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Observation of mobile poly(β-hydroxybutyrate) in the storage granules of *Methylobacterium* AM1 by in vivo ^{13}C-NMR spectroscopy

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^{13}C-NMR spectra of whole native cells of *Methylobacterium* AM1 show dominant signals belonging to poly(β-hydroxybutyrate) and trehalose. Fractionation of the cells demonstrates that the poly(β-hydroxybutyrate) is located in the storage granules and that the trehalose is located in the cytosol. The observation of relatively sharp poly(β-hydroxybutyrate) signals indicates that the polymer granule in this organism is in a mobile rather than truly solid state in vivo.

NMR; Polyhydroxybutyrate; Storage granule; Trehalose; (*Methylobacterium* AM1)

1. INTRODUCTION

Poly(β-hydroxybutyrate) (PHB) is an endogenous material serving as a reserve of carbon and energy in a range of bacteria [1]. It is a biodegradable thermoplastic which is being intensively studied as an alternative to the established petrochemical-based plastics [2]. Within bacterial cells, PHB occurs as granules which are observable in transmission electron micrographs of transverse sections of cells [3] and in electron micrographs of freeze-etched samples [4]. The generally accepted view seems to be that PHB within granules is solid [5,6] but there have been almost no studies on the state of the polymer in genuinely native granules; virtually all previous studies by X-ray diffraction, electron microscopy or CPMAS NMR spectroscopy have been on chemically isolated polymer, or preparations of dried granules or dried cells [7–10].

The facultative methylotroph *Methylobacterium* AM1 is known to produce PHB [11] and to contain granules [12]. We now present ^{13}C-NMR evidence that the bulk of the PHB in the live organism is in a remarkably mobile state. Our conclusion is based on the fact that the ^{13}C-NMR signals in solids are massively broadened and so do not yield a conventional 'high-resolution' spectrum [13]. Furthermore, we have separated particulate and cytosolic components of the bacterial cells and have shown that the PHB signals are associated solely with the particulate component, i.e. the granules.

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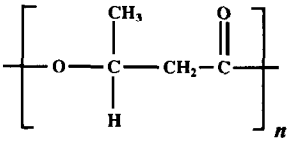
2. EXPERIMENTAL

All chemicals were AR grade. Lysozyme and DNase were obtained from Sigma; β-hydroxybutyric acid and 'authentic' PHB were obtained from Aldrich (Gillingham, Dorset, England).

Methylobacterium AM1 (NCIB 9133) was obtained as a freeze-dried sample from NCIMB (Torry Research Station, Aberdeen). Bacteria were resuspended in a small volume of medium and transferred directly to large flasks of a minimal medium (pH 7) containing (in g/l): Na_2HPO_4, 3; KH_2PO_4, 3; $(NH_4)_2SO_4$, 3; $MgSO_4 \cdot 7H_2O$, 0.2; and trace elements. 0.5% (v/v) methanol was the sole carbon and energy source. Cultures



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were incubated at 27°C in an orbital incubator at 100 rpm. Cells were collected by centrifugation at 4000 × *g* at 4°C for 15 min.

Granules and cytosol were prepared by the method of Lundgren et al. [14]. Cells (1.5 g wet wt) were resuspended in 0.05 M Tris-HCl (pH 8, 20 ml) with lysozyme (22 mg) and DNase (0.25 mg), and incubated at room temperature (24°C) with gentle stirring for 30 min. The suspension was then cooled in ice and subjected to ultrasonic treatment (20 kHz, 60 W average power) for 3 1-min periods while surrounded by ice. The lysate was layered on glycerol and spun at 9000 × *g* at 4°C for 15 min. A fixed-angle rotor was used and the small fraction of unbroken cells pelleted rapidly onto the side of the sample tube. The supernatant was removed and examined by NMR as described below. The PHB granules which collected on the surface of the glycerol were resuspended in 0.05 M Tris-HCl (pH 8) and dialysed for 24 h at 4°C against 0.02 M Tris-HCl (pH 8). The final granule preparation after dialysis was spun down using an Eppendorf centrifuge and resuspended in Tris-HCl (0.02 M) with 20% D_2O (pH 8) or 0.86% NaCl, 20 mM phosphate buffer (pH 7) with 20% D_2O.

100.6-MHz natural-abundance ^{13}C-NMR spectra were acquired on a Bruker 400 MHz spectrometer in 10-mm tubes. Spectra of whole cells of *Methylobacterium* AM1 were obtained from 2 g wet wt of cells resuspended in isotonic saline with 20% D_2O at pH 7. Cytosol spectra were from a sample consisting of 2.5 ml cytosol and 0.5 ml D_2O (pH 8). Granule spectra were from granules resuspended in 0.02 M Tris-HCl (pH 8) with 20% D_2O (pH 8), or 20 mM phosphate buffer (pH 7) with 0.86% NaCl, 20% D_2O. All spectra were acquired at 30°C using Waltz-16 broad-band proton decoupling, $\pi/2$ pulses, repetition time of 1.557 s and acquisition time of 0.557 s. A sweep width of 29411.8 Hz was used with 32K data points, giving a digital resolution of 1.8 Hz/point. Chemical shifts are quoted in ppm relative to TMS; shifts in aqueous media were referenced to methanol (50.2 ppm) or trehalose-C_1 (94.0) ppm, as appropriate.

3. RESULTS

Spectra of native *Methylobacterium* AM1 cells in suspension show signals due to trehalose and polysaccharide [15] together with four peaks that we assign to PHB (fig.1a). The PHB shifts agree with those we measured for commercial PHB in chloroform solution, but are not consistent with the monomer (table 1). The dimer or trimer would give more than four main resonances, while longer oligomers are water-insoluble [9]. It follows that the signals observed in vivo are from polymer in a non-aqueous environment; the assignments are listed in table 1.

We used a non-solvent extraction technique to separate PHB granules from cytosol, a method which is believed to produce native granules [14]. A spectrum of isolated granules is shown in fig.1b, while that of a portion of the cytosol is in fig.1c. PHB signals are now observed in the granule

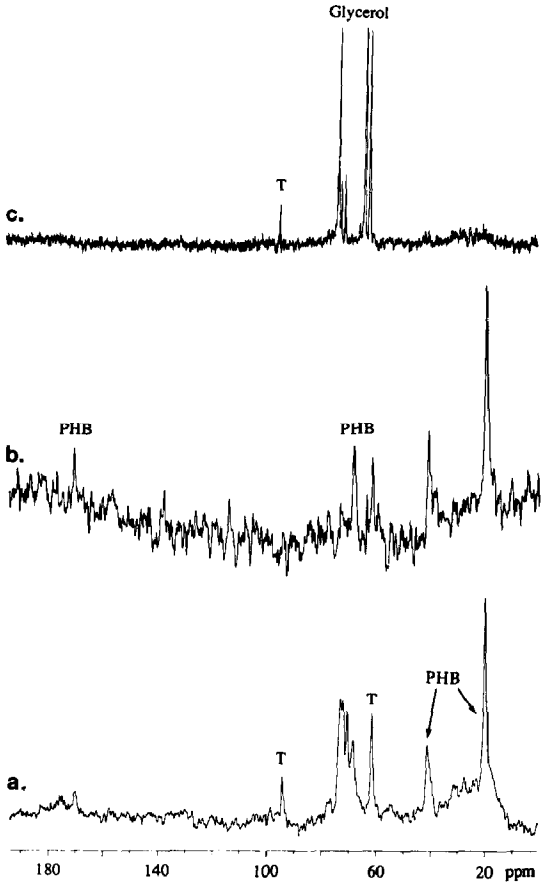

Fig.1. (A) Natural-abundance 100 MHz ^{13}C-NMR spectrum of intact cells of *Methylobacterium* AM1, 30°C, 1000 transients, 40 Hz exponential line broadening; (b) particulate fraction, resuspended in phosphate saline buffer, 5000 transients, 40 Hz exponential line broadening; (c) portion of cytosol diluted with Tris buffer, 5000 transients, 10 Hz exponential line broadening. T, trehalose.

preparation but not in the cytosol. There was no difference in the spectra of granules obtained in phosphate or Tris buffer. Trehalose is observed predominantly in the cytosol as expected. There is a signal in the granule preparation at 61.2 ppm which may be due to residual Tris buffer. The dominant signals in the cytosol spectrum are due to the glycerol and Tris used in the granule-cytosol preparation; glycerol was removed from the granule fraction by dialysis prior to spectroscopy.

The PHB signals have relatively large linewidths (30–70 Hz) as a result of rapid T_2 relaxation, and so are best observed in spectra which have considerable exponential line broadening applied. The cytosol spectrum, when identically line broadened,

Table 1

^{13}C chemical shifts for PHB and monomer[a]

Carbon	Monomer[b]	PHB	
		In $CDCl_3$	In vivo
CH_3	22.8	19.8	20.0
CH_2	47.5	40.8	41.1
CH	66.7	67.6	68.2
C=O	181.3	169.1	169.7

[a] In ppm relative to TMS
[b] In 20% D_2O, pH 7

shows no PHB resonances but the trehalose signals are lost in the base of broadened glycerol peaks. Fig. 1c shows a cytosol spectrum processed with less line broadening. Most of the trehalose resonances are clearly visible around 94 and 70–75 ppm; only the signal at about 73 ppm is obscured by the large glycerol signal. Spectra of cytosol which has been prepared from bacteria grown on ^{13}C-labelled methanol and concentrated by freeze-drying still show a complete absence of PHB signals; separation of trehalose and PHB components is effectively complete.

4. DISCUSSION

We have observed PHB in vivo. By the nature of the experiment, the corollary is that the PHB observed is relatively mobile. The fact that we readily observe natural-abundance signals with remarkably few transients demonstrates that we are detecting a high concentration. Furthermore, the signals are associated with the particulate fraction of the cells and are highly likely to be associated with the granules for which the method of isolation was designed. Using NMR to determine the PHB concentration observed in vivo in stationary-phase cells, we obtain a PHB concentration of 8% dry wt, in agreement with the chemical determination of total PHB in *Methylobacterium* AM1 by Peel and Quayle [11]. We are therefore observing the bulk of the PHB in these cells. We will report elsewhere the use of NMR to quantify PHB and follow its biosynthesis and consumption under batch-growth conditions.

The question arises as to the precise state of the PHB in this organism. T_1 values for the protonated carbons are in the range 300–600 ms, and only the methyl carbon has a substantial NOE; the T_2 values (5–20 ms) are consistent with the observed linewidths. Taken together, these measurements suggest that the polymer mobility is well outside the extreme-narrowing limit, but also that it is above its effective glass transition temperature [16]. Our results invite the question of whether the PHB in storage granules are generally mobile in vivo or whether *Methylobacterium* AM1 is atypical in this respect. This is unclear at present, but two broad PHB resonances have been observed in intact *Rhodopseudomonas sphaeroides* cells [17]. An investigation of PHB in the industrially important *Alcaligenes eutrophus* would be especially interesting.

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