The Poly-β-hydroxybutyrate Granule in Vivo

A NEW INSIGHT BASED ON NMR SPECTROSCOPY OF WHOLE CELLS

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High resolution 13C NMR spectroscopy of live cells has been used to show that poly-β-hydroxybutyrate (PHB) is predominantly in a mobile state within the storage granules of Alcaligenes eutrophus, Methylobacterium extorquens, and Methylobacterium AM1. Comparison of chemical and NMR analysis of PHB indicates that about 70% of the polymer in A. eutrophius gives sharp observable resonances. Temperature-dependent line widths and relaxation rates together with nuclear Overhauser effect measurements demonstrate that the observed material is effectively a mobile amorphous elastomer that is well above its glass transition temperature. The hydroxyvalerate-hydroxybutyrate copolymer produced by propionate-fed A. eutrophius has virtually the same mobility as the homopolymer. Evidence is presented indicating that water is an integral component of the PHB granule and that this component acts as a plasticizer for the polymer. These observations strongly suggest that the enzyme(s) responsible for PHB biosynthesis and consumption operate only on mobile hydrated material and that the solid granules characteristic of dried cells are partially artifactual. This model is supported by a reinterpretation of previously inexplicable biochemical results.

We present here a new insight into the physical state of poly-β-hydroxybutyrate (PHB,1,1) in vivo; this new understanding, which is based on NMR observations of PHB in live cells, appears to reconcile many of the long-standing puzzles and contradictions associated with the enzymology of this important and intriguing material. PHB is an endogenous biopolymer that is used as a carbon and energy reserve in a wide range of bacteria (1,2). It is also a biodegradable thermoplastic that has potential as a biotechnological alternative to the established plastics based on petrochemicals (1,3–5). However, there are many problems to be resolved en route to commercialization, not the least of which is an understanding of the enzymology of its biosynthesis and degradation.

PHB is found in cells as “granules” within the cytoplasm (6); it is biosynthesized by PHB synthase, which polymerizes the four-carbon monomer unit, and is degraded by a depolymerase (7,8). The isolated polymer is a solid, melting at around 180 °C, and the generally accepted view (3,5,9) based on physical studies of dried cells or dried granules (10–12) seems to be that it is also solid in vivo. This view has prevailed despite the fact that it leads to numerous difficulties in understanding the enzymology of PHB. The most obvious of these is the problem of how it is possible for the synthase and depolymerase to operate so efficiently on a close packed solid. In addition, isolated granules are notoriously labile; their ability to serve as substrates for added depolymerase is lost upon freezing and thawing, prolonged storage at 4 °C, or even close packing by centrifugation (8). This deactivation is always irreversible. Furthermore, some treatments reduce the extent of degradation by depolymerase without affecting the rate of degradation.

Many of these difficulties would vanish if PHB were normally in a mobile enzymically accessible state in vivo but were solidified irreversibly by the treatments mentioned above. Although this may seem unlikely at first sight, there have been virtually no physical studies on the state of the polymer in native granules or in vivo; however, native granules are sudanophilic (6), a property apparently not associated with solids (13).

We have previously used 13C NMR spectroscopy of whole cells to show that the bulk of PHB in Methylobacterium AM1 is mobile in vivo and is associated with the granules (14). The basis of this conclusion was that the PHB is readily visible by a “standard” NMR experiment despite the fact that solids do not give high resolution spectra under these conditions (15). Our results raised the question of whether this mobility is a general phenomenon or is restricted to Methylobacterium AM1. We have now extended our NMR studies to the industrially important organism Alcaligenes eutrophus and to the hydroxyvalerate copolymer, 2 (16). We show here that mobility does appear to be a general property of these polyhydroxalkanoates in vivo and that inactivation of the granule is indeed often accompanied by solidification.

The thrust of this work has been to try to understand the enzymology of the PHB granule rather than to provide a detailed description of the spin physics of the polymer. Consequently the quantitative results presented here should be regarded as preliminary and tentative.

MATERIALS AND METHODS

Methylobacterium AM1 (NCIB 9133), Methylobacterium extorquens (NCIB 9099), and Alcaligenes eutrophus A1H16 (NCIB 10442; ATCC 17699) were obtained as freeze-dried samples from the Na-

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The abbreviations used are: PHB, poly-β-hydroxybutyrate; NOE, nuclear Overhauser effect.
**NMR of Poly-β-hydroxybutyrate in Vivo**

General Properties of Spectra—The natural abundance $^{13}$C NMR spectrum of a suspension of *A. eutrophus* grown to stationary phase on a rich broth is shown in Fig. 1. This spectrum, which is the result of only 200 transients acquired at 30 °C, is dominated by four major signals that are readily assigned to PHB. The chemical shifts of these signals (Table I) are identical to those in the live cells and native granules of *Methylobacterium AM1* and *M. extorquens* and very similar to those of PHB in chloroform solution. The methylene resonance at 41.1 ppm is significantly shifted from its solid state position, 42.8 ppm (12), although the difficulties of comparing shifts in these very different systems should not be overlooked. As expected, PHB is not observed in cells that are harvested early in the growth cycle.

Inspection of the spectrum in Fig. 1 shows that the three main chain resonances are much less intense than the methyl signals due to a differential NOE. The NOEs for the main chain carbons are approximately 5% for the carbonyl carbon and 60% for the methylene and methane carbons; in contrast, the methyl carbon experiences a 100% NOE, close to the maximum 198%. Clearly (19) the observed signals arise from polymeric material rather than from small molecules, the Eppendorf centrifuge and resuspended with the required ratios of isotonic saline and acetone to give 0, 5, 25, and 100% acetone. The samples were left at room temperature (24 °C) for 5 min and then twice pelleted and resuspended in Tris buffer (2.8 ml). Following the second resuspension, 100 μl of D$_2$O was added to the suspension and the sample was examined by NMR. After NMR the granule suspensions were transferred to Soxhlet tubes and dried, and the samples were extracted with chloroform. The chloroform was dried down and the residue redissolved with 0.5 ml of deuterochloroform and examined by NMR. PHB and copolymer were extracted from whole cells in the same way. PHB content of dried cells was determined by the method of Braunegg et al. (18).

**RESULTS**

**Table I**

| Chemical shifts and assignments for PHB | Environment | CH$_2$ | CH$_3$ | CH | C=O |
|----------------------------------------|-------------|-------|--------|----|-----|
|                                       | ppm         |       |        |    |     |
| Methylobacterium AM1 in vivo           | 20.0        | 41.1  | 68.2   | 169.7 |
| *M. extorquens in vivo*                | 20.0        | 41.1  | 68.2   | 169.7 |
| *A. eutrophus in vivo*                 | 20.0        | 41.1  | 68.2   | 169.7 |
| Chloroform solution                    | 19.8        | 40.8  | 67.6   | 169.2 |
| Dried cells (CPMAS) (12)*              | 21.3        | 42.8  | 68.5   | 169.7 |
| Isolated solid polymer (12)            | 21.3        | 42.8  | 68.4   | 169.8 |

*Cross-polarization magic angle spinning.*
methyl side chain being free to rotate relatively independently.

The proportion of cellular PHB visible in the NMR spectrum is estimated to be about 70% by comparison of the content determined chemically (24 mg/100 mg of cells, dry weight), with that determined by NMR (16 mg/100 mg). The NMR assay was carried out by adding successive aliquots of known quantities of trehalose to the sample; following each addition a fully relaxed $^{13}$C spectrum was acquired without proton NOE, allowing reliable integrals of the trehalose C-1 carbon (94 ppm) and the PHB methyl carbon to be obtained. The trehalose signal intensity was proportional to the quantity added, ruling out any possible complications such as binding or metabolism of the added standard. By definition the PHB visible in these spectra is “mobile” on the NMR time scale rather than “solid.” We address below in qualitative terms the question of how mobile is “mobile.”

Similar results were obtained from cells that were grown to late exponential phase on a rich broth, then switched to a nitrogen-deficient medium containing acetate as the sole carbon source. The only difference is that the total polymer content of the cells as determined chemically was 38%; again, roughly two-thirds of the polymer was visible by NMR.

A. eutrophus grown on broth and then switched to a nitrogen-deficient medium containing propionate as the sole carbon source produces the $\beta$-hydroxybutyrate-$\beta$-hydroxyvalerate random copolymer, 2 (16), which is also readily observed in vivo by NMR. An example is shown in Fig. 2. All the major expected signals are resolved and easily assigned; as with PHB there is apparently a better match with the chemical shifts in solution than with those in the solid after allowing for an apparent referencing discrepancy of 1–2 ppm (Table II) (20). The small chemical shifts that result from dyad and triad sequence sensitivity are within the in vivo line width and are not resolved. Integration of a fully relaxed spectrum gives a butyrate:valerate composition of 44:56.

**Variable Temperature and Relaxation Experiments**—Having established the general features of the PHB resonances in A. eutrophus, Methylobacterium AM1, and M. extorquens, we wished to probe the polymer mobility in these organisms in more detail. We therefore measured spectra for all three species in the temperature range 5–30 °C, also acquiring as much information on relaxation times and line widths as was reasonably practical. Representative spectra are shown in Figs. 3 and 4, and the results are summarized in Tables III and IV.

When a suspension of Methylobacterium AM1 cells is cooled, the PHB signals broaden smoothly and disappear (Fig. 3). In the same sample the line widths of intracellular trehalose signals and external benzene remain unaffected. The line broadening is fully reversible, the PHB resonances being restored unchanged if the sample is warmed back to 30 °C within a relatively short time. Irreversible loss of signals does occur upon prolonged storage at low temperature; these experiments are described under “Inactivation Experiments.”

The PHB and copolymer signals in A. eutrophus behave similarly (Tables III and IV). Cooling the cells to 5 °C abolishes all the polymer signals, while warming them back to 30 °C fully restores the original spectrum. The PHB signals at intermediate temperatures show similar line widths and

![Diagram](image-url)
relative intensities in *A. eutrophus* and *Methylobacterium AM1*; the large experimental error that is inherent in this whole cell work precludes more accurate comparisons. Fig. 4 shows the methyl and methylene region of the copolymer spectrum in the temperature range 13–30 °C. There are several notable features in these spectra. First, it is clear that the apparent loss of intensity is due to a gradual broadening of the bulk signals rather than a shift in equilibrium between sharp, observable signals and broad, unobservable signals; second, it is clear that the main chain methylene resonances broaden at a higher temperature than those of the side chain; third, the behavior of the valerate and butyrate components is broadly similar, but the terminal carbon of the valerate side chain is the least susceptible to temperature. As expected for a polymer that is not in the extreme narrowing region (19, 21), cooling increases $T_1$ relaxation times for PHB in *A. eutrophus* (Table III); in the same sample cooling had the reverse effect on added external trehalose, a small molecule in the extreme narrowing region. The line widths and relaxation times of the copolymer (Table IV) are comparable with those of the homopolymer.

Inactivation Experiments—The results presented above and earlier (14) clearly demonstrated that the bulk of the PHB in *vivo* and in active granules is surprisingly mobile. It occurred to us that the ready and irreversible inactivation of isolated granules might be the result of polymer solidification. Accordingly, we isolated active granules from *Methylobacterium AM1* as before and subjected them to various standard types of inactivation treatment (7, 8). One preparation was stored for several weeks at 4 °C, and a set of samples was treated with various concentrations of aqueous acetone for 5 min. Representative results are shown in Fig. 5; for clarity only the PHB methyl resonance region is plotted, but the remainder of the spectrum behaves similarly. Treatment with 5% acetone has only a small effect, but the bulk of the PHB spectrum is abolished by 25% acetone. No PHB signals at all are visible after treatment with 100% acetone. Similarly, 4 °C storage slowly causes loss of signal, while freezing causes complete loss of signal overnight.

The loss of intensity is not due to loss of PHB. Dissolution of the granules with chloroform leads to recovery of all the missing PHB signal intensity. Therefore these treatments do indeed convert the PHB from a mobile, visible state to an invisible and presumably solid state.

The dramatic loss of PHB signals from the spectrum between 30 and 5 °C is not associated with a phase change. If it were, then we would expect the integrated intensity of the signals to decrease with temperature; in the narrow temperature range accessible for such measurements the integrals do not decrease. We might also expect to observe some hysteresis effects upon rapidly changing the temperature, but these are not seen. The dramatic change in appearance is merely a

![Fig. 4. Expansions of the 13C spectra of propionate-grown *A. eutrophus* cells as a function of temperature. Spectra were plotted with the 3.5-ppm methyl resonance at a constant height to emphasize differential line broadenings. Acquisition parameters were the same as for Fig. 2, but only 5-Hz line broadening was applied.](image-url)

![Fig. 5. Methyl region of *Methylobacterium AM1* granules.](image-url)

**TABLE III**

| Temperature (°C) | $\text{CH}_3$ | $\text{CH}_2$ | CH | C=O | Line width (Hz) | Relaxation time (s) |
|-----------------|------------|-------------|----|-----|----------------|-----------------|
| 30              | 30         | 60          | 50 | 50  |                |                 |
| 21              | 35         | 75          | 80 | 50  |                |                 |
| 16              | 60         | 300         |    |     |                |                 |
| 13              | 200        |             |    |     |                |                 |
| 21              | 0.57       | 0.24        | 0.39 | 3.23 |                |                 |

**TABLE IV**

| Butyrate units | V-Et | B-Me | V-Et | Line width (Hz) | Relaxation time (s) |
|----------------|------|------|------|----------------|-----------------|
| Butyrate units | 75   | 150-200 | 155 | 90  |                |                 |
| Valerate units | 70   | 120  | 150-200 | 145 | 90  |                |                 |
| Butyrate units | 0.53 | 0.40 | 0.74 | >1  |                |                 |
| Valerate units | 0.90 | 0.27 | 0.31 | 0.68 | >1  |                |                 |

*PHV, poly-3-hydroxyvalerate.*
for the result of where the polymer lies on the $\tau_r-T_1-T_2$ curves (19), where $\tau_r$ is rotational correlation time. The 50-Hz line width observed at 30°C corresponds to a $T_2$ of 6 ms (Vhear); if cooling increases $\tau_r$ by a factor of three, then $T_2$ decreases to 2 ms, and the line width increases to an almost unobservable 150 Hz. A similar shift of $T_2$ from 60 to 20 ms would increase line widths from 5 to 15 Hz, a virtually undetectable change in these whole cell systems.

Within the precision of these preliminary experiments PHB mobility is the same in all three bacterial species that we have investigated and appears to be the same at all stages of growth. The hydroxylated copolymer has a similar mobility to that of the homopolymer by our NMR criteria; however, there are indications from the line widths that the copolymer backbone is slightly less mobile than in the homopolymer, even though the latter has the higher $T_g$ (effective glass transition temperature). Precisely the same effects of ethyl versus methyl side chains are found in synthetic copolymers (22). The methyl and ethyl side chains are clearly less constrained motionally than the main chain carbons. This is apparent from the larger NOE, lack of temperature dependence in $T_1$, and lower temperature at which the signals disappear from the spectrum.

It is worth pointing out that gross tumbling of entire granules within the cytoplasm cannot generate the molecular mobility required by these spectra. The correlation time for granule tumbling is likely to be in the millisecond range or slower rather than in the necessary submicrosecond range.

**DISCUSSION**

**NMR Evidence**—It is clear from our results that the bulk of PHB in vivo is not solid; solids do not give high resolution NMR spectra under our acquisition conditions (19, 21). Equally important, the line widths and NOEs are inconsistent with the idea that the PHB is a mobile liquid or is in solution; certainly PHB dissolved in chloroform is much more mobile than we are observing in vivo (16). The NMR properties of PHB in vivo match precisely those of an amorphous elastomer that is well above its $T_g$; the small NOE for main chain carbons, long $T_1$, slower relaxation at lower temperatures, broad lines, and dramatic broadening over a small temperature range are well documented features of such elastomeric polymers (21).

$T_g$, the temperature at which the high resolution NMR spectrum appears, is always 30–90°C higher than the $T_g$ established by other physical methods (21). This is because the NMR experiment requires local molecular motion to be occurring on a time scale of $10^7$ s⁻¹ or faster, whereas bulk properties tend to be determined by slower motions. Thus at temperatures just above $T_g$ a material may appear mobile by bulk techniques but not by NMR. We conclude then that the effective $T_g$ for PHB in vivo is lower than −20°C and may be as low as −70°C. In principle it is possible to estimate $T_g$ from the temperature dependence of relaxation properties (the Williams-Landel-Ferry treatment, Ref. 22), but this approach is difficult to apply in a living cell system which is continuously metabolizing and where the accessible temperature range is so narrow; the spectrum disappears below 10°C, and cell viability disappears above 30°C. Nevertheless, where fresh and reproducible cell samples are constantly available, e.g., from continuous culture, this type of quantitative approach will be practical and attractive.

A Model for PHB in Vivo—The question inevitably arises of how a material such as PHB, with a melting point around 180°C when isolated, can be stored as a mobile elastomer within the granule. Clearly another component must be present and acting as a plasticizer. The protein and lipid that are known to be associated with the granule are possible candidates, but it is not easy to see how the relatively small quantities of these materials could have such a profound effect; no other organic components have been reported to be present in significant quantity, and certainly there is no evidence for additional organic components in the NMR spectra.

We believe that water is the most likely plasticizer. It is certainly known to plasticize and therefore mobilize other hydrophobic polymers. For example, the $T_g$ for amorphous nylon-66 is reduced from 97 to 6°C by adsorption of water (23). This plasticization has a profound effect on the mobility of nylon chains as detected by NMR, and we believe that we are observing precisely the same effect in this work. We discuss below the biological evidence in support of water as the plasticizer. The mechanism of action of water plasticization is not entirely clear but presumably involves either hydrogen bonding or dipole-dipole interactions with the ester groups, thereby inhibiting chain-chain proximity; there may even be localized pockets of water.

If the PHB is indeed present largely as an amorphous elastomer, this implies that there is a good deal of mobility for individual segments of chain but little or no long-range translational freedom. The main chain CH and CH₂ carbons of PHB have relaxation times that differ by a factor of 1.6 rather than the value of 2 expected for isotropic motion. It is clearly not possible to ascribe a single conformation to such flexible material; however, the difference in chemical shift between the methylene carbon in the crystal and solution in vivo closely matches the chemical shift difference between amorphous and crystalline polyethylene in the solid state.

Griebel and Merrick (24) postulated that “PHB is hydrolyzable only while it is present in a particular conformational state.” We propose that in fact the hydrated amorphous elastomeric form of PHB is the only form that is an accessible substrate for depolymerase and also that it is thermodynamically unstable with respect to the solid. Disruption of the environment rapidly leads to irreversible solidification of the granule, presumably by extrusion of water. We have proved experimentally that this solidification occurs upon treatment with acetone (a hygroscopic solvent) and after extended periods of cooling. The solidified material is no longer a substrate for depolymerase, explaining why granule inactivation is always irreversible (see below). The notion that the enzyme operates on a mobile material in a partially aqueous environment seems intuitively to be more reasonable than operation on the surface of a solid. Water is of course the second substrate for any enzyme that converts ester groups to alcohol and acid.

How the hydrated metastable state is maintained in vivo remains a mystery; the presence of an aqueous environment is, by itself, an inadequate explanation. We have no positive evidence addressing the question of whether the synthase and depolymerase are distributed throughout the PHB mass or concentrated at the surface, although the latter appears intuitively to be more likely. It is notable that “native” granules are not good substrates for added depolymerase unless activated by added trypsin (7, 8). One interpretation of this is that the PHB chain ends are normally protected by attachment to the active site of the synthase and are only available to depolymerase after release from the synthase.

What about the 30% of PHB detected chemically but not spectroscopically? One possibility is that it is qualitatively in a similar state to the visible 70% but that it has a $\tau_r$ that renders the lines too broad to detect. We tend to favor this model in which there is a spread of $\tau_r$ (possibly reflecting a
spread of molecular weight) straddling the borderline between observable and unobservable. There is an obvious set of alternative models in which 30% of the material is truly solid. This solid could form the core of each granule, be distributed as small crystallites throughout each granule, or even be segregated into a separate class of granules. There is some experimental NMR evidence against these solid models in our biochemical work referred to below, but it is not conclusive. 

Other Evidence—In retrospect, we find that previously published evidence supports the idea of PHB mobility. These observations (discussed in the following paragraphs) were puzzling and inexplicable in terms of the conventional solid granule hypothesis, but all are readily explained by a mobile PHB model.

1) PHB granules have been observed to coalesce during cell growth.2 This is hardly the behavior expected of solid particles.

2) Treatment of granules with acetone not only inactivates granules but also leads to morphological changes which have been interpreted as formation of crystalline bundles and fibers (10). Clearly, crystallization is occurring after dehydration of the granule by acetone. Incomplete solidification by brief treatment with acetone or other reagents (24) would lead to a mixture of native and solid PHB, explaining why the rate of depolymerization was unaffected even when the extent was reduced.

3) Granules stored at 4°C for 3 weeks shrink physically (10) as well as losing activity (7, 8); also, in contrast with native granules, their morphology is now resistant to acetone treatment (10). If solidification and water extrusion have occurred upon storage, given that PHB is insoluble in acetone, then we would expect mild acetone treatment to have no further effect on these granules.

4) Native granules are effectively stained by Sudan Black and Sudan III in situ (6), while granules that have been isolated by treatment with hypochlorite at pH 9.8 are not stained. Moreover, granules stained in situ are decolorized during hypochlorite-mediated extraction. It is generally agreed that these “stains” do not stain solids; rather, they are actually dyes that dissolve in mobile lipid environments (13). The original explanation of these granule results was therefore that the stains were dissolving in a granule component that is distinct from the PHB; a putative outer membrane which is destroyed by mild alkali treatment was the conventional site. We reinterpret the results as demonstrating that the stains dissolve directly in the native mobile PHB phase; inactivation as a result of solidification is then inevitably accompanied by loss of stain.

Finally, we turn to the question of the NMR-invisible material. In unpublished experiments monitoring PHB biochemistry by NMR (to be described in detail elsewhere), we have never seen any evidence either for the immobilization of PHB after biosynthesis or for its mobilization during consumption. This tends to weigh against the solid models outlined in the previous paragraphs, but clearly more work could be done in this area to clarify the situation. X-ray diffraction of native granules is an obvious possibility.

CONCLUSIONS

To the best of our knowledge, this is the first study of the physical chemistry of PHB in vivo. We believe that the results have given considerable insight into the enzymology of this important material, and, although we have investigated only three microbial species in this work, it seems likely that the conclusions are applicable to many biopolymers in vivo. For example, PHB has also been observed by NMR in Rhodopseudomonas sphaeroides (25). Interestingly, it has recently become clear from NMR measurements that glycogen, the mammalian storage polymer in liver, is also much more mobile than previously realized (26), and we see very similar polysaccharide signals in M. extorquens and Methylobacterium AM1 (27) and in Klebsiella species (28).

Many studies of Gram-negative cells and granules have elucidated details of the properties of solid PHB; this solid is of course the technologically important form of the polymer, so it is important to understand it as fully as possible. From the biological point of view, however, the solid appears to be largely a misleading artifact of the experimentalist’s making.

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