour at 1 °C and 12 watts, five ml of RO_{Green} protein fraction (108 mg protein) was applied. Thereafter focussing was continued for another 3½ hours at 1 °C and 12 watts. Fractions were assayed for aminotransferase as given in section 2.5.3. B.

2.9. Other methods

Glutamate 1-semialdehyde was synthesized by reductive ozonolysis of vinyl Gaba (4-amino-hex-5-enoic acid) as described in (6). m-Carboxyphenyl pyridoxamine 5' phosphate was synthesized as described in (14) by NaBH₄ reduction of the Schiff's base produced between gabaculine and pyridoxal 5' phosphate. Protein concentrations were estimated by Bio-Rad protein assay (Bio-Rad Laboratories GmbH, Germany).

Figure 3. Analysis of ¹⁴C-labelled products formed in the enzyme assays by High Pressure Liquid Chromatography.

Assays were performed in the presence of 2 mM ATP, 2 mM NADPH, 1 μ Ci of ¹⁴C(U) glutamate and 10A₂₆₀ units of RNA as described in section 2.5.2.

A. The assay contained proteins (0.5 mg) that bind to Matrex gel Red A (IM_{Red}) and 25 μ M pyridoxamines 5' phosphate.

B. A + 0.5 mg of glutamate 1-semialdehyde aminotransferase containing protein fraction (RO_{Green}).

C. The assay contained proteins (0.5 mg) that bind to Blue-Sepharose (IM_{Blue}).

D. C + 0.5 mg of glutamate 1-semialdehyde aminotransferase containing protein fraction (RO_{Green}) + 25 μ M pyridoxamine 5' phosphate.

The acid soluble products formed after the incubations were analysed as given in section 2.6.

Figure 4. The effect of NADPH on the accumulation of glutamate 1-semialdehyde and an unidentified intermediate in the dehydrogenase assays.

The assays were performed using $1M_{blue}$ protein prepared from 24 h greened *Cyanidium caldarium* cells and analysed by High Pressure Liquid Chromatography.

3. RESULTS

3.1. Identification of tRNA^{Glu} from *Cyanidium* as a cofactor for glutamate 1-semialdehyde synthesis

Cyanidium tRNA isolated from the chlorophyllin Sepharose bound material could be ligated to glutamate using ATP, Mg^{2+} and a protein preparation from barley containing glutamic acid tRNA ligase. With the addition of NADPH and the dehydrogenase, this RNA supported the conversion of glutamate to glutamate 1-semialdehyde. The separation profile of *Cyanidium* tRNAs extracted from the $1M_{green}$ fraction, by High Pressure Liquid Chromatography is shown in Figure 2. There are several peaks of ultraviolet light absorption in the elution profile. For glutamate acceptor activity, one major peak eluting

Figure 5. The effect of increasing tRNA concentration on the glutamate 1-semialdehyde formation by Blue Sepharose-bound protein ($1M_{blue}$) and Matrex gel Red A agarose-bound protein ($1M_{red}$).

Protein fractions prepared from 24 h greened *Cyanidium caldarium* cells were used in the dehydrogenase assays.

with a retention time of 180 minutes and a minor peak around 155 minutes were found when analysed with the greening barley chloroplast glutamic acid tRNA ligase. The ability to synthesize glutamate 1-semialdehyde was associated with the trailing side of the major glutamate acceptor peak when tested with the ligase, dehydrogenase, ATP, Mg^{2+} and NADPH.

3.2. Glutamic acid tRNA ligase

Both $1M_{blue}$ and $1M_{red}$ protein fractions from *Cyanidium caldarium* showed glutamic acid tRNA ligase activity (Table I). The ligation of glutamic acid to tRNA required ATP and the activity was comparable with that found in greening barley chloroplasts. Glutamine, glycine, leucine, aspartate and serine tRNA

Table II. Evidence for an acid soluble intermediate in addition to glutamate 1-semialdehyde in the conversion of glutamate to δ -aminolevulinate.

Assay with:	Conversion of ^{14}C glutamate (cps) into:		
	Glutamate 1-semialdehyde	Intermediate eluting with a retention time of 20.1 min	δ -aminolevulinate
1M _{Blue}	4666	4056	0
1M _{Blue} + RO _{Green}	670	2352	5381

synthetase activities were also observed in these two protein fractions using the total tRNA extracted from the 1M_{Green} fraction.

3.3. Dehydrogenase involved in δ -aminolevulinate synthesis

The dehydrogenase involved in δ -aminolevulinate synthesis in *Cyanidium caldarium* was analysed by studying the NADPH- and tRNA^{Glu}-dependent synthesis of glutamate 1-semialdehyde in a coupled assay with the ligase. Five peaks of radioactivity were resolved in the assays with 1M_{Blue} protein preparations using ^{14}C -glutamate as substrate and analyzing the reaction products by High Pressure Liquid Chromatography (Fig. 3). Three of these peaks with retention times 2.5, 14.4 and 20.1 minutes were produced only in the incubation containing tRNA. One of these with a retention time of 14.4 minutes, was identified as glutamate 1-semialdehyde by co-chromatography with the authentic compound. In contrast to barley both 1M_{Blue} and 1M_{Red} protein preparations from *Cyanidium caldarium* converted glutamate to glutamate 1-semialdehyde when incubated with the tRNA extracted from the 1M_{Green} fraction together with ATP, and Mg²⁺. The production of glutamate 1-semialdehyde was stimulated by the addition of NADPH to the incubation mixtures (Fig. 4). The conversion of glutamate to glutamate 1-semialdehyde increased when increasing concentrations of tRNA were added to the incubation mixtures (Fig. 5). With 1M_{Red} protein preparations, the stimulation of glutamate 1-semialdehyde levelled off beyond 10 A₂₆₀ units of tRNA, whereas in incubations with 1M_{Blue} protein preparations the stimulation con-

tinued up to 25 A₂₆₀ units of tRNA tested. It is concluded that a dehydrogenase is required to convert glutamate to glutamate 1-semialdehyde via glutamyl tRNA in *Cyanidium caldarium*.

3.3.1. Evidence for an intermediate in addition to glutamyl tRNA^{Glu} and glutamate 1-semialdehyde in the δ -aminolevulinate synthetic pathway

In incubations using 1M_{Blue} proteins in the absence of RO_{Green}, glutamate was converted into an additional compound. It had a retention time 20.1 minutes, which is greater than that of glutamate 1-semialdehyde and smaller than δ -aminolevulinate (Fig. 3C). Table II gives the quantities of ^{14}C -glutamate converted into this compound, glutamate 1-semialdehyde and δ -aminolevulinate when an aliquot of the RO_{Green} protein was included in the assay mixture. These results indicate that the compound eluting with a retention time of 20.1 minutes is an intermediate in the δ -aminolevulinate synthetic pathway. This intermediate is not produced in incubations using 1M_{Red} proteins. A compound with retention time of 20.1 minutes was also generated by incubating ^{14}C - δ -aminolevulinate with the RO_{Green} protein preparations in the absence of ATP, NADPH or tRNA.

The pH dependence of the formation of glutamate 1-semialdehyde and the intermediate by 1M_{Blue} proteins is given in Figure 6. Almost all product accumulation is blocked below pH 6.5 and a maximum accumulation is obtained between pH 7.0 and 8.0 (highest pH tested).

The bulk of the radioactive glutamate added was converted into a compound eluting with a retention time of 2.5 minutes, when tRNA,

ATP, NADPH and Mg^{2+} were included in the assay. Attempts to convert this compound to glutamate 1-semialdehyde, δ -aminolevulinate or the intermediate eluting at 20.1 minutes were unsuccessful.

3.4. Glutamate 1-semialdehyde aminotransferase

Glutamate 1-semialdehyde aminotransferase of *Cyanidium caldarium* did not bind to the three affinity columns used and was collected in the RO_{Green} protein preparation together with δ -aminolevulinate dehydratase and porphobilinogen deaminase activities. One to 5 mM levulinate was therefore always added to enzyme assays for glutamate 1-semialdehyde aminotransferase to prevent conversion of δ -aminolevulinate. The formation of δ -aminolevulinate by glutamate 1-semialdehyde aminotransferase was enhanced by the addition of up to 500 μ M pyridoxamine 5' phosphate to the assay mixtures. This was evident whether assayed by the appearance of counts in δ -aminolevulinate from ^{14}C -glutamate in the coupled assay with ligase and the dehydrogenase, or by the production of δ -aminolevulinate directly from chemically synthesized glutamate 1-semialdehyde (Fig. 7). Pyridoxal 5' phosphate also stimulated δ -

aminolevulinate production from glutamate 1-semialdehyde in the aminotransferase assays when added in low concentrations (up to 100 μ M), whereas higher concentrations were inhibitory (Fig. 8).

Low concentrations of gabaculine inhibited glutamate 1-semialdehyde aminotransferase of *Cyanidium caldarium* (Fig. 9) with 50% inhibition at 20 μ M. Metacarboxyphenyl pyridoxamine 5' phosphate the gabaculine-pyridoxal 5' phosphate adduct which is expected to inactivate this aminotransferase (13), was, however, much less inhibitory (Fig. 9).

When the RO_{Green} protein fraction from *Cyanidium caldarium* was subjected to isoelectric focussing, glutamate 1-semialdehyde aminotransferase focussed in the pH range of 4.4 to 5.9 (Fig. 10).

4. DISCUSSION

It is known from previous studies that the red algae *Cyanidium caldarium* synthesizes its δ -aminolevulinate from glutamate by the tRNA^{Glu}

Figure 6. The pH dependence on the formation of glutamate 1-semialdehyde and an unidentified intermediate by the Blue Sepharose-bound protein.

1M_{Blue} fraction from 24 h greened *Cyanidium caldarium* cells was used in dehydrogenase assays.

Figure 7. The effect of μ Molar concentrations of pyridoxamine 5' phosphate on glutamate 1-semialdehyde aminotransferase of *Cyanidium caldarium*.

The aminotransferase activities were assayed with RO_{Green} protein from 24 h greened cells using chemically synthesized glutamate 1-semialdehyde as well as enzymatically generated ^{14}C -glutamate 1-semialdehyde as given in section 2.5.3.

Figure 8. The effect of mMolar concentrations of vitamin B₆ cofactors on glutamate 1-semialdehyde aminotransferase of *Cyanidium caldarium*.

The aminotransferase was assayed as given in section 2.5.3. B using a RO_{Green} protein from 24 h greened cells.

mediated pathway (11, 18). The results presented in this paper confirm this observation. Partial purification of the enzymes ligase, dehydrogenase and glutamate 1-semialdehyde aminotransferase as well as the tRNA^{Glu} involved in the conversion of glutamate to δ -aminolevulinate in *Cyanidium caldarium*, have been presented here. By combining the appropriate components in the in vitro assays, it was demonstrated that the conversion of glutamate to δ -aminolevulinate in *Cyanidium caldarium* proceeds via the intermediates glutamyl tRNA^{Glu} and glutamate 1-semialdehyde.

When components involved in δ -aminolevulinate synthesis in *Cyanidium caldarium* were analysed by serial affinity chromatography, the glutamic acid tRNA ligase and the dehydrogenase activities were both found to bind to the Blue Sepharose as well as Matrex gel Red A agarose. This differs from earlier results with greening barley (16), *Scenedesmus obliquus* (1), *Synechococcus* (3) and *Methanobacterium thermoautotrophicum* (5). The ligase and the dehydrogenase of these organisms were con-

fined to the Blue Sepharose bound protein with the exception of *Scenedesmus obliquus*, where the ligase activity was also found in both Blue Sepharose and Matrex gel Red A agarose-bound protein fractions.

Greening cells of *Cyanidium caldarium* produce an NADPH-dependent dehydrogenase which converts glutamyl tRNA^{Glu} to glutamate 1-semialdehyde. Two compounds in addition to glutamyl tRNA^{Glu} and glutamate 1-semialdehyde are produced from glutamate in the ligase-dehydrogenase coupled assays using the Blue Sepharose-bound protein fraction from *Cyanidium caldarium*. The formation of these two additional compounds is dependent on the tRNA fraction from the chlorophyllin Sepharose-bound material. One of these compounds requires the further presence of NADPH for its formation and is considered to be an intermediate in the pathway to δ -aminolevuli-

Figure 9. The effect of gabaculine and m-carboxyphenyl pyridoxamine 5' phosphate (CPP_p) on glutamate 1-semialdehyde aminotransferase of *Cyanidium caldarium*.

The aminotransferase was assayed as given in section 2.5.3. B using RO_{Green} protein from 72 h greened *Cyanidium caldarium* cells.

Figure 10. Purification of *Cyanidium caldarium* glutamate 1-semialdehyde aminotransferase by isoelectric focussing.

RO_{Green} proteins from 24 h greened cells were fractionated as given in section 2.8.

nate. It is converted to δ -aminolevulinate by an enzyme in the RO_{Green} protein fraction obtained from greening *Cyanidium caldarium*. With regard to the identity of this intermediate and its position in the δ -aminolevulinate pathway two possibilities merit consideration.

1. The intermediate is 4,5-diaminovalerate, a compound previously proposed as an intermediate in the conversion of glutamate 1-semialdehyde to δ -aminolevulinate (cf. 8).
2. The intermediate is a precursor which is converted into glutamate 1-semialdehyde without further reduction. Possible candidate, azetidinol propionate which undergoes a hydrolytic rearrangement to glutamate 1-semialdehyde, has been suggested previously (cf. 13).

The additional compounds formed in the dehydrogenase assays using *Cyanidium* protein fractions have to be chemically identified before their positions in the δ -aminolevulinate synthetic pathway are assigned.

Glutamate 1-semialdehyde aminotransferase of *Cyanidium caldarium* was partially purified by gel filtration and passed through three affinity columns which bind other proteins. The aminotransferase was further purified by isoelectric focussing. Pyridoxal 5' phosphate and pyridoxamine 5' phosphate at low concentrations (up to 10 μ M) stimulate the activity of the partially purified glutamate 1-semialdehyde aminotransferase of *Cyanidium caldarium*. At high concen-

trations (tested up to 5 mM) only pyridoxamine 5' phosphate gave a stimulation. Concentrations of pyridoxal 5' phosphate from 0.5 to 5 mM inhibited the activity of glutamate 1-semialdehyde aminotransferase of *Cyanidium caldarium*. It is conceivable that in assays with high concentrations, pyridoxamine 5' phosphate acts as a substrate donating amino groups rather than a cofactor. With respect to the effect of these vitamin B₆ cofactors, the glutamate 1-semialdehyde aminotransferase of *Cyanidium caldarium* is different to that isolated from the stroma of greening barley chloroplasts and is similar to the corresponding enzyme from *Synechococcus* (7).

In earlier studies it was shown that gabaculine is a potent inhibitor of chlorophyll and phycocyanin synthesis in *Cyanidium caldarium* (9) as well as in the cyanobacterium *Synechococcus* 6301 (10). From the present results, this is explained as being due to an inhibitory effect of gabaculine on the enzyme glutamate 1-semialdehyde aminotransferase. The mechanism of inactivation of aminotransferases by gabaculine is generally thought to be due to tight binding of a stable gabaculine-pyridoxamine adduct, m-carboxyphenyl pyridoxamine 5' phosphate to the enzyme. It has been shown that m-carboxyphenyl pyridoxamine 5' phosphate binds to the pig brain γ -aminobutyrate transaminase apoprotein with ten fold higher affinity than the pyridoxal 5' phosphate (14). Compared to gabaculine, m-carboxyphenyl pyridoxamine 5' phosphate is less effective as an inhibitor of the *Cyanidium* glutamate 1-semialdehyde aminotransferase studied in the presence of 100 μ M pyridoxamine 5' phosphate. This may be due to the fact that the *Cyanidium* glutamate 1-semialdehyde aminotransferase has a higher affinity for pyridoxamine 5' phosphate than the gabaculine-pyridoxal phosphate adduct. The inhibitory effect of gabaculine on the *Cyanidium* enzyme was overcome when an excess of pyridoxamine 5' phosphate was included in the assay mixtures.

Fractionation of the *Cyanidium caldarium* RNA obtained from the chlorophyllin Sepharose-bound material by High Pressure Liquid Chromatography revealed two peaks of glutamate acceptor activity. The major peak of glutamate acceptor activity does not exactly coincide

with the peak of glutamate 1-semialdehyde synthesis (Fig. 2). Since the tRNAs located in the cytoplasm and the chloroplast are both present in the preparation analyzed, this indicates the presence of another glutamate acceptor tRNA, possibly a cytoplasmic tRNA^{Glu} co-eluting with the tRNA^{Glu} participating in glutamate 1-semialdehyde synthesis.

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