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Bacteriochlorophyll *c* formation via the C_5 pathway of 5-aminolevulinic acid synthesis in *Chloroflexus aurantiacus*

Tamiko Oh-hama, Patricio J. Santander, Neal J. Stolowich and A. Ian Scott

Center for Biological NMR, Department of Chemistry, Texas A&M University, College Station, TX 77843-3225, USA

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Biosynthesis of 5-aminolevulinic acid (ALA) in *Chloroflexus aurantiacus*, a thermophilic bacterium forming bacteriochlorophyll *c*, is shown to proceed via the C_5 pathway by demonstrating (1) the specific labeling of its chlorin ring with [1-^{13}C]glutamate and (2) the enzyme activity to produce ALA from glutamate in a cell-free extract. From the phylogenetic distribution it is suggested that ALA synthase distributed in some aerobic eubacteria could be monophyletic in origin.

5-Aminolevulinic acid synthesis; C_5 pathway; Bacteriochlorophyll *c*; ^{13}C NMR; *Chloroflexus aurantiacus*

1. INTRODUCTION

The thermophilic green non-sulfur bacterium, *Chloroflexus aurantiacus*, resembles green sulfur bacteria in possessing bacteriochlorphyl *a* and *c* [1] and purple non-sulfur bacteria in its aerobic and anaerobic light/dark metabolism [2,3]. Unlike purple non-sulfur bacteria, however, the Calvin cycle is not used to fix CO_2 into cell material under autrophic growth conditions [4]. According to Woese's classification based on 16 S ribosome RNA sequence studies, this group represents an extremely deep branching from the common line of eubacterial descent [5].

Two pathways of ALA synthesis are known for biosynthesis of tetrapyrrole compounds; viz. ALA synthase (EC 2.3.1.37) which catalyzes condensation of glycine and succinyl-CoA to form ALA, and the C_5 pathway in which the intact carbon skeleton of glutamate is converted by a multienzyme pathway into ALA [6]. The former was first demonstrated in purple non-sulfur bacteria [7] and the latter in higher plants [8] and later in green sulfur bacteria [9,10]. It would be of interest to establish the ALA synthesis pathway in *Chloroflexus*, because of its unique phylogenetic position and metabolism. Here, we report the exclusive operation of the C_5 pathway in *Chloroflexus* [11].

Correspondence address: T. Oh-hama, Center for Biological NMR, Department of Chemistry, Texas A&M University, College Station, Texas 77843-3225, USA

Abbreviations: ALA, 5-aminolevulinic acid; tricine, *N*-tris-(hydroxymethyl)-methylglycine; NMR, nuclear magnetic resonance; HPLC, high pressure liquid chromatography

2. MATERIALS AND METHODS

2.1. *Culture of* Chloroflexus *cells*

Chloroflexus aurantiacus, strains J-10-fl (ATCC 29366) was maintained in cotton-plugged Roux culture bottles filled to the neck with medium D, supplemented with 0.02% NH_4Cl, 0.05% yeast extract and 0.05% glycylglycine (pH 8.2) [3] under exposure to a 60 W tungsten lamp at 46-47°C. To obtain cells forming bacteriochlorophyll *c* at high levels, the reddish yellow cells, which had been grown as described above, were transferred to the above defined medium further enriched with 0.3% casein hydrolyzate. The culture was kept anaerobic by constant bubbling with N_2 containing 2% CO_2 and stirred with magnetic stirrer under illumination with a 150 W photo-flood lamp at 55°C for 2-4 days.

Labelling of bacteriochlorophyll *c* with [2-^{13}C]glycine or [1-^{13}C]glutamate was performed as previously described [9]: the cells were grown anaerobically as described above in medium (900 ml) containing either 1.0 mM [2-^{13}C]glycine or 0.5 mM [1-^{13}C]glutamate. During a 4 day culture, about 3.5 μmol of bacteriochlorophyll *c* were formed in each bacterial suspension, corresponding to a 15-fold increase of pigment in culture.

2.2. *Isolation of methyl bacteriopheophorbide* c

The isolation of the bacteriochlorophyll *c* and the transformation into the corresponding methyl bacteriopheophorbide (Fig. 1), as well as the chromatographic purification of these compounds with Sepharose CL-6B were carried out as previously described [9]. The sample prepared with [^{13}C]glutamate was further purified by HPLC on an Alltech C-18 column (10 × 250 mm) eluting with methanol/H_2O (87:13) at 3 ml min^{-1}. The bacteriopheophorbide was detected at 378 nm with a retention time of 50.2 min.

2.3. *Assay for* C_5 *pathway activity*

The 2 day anaerobically cultured cells (~2 g wet cells) were washed once with the cell extraction buffer (0.1 M Tricine buffer (pH 7.9) containing 20 mM $MgCl_2$, 1 mM dithiothreitol, 0.3 M glycerol and 200 μM pyridoxal phosphate) and disrupted in the same buffer by sonication with a Heat System-Ultrasonics model W200R for 5-7 min at 5-10°C. The disrupted cells were centrifuged (24000 × g, for 20 min at 2°C) and the supernatant subjected to gel filtration on Sephadex G-25 using the extraction buffer described above. Protein concentration of the cell extract was determined using bovine serum albumin as

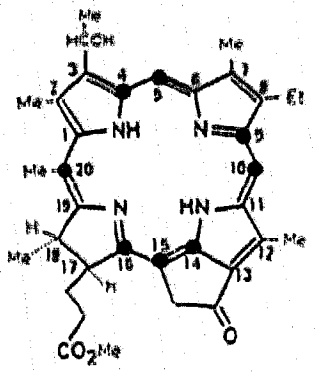

Fig. 1. Structure and numbering system of bacteriopheophorbide *c* methylester obtained from *Chloroflexus aurantiacus*. Carbon atoms derived from C-5 of ALA are marked with solid circles.

standard [12]. A test tube containing the above cell extract (0.7 ml) and the additives shown in Table I was maintained at 45°C. After termination, ALA produced was assayed as described previously [13].

2.4. *Chemicals*

L-[1-^{13}C]glutamic acid (99 atom% ^{13}C) and [2-^{13}C]glycine (90 atom% ^{13}C) were obtained from MSD Isotopes, Montreal. ALA, ATP, NADPH, dithiothreitol, levulinic acid, pyridoxal phosphate, Sephadex G-25 and Sepharose CL-6B were products of Sigma, St. Louis.

3. RESULTS

3.1. ^{13}C-NMR spectrum of bacteriopheophorbide c

The chlorin ring is derived from 8 molecules of ALA (Fig. 1). Incorporation of either [1-^{13}C]glutamate or [2-^{13}C]glycine into the tetrapyrrole via [5-^{13}C]ALA should result in the enrichment of four *meso*-carbons (C-5, C-10, C-15 and C-20) and 4 α-pyrrolic carbons (C-4, C-9, C-14 and C-16) with ^{13}C. Tentative assignments of these 8 carbons as well as 3 downfield carbon resonances in the natural abundance ^{13}C-NMR spectrum of 12-methylbacteriopheophorbide *c* methyl ester obtained from *Chloroflexus* (Fig. 2A) were based on direct comparison with the spectrum of the 12-ethyl homologue from *Prosthecochloris* [9], which, with the notable exception of C-12 and C-13, display nearly equivalent chemical shifts. A similar upfield shift of

Table I

Substrate and cofactor requirements for ALA formation in cell extracts of *C. aurantiacus*

	nmol of ALA formed	% of Control
Complete	3.2	100
- ATP	0.0	0
- NADPH	0.3	10
Glutamic acid	0.6	20

The complete reaction mixture (1.0 ml) contained 5 mM glutamate, 5 mM ATP, 1 mM NADPH, 5 mM sodium levulinate (a competitive inhibitor of ALA dehydratase) and 0.7 ml cell extract containing 2.3 mg protein per ml, incubated for 20 min at 45°C.

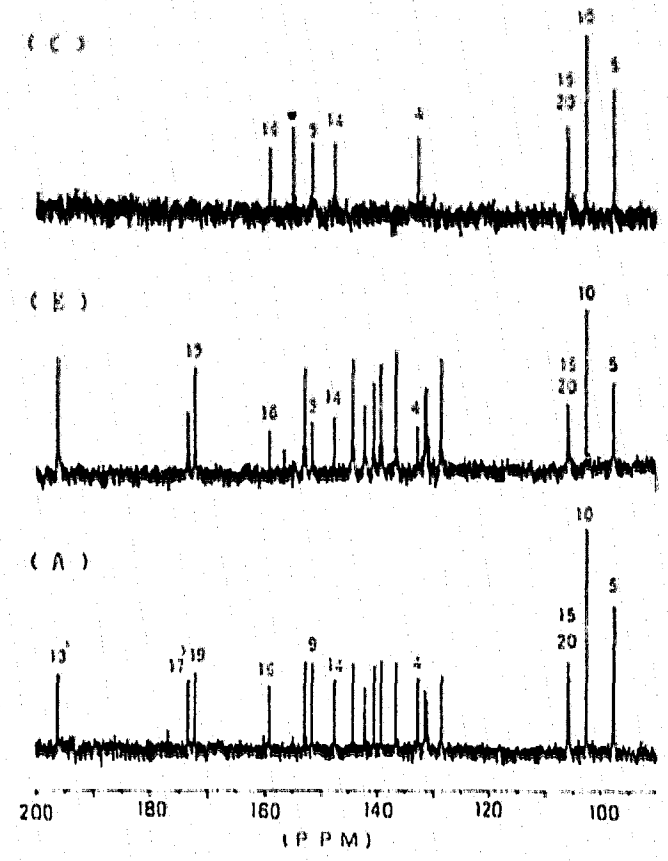

Fig. 2. ^{13}C-NMR spectra (125.7 MHz, Bruker AM500) of methyl bacteriopheophorbide *c* from *C. aurantiacus* grown (A) without any carbon enrichment, (B) with 90% enriched [2-^{13}C]glycine, and (C) with 99% enriched [1-^{13}C]glutamate. Samples dissolved in $CDCl_3$ were contained in 5 mm tubes. (A) 5.9 mM, (B) 3.6 mM, and (C) 0.4 mM; 25000 pulses each. A resonance marked with ● in (C) does not arise from bacteriopheophorbide *c*. Note that spectrum (C) was obtained at a concentration of ~10% of those used for spectra (A) and (B). Measurement conditions: pulse width, 5.0 μs; repetition time, 1.5 s; acquisition time, 0.623 s; line broadening, 3.0 Hz.

C-12 upon replacement of ethyl by methyl at C-12 has been previously demonstrated in methyl bacteriopheophorbide *d* [14].

Direct confirmation of these assignments, as well as their biosynthetic origin, were subsequently determined via ^{13}C isotopic labeling experiments. Growth of *Chloroflexus* on [2-^{13}C]glycine resulted in no enrichment in the ^{13}C NMR spectrum of bacteriopheophorbide *c* (Fig. 2B). Incubation of *Chloroflexus* in the presence of [1-^{13}C]glutamate, however, resulted in uniform enrichment of 8 carbons of bacteriopheophorbide *c* (Fig. 2C) predicted to be derived from C-5 ALA, thereby revealing the exclusive operation of the C_5 pathway for bacteriochlorophyll formation in *Chloroflexus* cells. Additionally, while the C-5 ALA-derived labeling pattern shown in Fig. 1 predicts that the enriched signals should be observed as *J*-coupled doublets, the limited enrichment (~10%) lowers the

probability of two adjacent ^{13}C-enriched carbons in the same molecule. On closer inspection *J*-coupled satellites are visible near the baseline of most of the enriched carbon signals in Fig. 2C.

3.2. *ALA-forming activity*

The enzyme reaction of ALA synthesis from glutamate via the C_5 pathway has been studied in higher plants, algae, photosynthetic and non-photosynthetic eubacteria, and an archaebacterium [6,15]. The reaction requires glutamate as substrate and ATP, Mg^{2+} and NADPH. Incubation of the cell-free extract of *C. aurantiacus* with these cofactors supported the formation of ALA (Table I). Elimination of any of the above components resulted in great loss of activity. These results provide evidence that in *C. aurantiacus*, ALA is formed by the C_5 pathway by a mechanism similar to that previously described [6,15].

4. DISCUSSION

Two pathways for the biosynthesis of ALA in nature are known; the Shemin pathway, which utilizes succinyl-CoA and glycine, and the C_5 pathway which utilizes glutamate and has been shown to require ATP, Mg^{2+}, and NADPH. In this study, ALA formation in cell free extracts from *Chloroflexus aurantiacus* was shown to require glutamate, ATP, and NADPH. Direct verification of in vivo operation of the C_5 pathway was further demonstrated by the incorporation of [1-^{13}C]glutamate, but not [2-^{13}C]glycine, into C5-ALA-derived carbon positions of 12-methyl-bacteriopheophorbide *c* isolated from *C. aurantiacus* cultures. Additional evidence for the exclusive operation of the C_5 pathway in *Chloroflexus* has been recently reported including gabaculine sensitivity [16] and conversion of [3,4-$^3H_2$]glutamate into ALA [17].

We previously proposed that the C_5 pathway is the ancestral form of ALA biosynthesis and occurs in the prokaryotes which do not have a complete citric acid cycle, i.e. lacking 2-oxoglutarate dehydrogenase, while the ALA synthase pathway evolved later in the aerobic bacteria which developed as the oxygen levels in the earth's atmosphere rose [13]. Weitzman [18] has hypothesized that the final stage in the evolution of the citric acid cycle in early anaerobic prokaryotes is the evolution of a lipoate-containing oxoglutarate dehydrogenase complex, thus filling the gap between oxoglutarate and succinyl-CoA. The demonstration of the operation of the C_5 pathway in *Chloroflexus* therefore serves to add strength to the previous proposal, since *Chloroflexus* represents an ancient phenotype [5] and lacks the lipoate mediated enzymes [19].

However it must be noted that *Escherichia coli* and *Bacillus subtilis*, both of which have the C_5-pathway of ALA synthesis [22,23], also contain the 2-oxoglutarate dehydrogenase complex [20,21]. We have previously proposed [13,24] that the Shemin pathway has been evolved toghether with the enzymes in the citric acid cycle, especially 2-oxoglutarate dehydrogenase, under an aerobic environment. During the anaerobic growth of this facultatively anaerobic organism, the citric acid cycle enzymes are all repressed to some extent; the 2-oxoglutarate dehydrogenase complex is almost completely repressed [18,21]. It would be interesting that ALA synthase and the dehydrogenase activity were suppressed together in anaerobically grown cells having dual pathways of ALA synthesis. A similar situation has occurred in another γ-purple bacterium, *Chromatium vinosum*, in which there is a preliminary report of ALA synthase activity [27]. Although 2-oxoglutarate activity has been demonstrated in this organism [25] the enzyme seems, by in vivo tracer experiments in acetate medium [24,26], to be suppressed in the organism, in which bacteriochlorophyll was formed by the C_5-pathway [24].

In 11 groups of eubacteria and archaebacteria classified by Woese [5], distribution of the Shemin pathway of ALA synthesis seems to be confined within the Gram-negative bacteria and actinomycete high G+C Gram-positive bacteria (cf. review [6]). The 2 groups are placed phylogenetically closer than the others [5]. An acticipation of the monophyletic origin of ALA synthase would be realized by comparison of the ALA gene sequence in actinomycetes with that in α-purple bacteria; the latter has been characterized in *Bradyrhizobium* [28].

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REFERENCES

[1] Gloe, A. and Rish, N. (1978) Arch. Microbiol. 118, 153–156.
[2] Sierevåg, R. and Castenholz, R. (1979) Arch. Microbiol. 120, 151–153.
[3] Pierson, B.K. and Castenholz, R. (1974) Arch Microbiol. 100, 283–305.
[4] Holo, H. (1989) Arch. Microbiol. 151, 252–256.
[5] Woese, C.R. (1987) Microbiol. Rev. 51, 221–271.
[6] Kannangara, C.G., Gough, S.P., Bruyant, P., Hoober, J.K., Kahn, A. and von Wettstein, D. (1988) Trends Biochem. Sci. 13, 139–143.
[7] Kikuchi, G., Kumar, A., Talmage, P. and Shemin, D. (1958) J. Biol. Chem. 233, 1214–1249.
[8] Beale, S.I. and Castelfranco, P.A. (1974) Plant Physiol. 53, 297–303.
[9] Oh-hama, T., Seto, H. and Miyachi, S. (1986) Eur. J. Biochem. 159, 189–194.
[10] Smith, K.M. and Huster, M.S. (1987) J. Chem. Soc. Chem. Commun. 14–16.
[11] Since the submission of this manuscript a similar report describing the operation of the C_5 pathway in *Chloroflexus* has been independently described by Swanson, K.L. and Smith, K.M. (1990) J. Chem. Soc. Chem. Comm. 1696–1697.
[12] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[13] Oh-hama, T., Stolowich, N.J. and Scott, A.I. (1988) FEBS Lett. 228, 89–93.

[14] Smith, K.M. and Goff, D.A. (1985) J. Chem. Soc. Perkin Trans. 1, 1099–1113.
[15] Rudiger, W. and Schoch, S. (1988) in: Plant Pigments, Goodwin, T.W. ed.) Academic Press London, pp. 1–59.
[16] Kern, M. and Klemme, J.-H (1989) Z. Naturforsch. 44c, 77–80.
[17] Avissar, Y.J., Ormerod, J.G. and Beale, S.I. (1989) Arch. Microbiol. 151, 513–519.
[18] Weitzman, P.D.J. (1985) in: Evolution of Prokaryotes (Schleifer, K.H. and Stackebrandt, E. eds.) Academic Press London, pp. 253–275.
[19] Holo, H. and Sierevåg, R. (1986) Arch. Microbiol. 145, 173–180.
[20] Weitzman, P.D.J. (1981) Adv. Microbiol. Physiol. 22, 185–244.
[21] Spencer, M.E. and Guest, J.R. (1987) Microbiol. Sci. 4, 164–168.
[22] Li, J.-M., Brathwaite, O., Cosloy, S.D. and Russel, C.S. (1989) J. Bacteriol. 171, 2547–2552.
[23] O'Neill, C.D., Chen, M.W. and Söll, D. (1989) FEMS Microbiol. Lett. 60, 255–260.
[24] Oh-hama, T., Seto, H. and Miyachi, S. (1986) Arch. Biochem. Biophys. 246, 192–198.
[25] Beatty, J.T. and Gest, H. (1981) Arch. Microbiol. 129, 335–340.
[26] Nicolay, K., van Gemerden, H., Hellingwerf, K.J., Konings, W.N. and Kaptein, R. (1983) J. Bacteriol 155, 634–642.
[27] Burnham, B.F. (1970) Methods Enzymol. 17, 195–200.
[28] McClung, C.R., Somerville, J.E., Guerinot, M.L. and Chelm, B.K. (1987) Gene 54, 133–139.