Volume 228, number 1, 89–93 FEB 05544 February 1988

5-Aminolevulinic acid formation from glutamate via the C_5 pathway in *Clostridium thermoaceticum*

T. Oh-hama, N.J. Stolowich and A.I. Scott

Center for Biological NMR, Department of Chemistry, Texas A&M University, College Station, TX 77843, USA

Received 20 October 1987; revised version received 9 December 1987

A cell-free extract of the anaerobic eubacterium, *Clostridium thermoaceticum*, catalyzes the synthesis of 5-aminolevulinic acid (ALA) from glutamate via the C_5 pathway. The enzyme reaction resembles that of higher plants and algae in cofactor requirements and sensitivity to ribonuclease. From the phylogenetic distribution it is proposed that the C_5 pathway evolved earlier than the ALA synthase pathway.

5-Aminolevulinic acid synthesis; C_5 pathway; Corrinoid synthesis; (*Clostridium thermoaceticum*)

1. INTRODUCTION

ALA, the common biosynthetic precursor to heme, chlorophyll, corrins and bile pigments can be formed either by condensation of succinyl-CoA and glycine catalyzed by ALA synthase (EC 2.3.1.37), or from the intact carbon skeleton of glutamate (C_5 pathway). ALA synthase activity has been demonstrated in a variety of photosynthetic and non-photosynthetic bacteria and animals [1], while the distribution of the C_5 pathway has been considered to be restricted to higher plants, algae and cyanobacteria, i.e. oxygen-evolving photosynthetic organisms [2].

Recently, bacteriochlorophyll formation in the anaerobic photosynthetic bacterium, *Chromatium* [3] and two green sulfur bacteria [4,5] has been shown to proceed via the C_5 pathway, a route which is followed in *Methanobacterium* [6], an anaerobic archaebacterium.

Correspondence address: T. Oh-hama, Center for Biological NMR, Department of Chemistry, Texas A&M University, College Station, TX 77843, USA

Abbreviations: ALA, 5-aminolevulinic acid; RNase, ribonuclease; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)-ethyl]glycine

A number of clostridia (metabolically simple anaerobes) produce corrins at high levels [7]. Since most of the work on corrinoid biosynthesis has been done with Propionibacteria [8], in which ALA is produced by ALA synthase [9], it would be of interest to establish if ALA is synthesized by the C_5 pathway in clostridia. Here, we report the exclusive operation of the C_5 pathway in *Clostridium thermoaceticum*, a thermophilic anaerobic bacterium [10].

2. MATERIALS AND METHODS

2.1. *Culture of* Clostridium *cells*

C. thermoaceticum (ATCC 39073) was maintained [11] with addition of 1 μM $NiCl_2$, under continuous bubbling with CO_2 at 55°C with transfers twice weekly. The cells used for isolation of cobester and preparation of cell extracts were grown in a medium (4 l) modified by reducing yeast extract to 1 g per l, at 58°C for 42–48 h (yield: 4 g wet cells per l). In tracer experiments with [^{13}C]- and [^{14}C]glycine, cells were grown in the medium (2 l) containing 1.0 mM [2-^{13}C]/[2-^{14}C]glycine (90 atom% ^{13}C/2 mCi ^{14}C per mol). Assimilation of the tracer was followed by measuring radioactivity remaining in the culture medium with a Packard model PL Tri-Carb liquid scintillation counter.

2.2. *Isolation of cobester [12]*

Corrinoids in sedimented cells were extracted with hot aqueous methanol containing KCN, the supernatant evaporated

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/88/$3.50

and the residue suspended in methanol containing 5% sulfuric acid (20 ml). The solution was heated under reflux under nitrogen with exclusion of light for 1 day, then evaporated to about 2 ml and neutralized with sodium bicarbonate solution. After addition of KCN, the solution was extracted with carbon tetrachloride. The latter phase was evaporated. The residue was dissolved in 0.5 ml hexane/2-propanol mixture (5:1, v/v) and applied to a Sepharose CL-6B (Pharmacia) column [13] which had been equilibrated with the same solvent. The column was successfully developed with hexane/2-propanol (saturated with KCN) mixtures (5:1, 5:2, 5:3, v/v). The first red band eluted off the column was dried and purified by thin-layer chromatograph (silica gel) using benzene/methanol (10:1, v/v) saturated with KCN. A single red band was scraped off and eluted with the same solvent. The ^{1}H- and ^{13}C-NMR spectra of the eluate were identical with those of cobester [14]. The amounts of cobester and corrinoids in the cell extract were determined using the millimolar absorbancy 12.1 at 591 nm [15] and 10.113 at 580 nm [16], respectively.

2.3. *Preparation of cell extract*

The sedimented wet cells (~15 g) were washed once with the cell extraction buffer [0.1 M Tricine buffer (pH 7.9) containing 25 mM $MgCl_2$, 5 mM dithiothreitol and 0.3 M glycerol] and kept at −20°C overnight. The freshly thawed cells were resuspended in 20 ml of the same buffer and disrupted by sonication with a Heat System-Ultrasonics model W200R for 10 min at 5–10°C. The supernatant fluid of the centrifuged suspension (24000 × *g*, 20 min at 2°C) was applied to a 2.6 × 12 cm column of Sephadex G-25 (Pharmacia). The column was equilibrated and eluted with the buffer described above. The protein fraction was dialyzed against the same buffer for 6 h. Protein concentration of the cell extract was determined using bovine serum albumin as standard [17].

2.4. *Assay for C_5-pathway activity [18,19]*

A flask containing the above reaction mixture (0.8 ml) and the additives shown in table 1 was successively evacuated and flushed with nitrogen several times, and maintained at 55°C. The reaction was terminated by addition of 40 μl of 70% perchloric acid.

The sediment was separated by centrifugation at 3000 × *g* and washed with 0.5 ml water. The combined supernatant fluid was loaded onto a Dowex 50W-X4, 200–400 mesh (Sigma) cation-exchange column (10 × 10 mm) twice prewashed with 2 ml of 1 N NaOH, then 3 ml Na citrate buffer (pH 3.1, 0.2 M Na^+). After loading, the column was washed again with 2 ml of the pH 3.1 Na citrate buffer, then with 1 ml water. The ALA in the column was eluted with 5 ml of 0.5 M NaOH adjusted to pH 6.8 with H_3PO_4. ALA in the eluate was converted into ALA-pyrrole and determined by measuring the absorbance at 552 nm, after addition of modified Ehrlich reagent [20]. The visible absorption spectrum of the Ehrlich reaction product was identical with that formed from authentic ALA.

2.5. *Chemicals*

ALA, ATP, NADPH, RNase type XII A, dithiothreitol, levulinic acid and pyridoxal phosphate were products of Sigma, St. Louis. [2-^{13}C]Glycine (90 atom%) was purchased from Merck, Sharp & Dohme Canada, Montreal; [2-^{14}C]glycine (52 mCi/mmol) was from Amersham International, England.

3. RESULTS

3.1. *^{13}C-NMR spectrum of cobester*

The corrin ring is built up from eight molecules of ALA. If [1-^{13}C]glutamate or [2-^{13}C]glycine is incorporated into the corrin ring via [5-^{13}C]ALA, the seven carbon atoms of cobester numbered in fig.1 should be enriched with ^{13}C [21,22] (see fig.2A).

Growing *Clostridium* cells were allowed to metabolize 1 mM [2-^{13}C]/[2-^{14}C]glycine (see section 2) for 42 h. During incubation, 40% of the added tracer was assimilated into the cells, and 2.8 μmol corrinoids were synthesized in the cells. As seen in fig.2B no enrichment occurred in the seven carbons derived from C-5 of ALA, showing that ALA synthase is inoperative. Resonances of seven peripheral methyl carbons derived from *S*-adenosylmethionine (see fig.1) were not enriched by C-2 of glycine (fig.2B). In the case of biosynthesis of chlorophylls incorporation of C-2 of glycine into the methyl carbons derived from methionine has been widely observed from angiosperms to photosynthetic bacteria with the exception of *Prosthecochloris* [4,5]. The result therefore suggests that the C_5 pathway is functioning in this bacterium. A similar experiment with glutamate was technically impossible, since glutamate was not taken up by the cells.

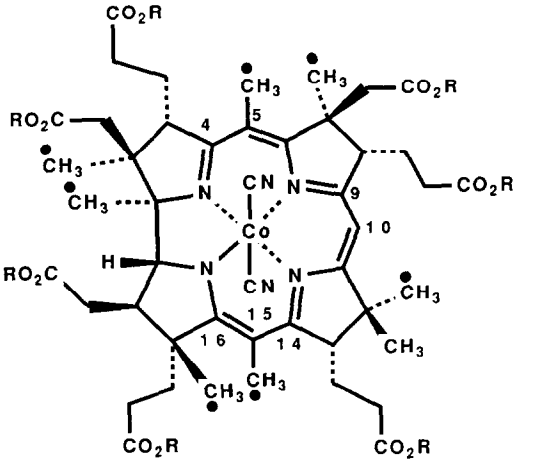

Fig.1. Structure and numbering system of heptamethyl dicyanocobrinate (cobester). R = CH_3, C-4, 5, 9, 10, 14, 15, and 16 are derived from C-5 of ALA [21] and the seven methyl groups marked with solid circles are derived from *S*-adenosylmethionine [22].

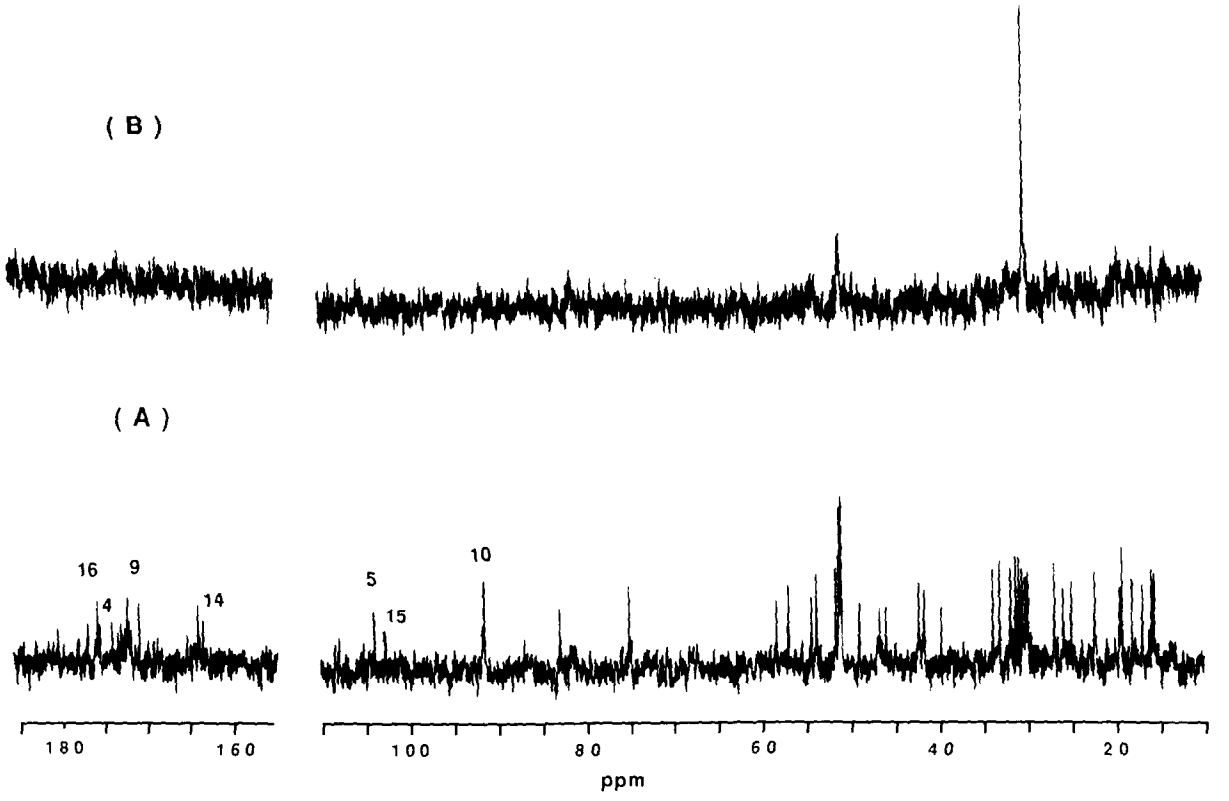

Fig.2. ^{13}C-NMR spectra (125.7 MHz, Bruker AM-500) of cobester from *C. thermoaceticum* grown (A) without any carbon enrichment; (B) with 90% enriched [2-^{13}C]glycine. (•) Methyl carbons derived from *S*-adenosylmethionine. Measurement conditions: pulse width, 6.5 μs; repetition time, 1.5 s; acquisition time, 0.623 s. Samples dissolved in benzene-d_6 (ICN Biomedicals) were contained in 5-mm tubes. (A) 5 mM, (B) 0.5 mM. (A) 27972 pulses, (B) 110000 pulses.

3.2. *ALA-forming activity*

The enzyme reaction of ALA synthesis from glutamate via the C_5 pathway has been studied in higher plants [23], algae [18,19] and an archaebacterium [6]. The reaction requires glutamate as substrate and ATP, Mg^{2+} and NADPH. RNA participates in the reaction sequence. The enzyme system was inactivated by RNase [6,24–26].

Incubation of the cell-free extract of *C. thermoaceticum* with glutamate, ATP, Mg^{2+}, NADPH and levulinic acid (a competitive inhibitor of ALA dehydratase) under nitrogen resulted in the formation of ALA. Elimination of any of the components described above resulted in almost complete loss of the activity with the cell extract which had been prepared by gel filtration and dialysis (table 1). RNase dramatically decreased the activity, the degree of inhibition varying with

Table 1

Substrate and cofactor requirements for ALA formation in cell extract of *C. thermoaceticum*

	ALA formed per assay	
	nmol	%
Complete	19	100
– ATP	0.8	4.2
– Glutamate	0.4	2.1
– NADPH	0.0	0.0
– Levulinic acid	1.5	7.9

The reaction mixture (1.0 ml) contained 3 mM glutamate, 5 mM ATP, 1 mM NADPH, 20 μM pyridoxal phosphate, 5 mM sodium levulinate and 0.8 ml cell extract containing 0.1 Tricine buffer (pH 7.9), 25 mM $MgCl_2$, 5 mM dithiothreitol, 0.3 M glycerol and 14 mg protein per ml, and incubated for 90 min under nitrogen at 55°C. Experimental variation was within 10% of the values shown

Table 2

Effect of RNase on ALA formation in cell extract of *C. thermoaceticum*

RNase added (ng/ml)	ALA formed per assay	
	nmol	%
0	14	100
10	4	29
100	0	0

Conditions for assaying ALA formation were the same as those corresponding to the first row in table 1, except that the cell extract used contained 17 mg protein per ml, RNase was added, and the mixture was preincubated for 15 min at 37°C. Experimental variation was within 10% of the values shown

the amount of RNase added (table 2). These results indicate that in *C. thermoaceticum*, ALA is formed by the C_5 pathway by a mechanism similar to that in chloroplasts in higher plants, algae and an archaebacterium.

4. DISCUSSION

There are groups of prokaryotes in which the 2-oxoglutarate-oxidizing enzyme system is lacking in the citric acid cycle [27]. In these bacteria the route which could supply succinate and succinyl-CoA has been sought for the formation of ALA and porphyrins. However, there is increasing evidence to show that the C_5 pathway of ALA synthesis is operating in such bacteria. Therefore, the source of glutamate synthesis rather than succinate should be sought. Bacteria in which the C_5 pathway is operating can be divided into two groups according to the pathway used for glutamate synthesis. Glutamate synthesis via the citrate pathway (oxaloacetate + acetyl-CoA $\longrightarrow$ citrate $\longrightarrow$ isocitrate $\longrightarrow$ oxoglutarate $\longrightarrow$ glutamate) has been shown in *C. thermoaceticum* [28], *Chromatium vinosum* [3] and a cyanobacterium, *Anacystis nidulans* [29]. A second pathway is a segment of the reductive tricarboxylic acid pathway (oxaloacetate $\longrightarrow$ malate $\longrightarrow$ fumarate $\longrightarrow$ succinate + CO_2 $\longrightarrow$ oxoglutarate $\longrightarrow$ glutamate). This pathway operates in green sulfur bacteria [4] and *Methanobacterium thermoautotrophicum* [30].

We previously proposed that the C_5 pathway is the ancestral form of ALA biosynthesis [31] while the ALA synthase pathway most probably evolved later in aerobic bacteria which developed during oxygenation of the Earth's atmosphere. The present demonstration of the C_5 pathway in *Clostridium* serves to reinforce this proposal, since clostridia are placed at the base of most phylogenic trees (e.g. [32]). According to this view, chloroplasts in plants must be considered to have retained the ancestral pathway of ALA biosynthesis, which can be explained by the endosymbiont hypothesis [33] that plastids share a common ancestry with cyanobacteria, thus lending further support to this hypothesis [34].

Acknowledgements: We thank the National Institutes of Health and Robert A. Welch Foundation for generous support of this work.

REFERENCES

[1] Granick, S. and Bealc, S.I. (1978) Adv. Enzymol. 46, 33–203.
[2] Castelfranco, P.A. and Beale, S.I. (1983) Annu. Rev. Plant Physiol. 34, 241–278.
[3] Oh-hama, T., Seto, H. and Miyachi, S. (1986) Arch. Biochem. Biophys. 246, 192–198.
[4] Oh-hama, T., Seto, H. and Miyachi, S. (1986) Eur. J. Biochem. 159, 189–194.
[5] Smith, K.M. and Huster, M.S. (1987) J. Chem. Soc. Chem. Commun., 14–16.
[6] Friedman, H.C. and Thauer, R.K. (1986) FEBS Lett. 207, 84–88.
[7] Friedman, H.C. and Cagen, L.M. (1970) Annu. Rev. Microbiol. 24, 159–208.
[8] Scott, A.I. (1979) in: Vitamin B_{12}: Proceedings 3rd European Symposium in Vitamin B_{12} and Intrinsic Factor (Zagalak, B. and Friederick, W. eds) pp.247–277, De Gruyter, Berlin.
[9] Menon, I.A. and Shemin, D. (1967) Arch. Biochem. Biophys. 121, 304–310.
[10] Ljungdahl, L.G. and Wood, H.G. (1982) in: B_{12} (Dolphin, D. ed.) vol.2, pp.165–202, Wiley, New York.
[11] Ljungdahl, L.G. and Andreesen, J.R. (1978) Methods Enzymol. 53, 360–372.
[12] Battersby, A.R., McDonald, E., Hollenstein, R., Ihara, M., Satoh, F. and Williams, D.C. (1977) J. Chem. Soc. Perkin Trans. 1, 166–178.
[13] Omata, T. and Murata, N. (1980) Photochem. Photobiol. 31, 183–185.
[14] Battersby, A.R., Edington, C., Fookes, C.J.R. and Hook, J.M. (1982) J. Chem. Soc. Perkin Trans. 1, 2265–2277.
[15] Santander, P.J. (1987) Dissertation, Texas A&M University.
[16] Ljungdahl, L.G., LeGall, J. and Lee, J.-P. (1973) Biochemistry 12, 1802–1808.

[17] Lowry, O.H., Roseborough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[18] Wang, W.-Y., Huang, D.-D., Stachon, D., Gough, S.P. and Kannangara, C.G. (1984) Plant Physiol. 74, 569–575.
[19] Weinstein, J.D. and Beale, S.I. (1985) Arch. Biochem. Biophys. 237, 454–464.
[20] Urata, G. and Granick, S. (1963) J. Biol. Chem. 238, 811–820.
[21] Scott, A.I., Townsend, C.A., Okada, I., Kajiwara, M., Cushley, R.J. and Whitman, P.J. (1974) J. Am. Chem. Soc. 96, 8069–8080.
[22] Scott, A.I. (1978) Acc. Chem. Res. 11, 29–36.
[23] Wang, W.-Y., Gough, S.P. and Kannangara, C.G. (1981) Carlsberg Res. Commun. 46, 243–257.
[24] Weinstein, J.D. and Beale, S.I. (1985) Arch. Biochem. Biophys. 239, 87–93.
[25] Huang, D.-D., Wang, W.-Y., Gough, S.P. and Kannangara, C.G. (1984) Science 225, 1482–1484.
[26] Schon, A., Krupp, G., Gough, S., Berry-Lowe, S., Kannangara, C.G. and Soll, D. (1986) Nature 322, 281–284.
[27] Smith, A.J. and Hoare, D.S. (1977) Bacteriol. Rev. 41, 419–448.
[28] Stern, J.R. and Bambers, G. (1966) Biochemistry 5, 1113–1118.
[29] Laycock, M.L. and Wright, J.L.C. (1981) Phytochemistry 20, 1265–1268.
[30] Zeikus, J.G. (1983) Adv. Microb. Physiol. 24, 215–299.
[31] Oh-hama, T., Seto, H. and Miyachi, S. (1987) in: Progress in Photosynthesis Research (Biggins, J. ed.) vol.4, pp.445–448, Martinus Nijhoff, Dordrecht.
[32] Schwartz, R.M. and Dayhoff, M.O. (1978) Science 199, 395–403.
[33] Sagan, L. (1967) J. Theor. Biol. 14, 225–274.
[34] Gray, M.W. and Doolittle, W.F. (1982) Microbiol. Rev. 46, 1–42.