Ribonuclease-sensitive δ-aminolevulinic acid formation from glutamate in cell extracts of *Methanobacterium thermoautotrophicum*

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Cell extracts of *Methanobacterium thermoautotrophicum*, which form δ-aminolevulinic acid (ALA) via the C_5 pathway, catalyzed the synthesis of ALA from glutamate in an ATP-dependent reaction. The activity was abolished by incubation of the extract with ribonuclease. The mechanism of ALA synthesis in this methanogenic archaebacterium thus appears to be similar to that found in algae and in higher plants.

C_5 pathway *Aminolevulinic acid synthesis* *(Archaebacteria)* *Tetrapyrrole biosynthesis*

1. INTRODUCTION

δ-Aminolevulinic acid (ALA) is synthesized in nature via two different pathways: in many bacteria (e.g. *Propionibacterium, Rhodopseudomonas*), in yeast, and in animals ALA is formed from succinyl-CoA plus glycine (Shemin pathway) [1]. In plants and cyanobacteria [2–7], in *Eubacterium limosum* [8], and in *Methanobacterium thermoautotrophicum* [9] the ALA carbon chain is derived from a 5-carbon precursor, almost certainly glutamate (C_5 pathway).

The enzymology of ALA synthesis via the C_5 pathway has so far been studied only in plants [10–12] where it has recently been shown that an RNA participates in glutamate activation [5,11–13]. There have up to now been no reports on ALA formation from glutamate in bacterial cell-free systems. Here, we describe such a system obtained from *M. thermoautotrophicum*. This anaerobic methanogenic archaebacterium grows at 65°C on H_2 and CO_2 as sole energy and carbon sources and is known to synthesize ALA via the 5-carbon pathway [9].

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2. MATERIALS AND METHODS

Ribonuclease from bovine pancreas (dry powder) was from Boehringer (Mannheim, FRG), ribonuclease inhibitor from human placenta (5700 units/ml; solution in 50% glycerol containing 45 mM potassium phosphate (pH 6.4), 5 mM dithiothreitol, and 1 mM EDTA) and levulinic acid (grade I) were from Sigma (St. Louis, MO, USA). *M. thermoautotrophicum* strain Marburg (DSM 2133) was from the Deutsche Sammlung von Mikroorganismen (Göttingen, FRG).

2.1. *Preparation of cell extracts*

M. thermoautotrophicum was grown in a 500 ml glass fermenter containing 400 ml mineral salts medium on 80% H_2 and 20% CO_2 at 65°C as described by Schönheit et al. [14]. At a cell concentration of approx. 2.4 g cells (dry wt)/l (corresponding to an absorbance at 578 nm of 6.0) the bacteria were harvested by centrifugation and washed once anaerobically in 150 ml imidazole

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HCl buffer, pH 7.5 (0.2 M), containing $MgCl_2$ (10 mM), dithioerythritol (5 mM), glycerol (3 M), and the redox indicator resazurin (0.6 mg/l). They were then resuspended in 20 ml of the same buffer. The cell extract was prepared from the cell suspension by two passages through a French pressure cell at 18000 psi (~1200 bar) followed by centrifugation for 15 min at 27000 × *g*. The pellet was discarded. All the above steps were performed at 4°C and under N_2 as gas phase. Protein was determined according to Bradford [15] using the Bio-Rad protein assay (Bio-Rad, München, FRG).

Routinely the cell extracts were assayed within 24 h after preparation.

2.2. *Enzyme assays*

Substrates, inhibitors, and cofactors were pipetted into 25 ml serum bottles which were then stoppered. The air was replaced by H_2 by repeated evacuation and refilling at 1.2 bar. Cell extracts and buffer for volume adjustment were added by injection from a syringe. After increase of the H_2 pressure to 1.8 bar the reaction was started by transferring the bottles from ice to a water bath at 55°C. At the times indicated in the tables and figures, 0.5–1.1 ml samples were withdrawn with a syringe, and the reaction stopped by the addition of 0.1 volume of 1 M citric acid. If the analysis for ALA was not performed immediately the samples were stored at −20°C.

2.3. *Purification and quantitation of ALA [16]*

Before analysis 1 vol. of 10% SDS was added to the samples. After heating for 2 min in a boiling water bath the clear solution was applied to columns (12 cm long, 5 ml graduated disposable tips for Gilson Pipetman pipettes) containing approx. 0.75 ml Dowex 50W–X8 (200–400 mesh) in the Na^+ form. The columns were washed with 2 ml of 0.05 M NaOH adjusted to pH 3 with citric acid and containing 25% (v/v) methanol and then twice with 1.5 ml H_2O. ALA was eluted with 2 successive volumes of 1.5 ml of 0.5 M NaOH that had been adjusted to pH 6.8 with H_3PO_4. To 1 ml of the eluate 50 μl ethylacetoacetate was added. The mixture was thoroughly agitated to dissolve all of the ester and then heated for 15 min at 95°C in order to form the ALA pyrrole. After cooling to 50°C, 1 ml freshly prepared Ehrlich's reagent (0.5 g *p*-dimethylaminobenzaldehyde dissolved in 21 ml of 100% glacial acetic acid and 4 ml of 70% perchloric acid) was added. After 5 min at 50°C to allow full colour development (at lower temperatures a white precipitate was sometimes formed) the absorbance at 552 nm was determined within the next 5 min. The ALA concentration was calculated from a standard curve (1 nmol corresponding to an ΔA_{552} of 0.035).

The colour formed by the material obtained in the enzyme assays had the same visible absorption spectrum as the colour generated starting with authentic ALA. Furthermore, the ALA pyrrole was identified by thin layer chromatography (silica gel 60 thin layer plates from Merck (Darmstadt, FRG): butanol/acetone/diethylamine/H_2O = 70:70:14:35; $R_f \approx 0.65$).

3. RESULTS AND DISCUSSION

Cell extract of *M. thermoautotrophicum* catalyzed the formation of ALA in an assay system containing glutamate, ATP, Mg^{2+}, NADP or NADPH, pyridoxal phosphate, and levulinic acid with H_2 as the gas phase. When levulinic acid (10 mM) was omitted from the assay system no product was detected (table 1) since ALA was further metabolized. Levulinic acid specifically

Table 1

Substrate and cofactor requirements for δ-aminolevulinic acid (ALA) formation in cell extracts of *M. thermoautotrophicum*

	ALA formed per assay	
	nmol	%
Complete	11	100
− glutamate	3.9	36
− ATP	1	10
− levulinic acid	1	10

The 1.05 ml assay mixture contained: 0.02 ml monosodium glutamate (0.2 M); 0.02 ml NADP (50 mM); 0.03 ml ATP (0.25 M); 0.01 ml pyridoxal phosphate (5 mM); 0.07 ml sodium levulinate (0.3 M); 0.5 μl ribonuclease inhibitor (5700 units/ml); and 0.9 ml of dialyzed cell extract, pH 7.5 (24.5 mg protein/ml). The gas phase was 100% H_2 at 1.8 bar. The assay mixture was incubated for 80 min at 55°C and then analyzed for ALA

prevents the conversion of ALA to porphobilinogen by inhibition of ALA dehydratase (see for example [2]). This enzyme is present in high specific activities in cell extracts of *M. thermoautotrophicum* [17].

3.1. *Specific activity of ALA forming system*

ALA formation in cell extracts of *M. thermoautotrophicum* proceeded linearly with time up to at least 2 h (fig.1) and continued for at least a further 2–3 h at decreased rates. The pH optimum was 7.5. The protein dependence was non-linear at low protein concentrations (fig.2). With 10 mg protein per ml a specific activity of approx. 10 pmol ALA formed/min per mg protein was observed at 55°C. From the growth rate (generation time = 2.5 h) and the tetrapyrrole content of *M. thermoautotrophicum* (approx. 0.7 μmol coenzyme F430 and 0.1 μmol corrinoids per g dry wt cells; the bacteria do not contain cytochromes) it was calculated that the exponentially growing cells synthesize at 65°C approx. 50 pmol ALA/min per mg protein. Thus the in vitro and in vivo specific activities were comparable. The specific activity of algae cell extracts is in the same range [16].

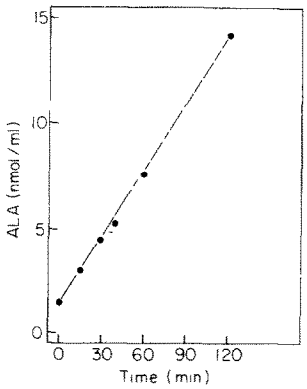

Fig.1. Time course of δ-aminolevulinic acid (ALA) formation in cell extracts of *M. thermoautotrophicum*. The 16.1 ml assay mixture contained: 0.15 ml monosodium glutamate (0.2 M); 0.15 ml NADP (50 mM); 0.23 ml ATP (0.25 M); 0.075 ml pyridoxal phosphate (5 mM); 0.53 ml sodium levulinate (0.3 M); 10 μl ribonuclease inhibitor (5700 units/ml); and 15 ml cell extracts, pH 7.5 (15 mg protein/ml). The gas phase was 100% H_2 at 1.8 bar. The assay mixture was incubated at 55°C. At the times indicated 1 ml samples were removed and analyzed for ALA.

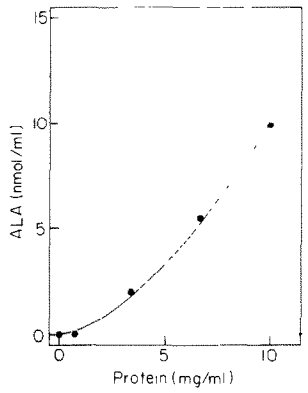

Fig.2. Effect of protein concentration on δ-aminolevulinic acid (ALA) formation in cell extracts of *M. thermoautotrophicum*. The 2.6 ml assay mixture contained: 0.02 ml monosodium glutamate (0.2 M); 0.02 ml NADP (50 mM); 0.03 ml ATP (0.25 M); 0.01 ml pyridoxal phosphate (5 mM); 0.07 ml sodium levulinate (0.3 M); 0.5 μl ribonuclease inhibitor (5700 units/ml); 0.1–1.5 ml cell extract, pH 7.5 (18 mg/ml); ad 2.6 ml with imidazole HCl buffer, pH 7.5 (0.2 M) containing $MgCl_2$ (10 mM), dithioerythritol (5 mM), and glycerol (3 M). The gas phase was 100% H_2 at 1.8 bar. The assay mixture was incubated for 120 min at 55°C and then analyzed for ALA.

The activity that catalyzes ALA synthesis was associated with the soluble cell fraction since it was present in the 163000 × *g* supernatant solution. Glycerol appeared to stabilize the enzyme system. No activity was observed when the extracts were incubated in the presence of air. Routinely the enzyme assay was performed with extracts prepared from cells which had not been frozen. However, cells stored at −50°C yielded similarly active extracts.

3.2. *Substrates and cofactor requirements*

At high protein concentrations the cell extracts formed ALA when glutamate, ATP, NADP (or NADPH) or pyridoxal phosphate were omitted. At low protein concentrations glutamate, ATP, and NADP (or NADPH) were stimulatory. Dialyzed extracts were dependent on the presence of glutamate and ATP (table 1).

It has been reported that 2-oxoglutarate rather than glutamate is the immediate precursor in the 5-carbon pathway of ALA formation [3,6]. When in cell extracts of *M. thermoautotrophicum*

glutamate was replaced by 2-oxoglutarate only 50% of the specific activity was observed.

The formation of ALA from glutamate requires a net reduction. The source of electrons in *M. thermoautotrophicum* has not been determined. The extracts were incubated under H_2 which is known to reduce coenzyme F420 which in turn reduces NADP [18]. Surprisingly, addition of NADP to the cell extracts resulted in higher ALA formation rates than addition of NADPH.

3.3. *Inactivation by ribonuclease*

The enzyme system mediating ALA formation from glutamate in plants has been shown to be inactivated by ribonuclease [5,11,12]. We therefore tested the effect of ribonuclease on the bacterial system. It was found, indeed, that bovine pancreatic ribonuclease dramatically decreased the activity. Preincubation of the cell extract of *M. thermoautotrophicum* with as little as 5 ng ribonuclease for 15 min at 35°C already resulted in a 47% lowering of the activity (table 2).

In some experiments the cell extracts lost activity upon overnight storage at 4°C. This activity loss could be prevented by the addition of ribonuclease inhibitor which was therefore routinely added to the freshly prepared cell extracts except in the experiment of table 2.

Table 2

Effect of ribonuclease on δ-aminolevulinic acid (ALA) formation in cell extracts of *M. thermoautotrophicum*

RNase added (ng)	ALA formed per assay	
	nmol	%
0	36	100
5	17	47
10	12	34
20	6	17
50	3	8
75	1	3

The 2.15 ml assay mixture contained: 0.02 ml monosodium glutamate (0.2 M); 0.02 ml NADP (50 mM); 0.03 ml ATP (0.25 M); 0.01 ml pyridoxal phosphate (5 mM); 0.07 ml sodium levulinate (0.3 M); 2 ml cell extract, pH 7.5 (15 mg protein/ml); and 1–15 μl ribonuclease (5 μg/ml). The gas phase was 100% H_2 at 1.8 bar. The assay mixture was preincubated for 15 min at 37°C and subsequently incubated for 120 min at 55°C and then analyzed for ALA

3.4. *Conclusion*

The results indicate that in *M. thermoautotrophicum* ALA is formed from glutamate in an ATP- and RNA-dependent reaction. The enzyme system in the archaebacterium thus appears to be similar to that in algae and in higher plants.

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