Biosynthesis of the Envelope Glycolipids during Heterocyst Development in *Anabaena cylindrica*

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Incorporation of sodium [1-¹⁴C]acetate into the heterocyst-specific glycolipids and glycerolipids of the cyanobacterium *Anabaena cylindrica* during induced heterocyst differentiation indicated that (1) the activity of key enzymes involved specifically in the biosynthesis of the very long chain polyhydroxyalkanes and hydroxyfatty acid moieties of the heterocyst glycolipid fraction is stimulated during heterocyst differentiation, and (2) in mature heterocysts, formation of the characteristic very long chain heterocyst glycolipids essentially ceases.

INTRODUCTION

When grown in the absence of a combined nitrogen source, vegetative cells of *Anabaena cylindrica* undergo characteristic morphological changes, associated with changes in their physiology, to give rise to the characteristic pattern of heterocysts observed in the cyanobacterial filament (Haselkorn, 1978; Fay, 1980). Prominent amongst the morphological changes is the development of the refractile multi-layered heterocyst envelope, the innermost layer of which (the 'laminated' layer) is composed of a heterogeneous family of structurally related glycolipids. The semi-crystalline arrangement of these has been suggested to present a barrier to the ready diffusion of oxygen from the growth medium into the heterocyst, thus protecting the oxygen-sensitive nitrogenase system (Winkenbach *et al.*, 1972). Deposition of the laminated layer appears to be a necessary requirement for development of heterocysts capable of aerobic dinitrogen fixation, since mutants of *Anabaena variabilis* deficient in heterocyst envelope glycolipids have no or very little nitrogenase activity when assayed aerobically (Haury & Wolk, 1978).

The glycolipids produced are apparently unique to cyanobacteria capable of nitrogen fixation and are absent from cyanobacterial species incapable of nitrogen fixation and a diverse range of eukaryotic algae (Nichols & Wood, 1968). In species such as *A. cylindrica* they are produced only when the organism is grown in a medium lacking an assimilable fixed nitrogen source, and are concentrated, if not located exclusively, in the heterocyst (Walsby & Nichols, 1969). The major components of this heterocyst glycolipid (HG) fraction are glycosides of C₂₆ and C₂₈ polyhydroxyalkanes, and glucose esters of C₂₆ and C₂₈ hydroxyfatty acids (Bryce *et al.*, 1972; Lambein & Wolk, 1973). The similarity in structure of these very long chain hydroxyfatty acids and polyhydroxyalkanes suggests a precursor-product relationship. The structure of these lipids is, however, in marked contrast to those of the polar glycerolipids of the heterocysts and vegetative cells in which monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), phosphatidylglycerol (PG) and sulphoquinovosyldiacylglycerol (SQDG) are the major lipids, containing principally C₁₆ and C₁₈ fatty acyl substituents (Winkenbach *et al.*, 1972; Zepke *et al.*, 1978).

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Abbreviations: DGDG, digalactosyldiacylglycerol; HG, heterocyst glycolipid; MGDG, monogalactosyldiacylglycerol; PG, phosphatidylglycerol; SQDG, sulphoquinovosyldiacylglycerol.

Since very long chain ($> C_{20}$) fatty acids and their derivatives are absent from the vegetative cells of *A. cylindrica*, biosynthesis of the aglycone moieties of the HG fraction must be induced during heterocyst differentiation. Heterocyst differentiation, induced by transfer of cells grown in medium containing combined nitrogen to a medium lacking a combined nitrogen source (Bradley & Carr, 1976) has been used to extend studies of the biosynthesis of the characteristic very long chain hydroxyfatty acids and polyhydroxyalkanes which constitute the aglycone moieties of the HG fraction and its relationship with glycerolipid fatty acid formation.

METHODS

Organism. *Anabaena cylindrica* 1430/2a was obtained from the Culture Centre of Algae and Protozoa, Cambridge, U.K.

Growth. Batch cultures were maintained in modified ASM-1 mineral salt medium, as described elsewhere (Abreu-Grobois *et al.*, 1977). Under aseptic conditions, after 40 h growth in this medium, batch cultures were supplemented with 5 M-NH₄Cl to give 5 mM-NH₄⁺ in the growth medium to suppress further heterocyst formation; these cultures are referred to as being grown in combined nitrogen medium. Batch cultures of 15 l were grown in 20 l vessels; for cultures grown in combined nitrogen medium, the vessel was modified to accept a pH electrode and the pH of the medium constantly monitored and maintained at pH 7.8 throughout growth by addition of 200 mM-KOH. Cultures were continuously aerated and agitated with compressed air [0.5 l air (l medium)⁻¹ min⁻¹] and grown under constant illumination (2500 lx) at 25 °C.

After 226 h growth in combined nitrogen medium, cells (2 l) were harvested by centrifugation at 4000 g for 10 min at 20 °C and transferred aseptically to nitrogen-free medium (2 l) and resuspended.

Total heterocyst frequency, chlorophyll content and nitrogen fixation assays. Total heterocyst frequency was determined by counting at $\times 400$ magnification under a light microscope. Chlorophyll content and nitrogen fixation assays were determined spectrophotometrically and by the GLC-acetylene reduction technique, respectively, as described previously (Krepski & Walton, 1979). All assays were carried out on cells harvested by centrifugation (as described above) from 200 ml growth medium.

Radioactive incubations. Cells from 200 ml growth medium were harvested by centrifugation as described above and resuspended in 2 ml of the appropriate growth medium containing 92.5 kBq sodium [1-¹⁴C]acetate (specific activity 2.1 TBq mol⁻¹) obtained from Amersham. The mixture was then incubated in a gyratory shaker (100 r.p.m.) at 25 °C under constant illumination (2500 lx) for 3 h. Incubations were terminated by addition of propan-2-ol (15 ml).

Extraction and analysis of lipids. Total lipid was extracted and examined by radio-TLC as described by Abreu-Grobois *et al.* (1977). HG fractions were isolated by preparative TLC and subjected to alkaline hydrolysis (2 M-methanolic KOH for 3 h at ambient temperature) and acid hydrolysis (refluxed with 2 M-H₂SO₄ in methanol/water (9:1, v/v) for 28 h). Fatty acid methyl esters from the HG ester fraction and the total lipid were prepared and analysed for radioactivity as described previously (Krepski & Walton, 1979) and the aglycone fraction of the glycoside HG components was analysed by radio-TLC (Abreu-Grobois *et al.*, 1977).

RESULTS AND DISCUSSION

Transfer of cells from a combined nitrogen medium to a nitrogen-free medium resulted in the expected increase in heterocyst frequency which was greatest for the first 30 h following transfer. During this period there was no significant increase in chlorophyll content of the culture (Fig. 1a), indicating that the observed increase in heterocyst frequency was due to induced differentiation of existing cells rather than new growth. The highest rate of nitrogenase activity was observed 55 h after transfer (Fig. 1b) and was coincident with the maximal heterocyst frequency (Fig. 1a). Increased labelling of the HG fraction was observed 6 h after transfer, at which time there was no significant increase in nitrogenase activity. Maximum synthesis of HG preceded the highest rates of nitrogen fixation by some 22 h and thereafter declined sharply, returning to levels comparable with that seen at transfer, while nitrogenase activity remained relatively high (Fig. 1b), supporting the proposal that presence of HG is an essential prerequisite for aerobic nitrogen fixation in physiologically mature heterocysts (Haury & Wolk, 1978).

The stimulatory effect on HG biosynthesis observed 6 h after transfer to nitrogen-free medium was accompanied by smaller increases in the level of labelling of the MGDG, SQDG and DGDG, and PG fractions; however, while HG labelling continued to increase until 28 h after transfer, labelling of the glycerolipids fell after 6 h and gradually returned to the levels observed at the time of transfer (Fig. 2). The labelling pattern and time course clearly indicate

Fig. 1. Effect of induced heterocyst differentiation on *A. cylindrica*. (a) Total heterocyst frequency in nitrogen-free medium (○) and combined nitrogen medium (●), and chlorophyll level in nitrogen-free medium (□) and combined nitrogen medium (■). (b) Nitrogenase activity in nitrogen-free medium (○) and combined nitrogen medium (●), and incorporation of sodium $[1\text{-}^{14}\text{C}]$ acetate into the heterocyst glycolipid fraction in nitrogen-free medium (□) and combined medium (■). The arrows indicate the times of transfer of cells from combined nitrogen medium to nitrogen-free medium.

Fig. 2. Incorporation of sodium $[1\text{-}^{14}\text{C}]$ acetate into the lipid fractions of *A. cylindrica* during induced heterocyst differentiation expressed as a percentage of that of a batch culture grown continuously in combined nitrogen medium. Radioactivity was measured as d.p.m. $(\text{mg chlorophyll})^{-1}$. ○, HG; ●, SQDG; □, MGDG; ■, DGDG plus PG.

Fig. 3. Incorporation of sodium [1-¹⁴C]acetate into heterocyst glycolipid fractions during induced heterocyst differentiation. (a) ○, Radioactivity in the HG glycoside fraction (expressed as a percentage of total HG radioactivity); ●, radioactivity in the HG glycoside aglycone fraction (expressed as a percentage of glycoside radioactivity). (b) ○, Radioactivity in the HG glycoside ester aglycone fraction (expressed as a percentage of total glycoside ester radioactivity); ●, radioactivity in the glycoside ester fatty acid fraction (expressed as a percentage of glycoside ester aglycone radioactivity).

that the increased labelling of the HG fraction was not due to a general stimulation of acyl lipid biosynthesis initiated by transfer, but was specifically associated with induced heterocyst differentiation.

The HG fraction has been shown in compositional and radiolabelling experiments to be a heterogeneous group of structurally related, very long chain polyhydroxyalkaneglycosides and hydroxyfatty acid glycoside esters (Lambein & Wolk, 1973; Abreu-Grobois *et al.*, 1977). During heterocyst differentiation induced by transfer to nitrogen free medium, incorporation of sodium [1-¹⁴C]acetate into the glycoside fraction of HG was maximal 6 h after transfer (85.5% of total HG radioactivity, Fig. 3a), returning during the following 136 h to the value observed at the time of transfer. Within the glycoside ester fraction the time course of radiolabelling of the fatty acyl and hydroxyfatty acyl residues, which constitute the aglycone moieties of the glycoside ester fraction, followed a similar pattern to that observed for the glycoside fraction of HG (Fig. 3b). At the time of transfer, 44% of the sodium [1-¹⁴C]acetate incorporated into the aglycone residues of the glycoside ester fraction was in the fatty acyl groups (Fig. 3b), the remainder being in hydroxyfatty acyl residues; the fatty acyl fraction contained no radioactive very long chain fatty acids and the distribution of label within the fatty acids (16:0, 82.8%; 16:1, 3.6%; 18:0, 5.2%; 18:1, 8.5%) was very similar throughout the time course of differentiation. Labelling of this fatty acyl fraction fell rapidly after transfer to nitrogen-free medium (Fig. 3b), indicating that biosynthesis of the very long chain hydroxyfatty acid moieties of the glycoside ester fraction is stimulated under these conditions.

The lipid moieties of the HG glycoside fraction contained 44% of the total glycoside radioactivity in the batch culture grown in the combined nitrogen medium at the time of transfer

Table 1. *Distribution of radioactivity amongst the aglycone moieties of the glycoside fractions of the heterocyst glycolipid after transfer from combined nitrogen medium to nitrogen-free medium*

Time after transfer to nitrogen-free medium (h)	Percentage distribution				
	Alkanetetraol	Alkanetriol	Alkanediol	Alkanol	'X'*
0	6.6	62.7	13.7	13.4	3.6
6	8.0	66.8	11.9	11.1	2.2
15	10.6	78.4	5.2	5.3	0.5
28	11.1	78.9	5.0	4.5	0.5
56	7.6	47.2	21.2	21.3	2.7
93	5.9	47.2	22.3	21.1	3.5
142	5.7	48.9	21.2	20.9	3.3

* Component 'X' was a minor component which chromatographed between the alkanol fraction and the solvent front on silicagel-G TLC.

(Fig. 3a). Following transfer to nitrogen-free medium, this proportion increased rapidly to reach 72% after 15 h and this level was then maintained throughout the time course of heterocyst differentiation. Thus, at the maximal rates of overall HG labelling (15–28 h, Fig. 1b) synthesis of the polyhydroxyalkane components was maximal. The labelling data indicates that transfer to nitrogen-free medium causes specific activation of the enzyme systems involved in the formation of the lipid moieties of the HG glycoside and glucose ester fractions.

Analysis of the distribution of radioactivity amongst the polyhydroxyalkane components of the HG glycoside fraction showed that during maximal synthesis of this fraction (15–28 h after transfer) radioactivity in 1,3,26,28-tetrahydroxyoctacosane (alkanetetraol) and 1,3,25-trihydroxyhexacosane (alkanetriol) accounted for 90% of the radioactivity in the lipid moieties (Table 1). From 56 h onwards, however, when overall labelling of HG was reduced (Fig. 1b), 40% of the aglycone radioactivity accumulated in fractions containing very long chain alkanols and alkanediols (Abreu-Grobois *et al.*, 1977; Mohy-ud-Dhin, 1980) as well as their long chain (C₁₆ and C₁₈) homologues (W. J. Krepski & T. J. Walton, unpublished observations), and there was a concomitant reduction in the labelling of the alkanetetraol and alkanetriol fractions. Thus, in the most active phase of HG biosynthesis, during the early phase of heterocyst differentiation, the characteristic very long chain alkanetriols, alkanetetraols and hydroxyfatty acids are major products, while after this phase of heterocyst differentiation, when the heterocyst is functionally mature as judged by maximal rates of nitrogen fixation, synthesis of the alkanol, alkanediol and fatty acid components of HG becomes significant.

The biosynthesis of the very long chain lipid moieties of the heterocyst glycolipid fraction is therefore under separate control mechanisms from those regulating glycerolipid fatty acid biosynthesis. These allow a sustained increase in generation of the lipid moieties of the HG fraction during an early phase of heterocyst differentiation in *A. cylindrica* and other cyanobacteria (Wahal *et al.*, 1974; Feige & Ritschel, 1978), which is characterized by formation of the C₂₆-triol and C₂₈-tetraol glycosides and the very long chain hydroxyfatty acid glucose esters. The regulatory mechanism also enables synthesis of HG to be shut down in filaments possessing physiologically mature heterocysts, and during this phase quantitatively minor very long and long chain HG components are produced. These results suggest that there is a minimum of two points at which biosynthesis of the lipid moieties of the HG glycoside fraction is regulated. In order to provide an adequate supply of very long chain precursors during deposition of the laminated layer, an enzyme system involved specifically in synthesis of very long chain acyl lipids, but separate from bulk fatty acid biosynthesis, e.g. a fatty acid elongase, must be stimulated during differentiation. In addition the observation that formation of polyhydroxyalkanes is reduced in filaments possessing mature heterocysts suggests that an aliphatic hydroxylation system is transiently activated during heterocyst differentiation.

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