Δ -AMINOLEVULINIC ACID FORMATION IN THE ARCHAEOBACTERIUM METHANOBACTERIUM THERMOAUTOTROPHICUM REQUIRES tRNA^{Glu}

by

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By combining three fractions separated from cell extracts of *Methanobacterium thermoautotrophicum* (strain Marburg) synthesis of δ -aminolevulinic acid from glutamate was obtained. One of the required fractions contained two tRNAs which could be charged with glutamate and separated by high pressure liquid chromatography. Both supported the synthesis of δ -aminolevulinate. The formation of δ -aminolevulinate from glutamate or from glutamate 1-semialdehyde was inhibited by gabaculine. The conversion of glutamate to δ -aminolevulinate in *Methanobacterium* thus displays the same features as found in the chloroplasts of plants.

1. INTRODUCTION

Biosynthesis of δ -aminolevulinic acid (ALA) proceeds by two different pathways. In certain bacteria (e.g. *Propionibacterium*, *Rhodopseudomonas*), in yeast, and in animals δ -aminolevulinate is formed from succinyl CoA and glycine (Shemin pathway) (12, 21). In plastids of higher plants and algae (2, 3, 5, 9, 11, 20, 26), as well as in cyanobacteria (2, 5), *Chromatium* (19), *Chlorobium* (24) and *Methanobacterium* (8), the ALA carbon chain is derived from glutamate (C_5 pathway). This pathway has also been suggested for *Eubacterium limosum* (10). It appears that bacteria which are capable of

acetyl-CoA oxidation via the citric acid cycle and those capable of fumarate respiration use the Shemin pathway for ALA synthesis while other bacteria use the C_5 pathway.

The enzymology of δ -aminolevulinate synthesis via the C_5 pathway has been especially studied in plants (6, 15, 16, 27). Activation of glutamate by ligation to a tRNA^{Glu} is required (16, 22) and glutamate 1-semialdehyde is a likely intermediate (14) (Figure 1).

Recently, ALA formation from glutamate in extracts of *M. thermoautotrophicum* (strain Marburg) was shown to be inhibited by ribonuclease (7), suggesting that ALA biosynthesis in

Abbreviations: ALA = δ -aminolevulinic acid; HPLC = high performance liquid chromatography.

Figure 1. Pathway proposed for conversion of glutamate to δ -aminolevulinate in plants.

1. glutamic acid tRNA ligase, 2. dehydrogenase system, 3. glutamate 1-semialdehyde aminotransferase. The compound made by the synthetic procedure described in (14) is converted into ALA by enzyme 3. The intermediate is considered to be glutamate 1-semialdehyde but final verification is still in progress.

this archaeobacterium proceeds along the same pathway as in the plastids of plants. In the present report we demonstrate that ALA formation from glutamate in *M. thermoautotrophicum* is dependent on tRNA^{Glu} from this organism.

2. MATERIALS AND METHODS

2.1. Materials

Chemicals were obtained from the following sources: MN-cellulose from Macherey-Nagel, Duren, FRG, gabaculine from Fluka, Buchs, Switzerland, $^{14}\text{C}(\text{U})$ -glutamate (specific activity 283 mCi/mmole) and $^3\text{H}(3,4)$ -glutamate (specific activity 52 Ci/mmole) from New England Nuclear, Dreieich, FRG, ODS-hypersil (5μ) from Shandon, Cheshire, UK, AH-sepharose-4B, Sephacryl S-300 and Blue-Sepharose CL-6B from Pharmacia, Uppsala, Sweden, Matrex gel Red A from Amicon, Oosterhout, Holland, resazurin and ribonuclease inhibitor from Sigma, St. Louis, USA, 4,5-dioxovaleric acid (synthesised as in ref.(4)) was a gift from Dr. DIETER DÖRNEMANN, Philipps University, Marburg. Econo-columns were obtained from Bio-Rad, Richmond, California, USA. *Methanobacterium thermoautotrophicum* (strain Marburg) (DSM 2133) was from the Deutsche Sammlung von Mikroorganismen, Göttingen, FRG.

2.2. Preparation of cell extracts

Methanobacterium thermoautotrophicum

was grown and the cells were harvested and stored frozen at -50°C as described previously (7). Cell extracts were prepared and fractionated as described before (16, 26) using 0.1 M-Tricine-NaOH (pH 9.0) buffer containing 0.3 M-glycerol, 1 mM-dithiothreitol and 25 mM- MgCl_2 flushed with N_2 . Crude extracts for direct enzyme assays were prepared under stringent anaerobic conditions as described in ref. 7. Cells were suspended in an oxygen-free buffer containing 0.2 M-imidazole-HCl (pH 7.5), 3 M-glycerol, 5 mM-dithiothreitol, 10 mM- MgCl_2 and 0.6 mg of resazurin per litre. This buffer was made anaerobic by alternating cycles of evacuating the bottle and flushing it with N_2 until the resazurin was decolorised. Cells were thereafter disrupted by passing them twice through a French pressure cell. After centrifugation, disrupted cells were transferred into N_2 -filled closed centrifuge tubes and centrifuged for 15 min at 17000 g. The supernatant fraction - which is referred to as the crude cell extract - was transferred into N_2 -filled, closed bottles and stored at 4°C .

2.3. Fractionation of cell extracts

Four columns were used in sequence (16, 26): 1. On a Sephacryl S-300 gel filtration column (5×49 cm) high and low molecular weight components were removed. 2. A Cibacron blue-Sepharose column (2.5×12 cm) served to adsorb the glutamyl tRNA synthetase and the dehydrogenase involved in ALA synthesis. 3. A Procion red-Agarose column (2.5×8 cm) removed fur-

ther components. 4. A chlorophyllin-Sepharose column (2.5×12 cm) was used to adsorb the tRNA molecules. The columns were equilibrated and run at 4 °C and three fractions collected. Fraction A designates the proteins that run through the columns, fraction B the proteins that bind to blue-Sepharose and fraction C the RNA which binds to chlorophyllin-Sepharose (16).

2.4. Enzyme assays with crude cell extracts

Enzyme assays were carried out under stringent anaerobic conditions in 10 ml serum bottles closed with butyl rubber stoppers and crimped with aluminum caps. The bottles were alternately evacuated and flushed six times with N₂ at a pressure of 0.5 atmospheres.

2.4.1. ALA synthesis from glutamate with crude cell extracts

The assay mixture of 1 ml contained 0.2 M-imidazole-HCl (pH 7.5), 3 M-glycerol, 5 mM-dithiothreitol, 10 mM-MgCl₂, 4 mM-monosodium glutamate, 7 mM-ATP, 1 mM-NADP, 50 μ M-pyridoxal phosphate, 20 mM-sodium levulinate, 40 mg protein and 0.6 μ g resazurin. The assay mixtures were incubated for 3 hours at 55 °C and analysed for ALA as described in section 2.7.

2.4.2. ALA synthesis from glutamate 1-semialdehyde using crude cell extracts

The assay mixture of 1 ml contained 0.2 M-imidazole-HCl (pH 7.5), 3 M-glycerol, 5 mM-dithiothreitol, 10 mM-MgCl₂, ca. 5 mM-glutamate 1-semialdehyde, 20 mM-sodium levulinate, crude extract corresponding to 16 mg protein and 0.6 μ g resazurin. The assay mixtures were incubated for 1 hour at 55 °C and analysed for ALA as described in 2.7. A parallel incubation in which the enzyme extract was omitted was analysed to determine the color generated by the glutamate 1-semialdehyde alone.

2.4.3. ALA synthesis from 4,5-dioxovalerate using crude cell extracts

The assay mixture of 1 ml contained 0.2

M-imidazole-HCl (pH 7.5), 3 M-glycerol, 5 mM-dithiothreitol, approximately 1 mM-4,5-dioxovalerate, 8 mM-L-alanine, 20 mM-sodium levulinate, cell extract corresponding to 16 mg protein and 0.6 μ g resazurin. The assay mixtures were incubated for 15 minutes at 55 °C and analysed for ALA as described in section 2.7.

2.5. Enzyme assays with cell extract fractions

Enzyme assays with fractions A, B and C referred to in 2.3 were carried out in capped plastic tubes with H₂ as gas phase. These conditions were not stringently anaerobic.

2.5.1. ALA synthesis from glutamate with cell extract fractions

The assay mixture of 1 ml contained 0.1 M-Tricine-NaOH (pH 7.9), 3 M-glycerol, 5 mM-dithiothreitol, 5 μ Ci of U-¹⁴C-glutamate (283 mCi/mmol), 10 mM-ATP, 25 mM-MgCl₂, 1 mM-NADPH, 50 μ M-pyridoxal phosphate, 20 mM-sodium levulinate, 0.3 mg of protein from fraction B, 1 mg of protein from fraction A and 0.6 to 21 A₂₆₀ units of tRNA from fraction C. The mixtures were incubated for 2 hours at 55 °C and then analysed for ¹⁴C-ALA as described in 2.6.

2.5.2. Charging tRNA with glutamate using fractionated cell extracts

The assay mixture of 0.5 ml contained 0.1 M-Tricine-NaOH (pH 7.9), 3 M-glycerol, 5 mM-dithiothreitol, 10 μ Ci of 3,4-³H-glutamate (52 Ci/mmol), 0.035 mM-L-glutamate 10 mM-ATP, 25 mM-MgCl₂, 0.163 mg of protein that binds to blue-Sepharose, and 0.1 to 0.5 A₂₆₀ units of the tRNA. The mixtures were incubated for 2 hours at 55 °C, and then analysed for trichloroacetic acid precipitable radioactivity as described in ref. 16.

2.6. Analysis of ¹⁴C-ALA

The incubations were terminated by transferring the tubes to ice, adding 100 μ g of ALA and 25 μ l of 70% perchloric acid. After 5 to 10 minutes the precipitated protein was removed

by centrifugation and the supernatant was loaded on a Dowex 50W- $\times$ 8 (200-400 mesh) column (0.5 $\times$ 1.5 cm in a Bio-Rad polypropylene Econo-column), equilibrated with 0.2 M-sodium citrate buffer at pH 3.1. The column was then washed with 3 ml of the same buffer and ALA was eluted with 5 ml of 0.2 M-sodium citrate buffer at pH 5.1. To convert the ALA to the ALA-pyrrole the pH of the eluate was adjusted to pH 6.9 with 0.5 M- Na_3PO_4 . Two hundred μl of ethylacetoacetate were added, and the mixture was heated for 20 minutes in boiling water. After cooling the pH was adjusted to 7.2 with 0.5 M- Na_3PO_4 , and the solution was extracted three times with equal volumes of chloroform to remove unreacted ethylacetoacetate. The pH of the solution was then lowered to 4.0 – 4.1 with H_3PO_4 and the ALA-pyrrole extracted three times with 3 ml diethylether. After addition of a drop of 25% ammonia the ether extract was evaporated to dryness under a stream of N_2 . The ALA-pyrrole was dissolved in acetone:25% ammonia (9:1) and transferred to thin layer (MN cellulose containing uv indicator) chromatography plates. The chromatograms were developed using 1-butanol:water:25% ammonia (100:17:2). The ALA-pyrrole spots were visualised under uv light, scraped off, and the cellulose suspended in 2 ml of water:methanol:25% ammonia (5:4:1). After centrifugation 1 ml of the supernatant containing the eluted ALA-pyrrole was counted for radioactivity and 75 μl was used to determine ALA-pyrrole concentration as described in (17). Normally the recovery was between 60 and 80%.

2.7. Analysis of cold ALA

Non-radioactive ALA was purified by a procedure modified from that described in (27). Reactions were terminated by transferring the serum bottles to ice. One ml of the incubated assay mixture was transferred into a glass tube and 100 μl of 1 M-citric acid and 1 ml of 10% sodium dodecyl sulphate was added. The tubes were then placed in a boiling water bath for two minutes to clarify the solution. Thereafter, the solutions were cooled to room temperature in a water bath and loaded on a Dowex 50W $\times$ 8 column (2.0 $\times$ 0.6 cm resin bed in Bio-Rad polypropylene Econo-column), pre-equilibrat-

ed with 0.05 M-sodium citrate buffer at pH 3.1 containing 25% (v/v) methanol. Air pressure from a syringe was used to facilitate movement of all solutions through the Dowex bed. The serum bottle was rinsed with water three times (2 ml) and this water was also applied to the column. Thereafter, the column was washed with 3 ml of the above pH 3.1 buffer. The column was washed with water (about 6 ml) until the eluate was nearly pH 7.0. Air pressure was used to flush the water from the column and then ALA was eluted in two successive elution runs. The eluting buffer (0.5 M-sodium phosphate buffer pH 6.8) was applied in two aliquots of 0.75 ml, allowed to drain through the column by gravity and the remaining interstitial one or two drops were flushed with air pressure after each run. The buffer effluents were pooled (1.5 ml). To 1 ml of this eluate 50 μl of ethylacetoacetate was added and the mixture was placed in a boiling water bath for 15 min. ALA-pyrrole formed was quantitated using Ehrlich's reagent (17).

3. RESULTS

Cell extracts of *Methanobacterium thermoautotrophicum* catalysed the formation of ALA from glutamate at a rate of approximately 10 pmoles $\cdot$ min $^{-1}$ $\cdot$ mg protein $^{-1}$. This specific activity correlated well with the in vivo rate of factor F 430 synthesis in the organism (7). The ALA forming activity in cell extracts rapidly decreased in the presence of air, indicating that one or more of the enzymes involved was sensitive to oxygen.

The extracts were fractionated by passing them sequentially through columns of Sephacryl S-300, Cibacron blue-Sepharose, Procion red Agarose and chlorophyllin-Sepharose as described in (16). Three fractions were obtained by this procedure. These are the Blue-Sepharose bound protein, protein that is not retained by the columns (run-through protein) and the tRNA fraction that binds to chlorophyllin-Sepharose. These fractions reconstituted the ALA synthesising system of *Methanobacterium thermoautotrophicum* (Table I). GTP, in addition to the other cofactors given in section 2.5.1 stimulated the conversion of glutamate to ALA. A similar

Table I. Reconstitution of ALA synthesis with three fractions isolated from the cell extracts of Methanobacterium thermoautotrophicum.

The tRNA fraction containing 21 A₂₆₀ units was included in assays except assay 3. Reconstituted assay mixture is as given in 2.5.1. The mixtures were incubated for 2 hours at 55 °C under H₂ as gas phase and then analysed for ¹⁴C-ALA as given in 2.6.

Assay				ALA formed per assay
				cpm*
1.	Reconstituted Assay			9000
2.	"	"	- Blue-Sepharose bound proteins	2000
3.	"	"	- tRNA fraction	1000
4.	"	"	- Run through proteins	2000
5.	"	"	+ GTP (10 mM)	14000

* Corrected for background counts in the order of 1000 cpm. 10000 cpm correspond to approximately 30 pmoles of ALA.

stimulation by GTP has been observed with the barley ALA synthesising system, but its nature is not yet understood. The fractionation procedure used with cell extracts of Methanobacterium thermoautotrophicum is essentially that developed for the barley chloroplast ALA synthesising system. As with the plant enzyme system, the ligase and the dehydrogenase involved in ALA synthesis are probably bound by the Blue-Sepharose, and the tRNAs necessary for the activation of glutamate was bound by the chlorophyllin-Sepharose. Furthermore, the protein fraction not retained by the columns (run-through protein) contained the glutamate 1-semialdehyde aminotransferase.

The activities in the reconstituted system were low but significant since the method used to determine ¹⁴C-ALA formation from ¹⁴C-glutamate (see 2.6) permitted very small amounts of ALA (2 to 5 pmoles per assay) to be determined reliably. The low activities are considered to be due to insufficient stringency of the anaerobic conditions during fractionation of the bacterial enzymes. Also, the chromatographic procedure as developed for the barley chloroplast enzymes was used without an attempt to optimise it for the fractionation of the bacterial proteins.

The Methanobacterium thermoautotrophicum proteins bound to Blue-Sepharose catalysed the aminoacylation of tRNA from this bacterium with glutamate (Figure 2). No glutamyl tRNA formation was observed when the

bacterial tRNA was replaced by barley chloroplast tRNA (isolated as in 16). Attempts to reconstitute the ALA synthesising system of Methanobacterium using corresponding fractions from the barley chloroplast or to reconstitute barley ALA synthesis with fractions from Methanobacterium, were unsuccessful. A possible explanation for this is that high temperatures (55 °C) are required for the activity of the bacterial components and these elevated temperatures destroy the activity of the barley components.

The Methanobacterium thermoautotrophicum tRNAs required for the reconstitution of ALA synthesis were isolated from the chlorophyllin-Sepharose bound fraction by phenol extraction and ethanol precipitation. This RNA was then fractionated by high pressure liquid chromatography as described in (16). Figure 2 illustrates that HPLC resolves two tRNAs that can be charged with glutamate. The tRNAs of both these peaks (I and II) reconstituted ALA synthesis when combined with the run-through and Blue-Sepharose bound protein fractions isolated by serial chromatography (Figure 2).

Experiments with crude bacterial cell extracts were performed under stringent anaerobic conditions in order to confirm the results obtained with the fractionated extracts. The two HPLC-purified RNA fractions tRNA I and tRNA II both stimulated ALA synthesis when added to crude cell extracts (Table II). It is therefore

Figure 2. HPLC fractionation of the tRNAs from *Methanobacterium thermoautotrophicum* and identification of the two glutamate acceptors tRNA I and tRNA II capable of reconstituting the archaeobacterial system forming ALA from glutamate.

RNA was isolated from the chlorophyllin-Sepharose bound material by phenol extraction and precipitation with ethanol. The RNA was injected into a 250×9 mm column packed with triethylmethylammonium chloride-coated 5 μ Hypersil ODS and separated as described in (16). One ml fractions were collected and their RNA precipitated with ethanol. Charging of the tRNAs with glutamate and ALA synthesis were determined as described in 2.5.2 and 2.5.1, respectively.

concluded that *Methanobacterium thermoautotrophicum* has two glutamate acceptor tRNAs that are used for ALA synthesis. This is in contrast to the barley chloroplast with three glutamate acceptor tRNAs, only one of which is active in ALA synthesis.

The crude cell extracts of *Methanobacterium thermoautotrophicum* had the ability to synthesise ALA from glutamate 1-semialdehyde or from 4,5-dioxovalerate (Table II). In greening barley, gabaculine inhibits ALA synthesis by irreversible inactivation of glutamate 1-semi-

aldehyde aminotransferase (14). In crude cell extracts of *Methanobacterium thermoautotrophicum* gabaculine inhibited ALA synthesis from glutamate and from glutamate 1-semialdehyde but not from 4,5-dioxovalerate (Table II). The gabaculine antagonist pyridoxal phosphate (25) did not relieve this inhibition. It is therefore concluded that as in greening barley the conversion of glutamate to ALA in *Methanobacterium* proceeds via glutamate 1-semialdehyde.

Table II. ALA formation in cell extracts of Methanobacterium thermoautotrophicum from glutamate and glutamate 1-semialdehyde.

With glutamate as substrate 40 mg of cell extract protein and 56 units of ribonuclease inhibitor were used in the assays. With glutamate 1-semialdehyde or 4,5-dioxovalerate as substrates 16 mg of cell extract protein was used in the assays. ALA values in B are corrected for colour generated by the glutamate 1-semialdehyde alone. tRNA I and tRNA II refer to RNAs eluted from the HPLC column between 180 and 190 min, and 225 and 235 min, respectively (see Figure 2). The respective complete assay mixtures are described in 2.4.1, 2.4.2, and 2.4.3.

Assays	ALA formed per assay
	nmoles
A. With glutamate as substrate	
1 Control	13
2 + tRNA I (2.3 A ₂₆₀)	19
3 + tRNA II (0.6 A ₂₆₀)	17
4 + gabaculine (500 nmol)	0.5
B. With glutamate 1-semialdehyde as substrate	
1. Control	7.7
2. + gabaculine (20 nmol)	< 0.1
3. 2 + pyridoxal phosphate (200 nmol)	< 0.1
C. With 4,5-dioxovalerate as substrate	
1. Control	19
2. + gabaculine (1000 nmol)	18

4. DISCUSSION

The results presented in this and the previous paper (7) indicate that the pathway of ALA formation from glutamate in *Methanobacterium thermoautotrophicum* is similar if not identical to that in plants. A tRNA chargeable with glutamate is required, which suggests that glutamate is activated by ligation to the tRNA before being reduced. The bacterium also contains glutamate 1-semialdehyde aminotransferase activity. In addition, ALA formation from glutamate and glutamate 1-semialdehyde is inhibited by gabaculine suggesting that glutamate 1-semialdehyde is an intermediate. The bacterial system differs, however, from the plant system by the presence of two active tRNA species.

In plastids from greening barley three tRNAs can be charged by glutamate (16, 22). Only one of these three tRNAs participated in ALA synthesis. This tRNA was therefore designated as δ -ALA-RNA. This designation is misleading since this tRNA has a glutamate anticodon, and therefore probably also functions in protein synthesis. The two other glutamate acceptor

tRNAs have in the meantime been sequenced and found to have glutamine anticodons (23). Glutamate ligated to these tRNAs is converted to glutamine by an amidotransferase present in the stroma of greening barley chloroplasts (23). A similar amidotransferase was found by WILCOX and NIRENBERG in several strains of *Bacilli* (28).

From the DNA sequence of the *Methanobacterium thermoautotrophicum* gene encoding methyl coenzyme M reductase it is known that both the glutamate codons GAA and GAG are used in protein synthesis by this organism (1). It is therefore not surprising that two tRNAs which can be charged with glutamate were active in ALA synthesis in *Methanobacterium thermoautotrophicum*. From the codon usage it can be inferred that the more abundant glutamate acceptor (tRNA I in Figure 2) is a tRNA^{Glu} with UUC as the anticodon while the other (tRNA II in Figure 2) carries the CUC anticodon.

It is still a matter of some controversy whether glutamate 1-semialdehyde or 4,5-dioxovaleric acid (DOVA) are free intermediates in ALA

formation from glutamate (13, 14, 18). Reduction of glutamyl tRNA almost certainly leads to the formation of glutamate 1-semialdehyde. During inter- or intramolecular transamination, DOVA is a possible intermediate. In this respect it is of interest that ALA formation from glutamate and from glutamate 1-semialdehyde rather than from 4,5-dioxovalerate was inhibited by gabaculine in crude cell extracts of *Methanobacterium thermoautotrophicum*. This finding appears to exclude free 4,5-dioxovalerate as an intermediate in this organism.

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