Synthesis of Poly(3-hydroxybutyrate) by *Ralstonia eutropha* in the Presence of ^{13}C-Labeled Ethylene Glycol

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Received March 2, 2000
Revised Manuscript Received May 26, 2000

Introduction

Poly(3-hydroxybutyrate) [P(3HB)] is a biodegradable polymer produced by many bacteria in response to growth restriction by a nutrient other than the carbon source.[1] It is a member of a group of polymers known as poly(hydroxyalkanoate)s [PHA]. P(3HB) is the most abundant and studied polymer[2] of the PHA family and generally regarded to have poor material properties. Attempts at improving the properties of PHAs have focused on incorporation of different monomers,[3−7] altering the chain alignment within the polymer,[8] introducing branching,[9] and chain functionalization followed by postprocessing.[7,10−12]

Diblock copolymers have also been produced by the use of poly(ethylene glycol) during fermentation.[13,14] The introduction of functionalized chain ends into PHA has been reported,[15−18] and this could facilitate subsequent modification, for example, in the production of block copolymers.

The authors have previously reported the biosynthesis of P(3HB) with the desired end-functionalization incorporated, by supplementation of the culture medium with various alcohols, diols, and polyols.[16−18] A carboxyl group belonging to the final 3HB monomer in a P(3HB) chain is normally found at the chain terminus.[14] However, this has been altered in vivo by the inclusion of hydroxy-containing compounds, which become incorporated at the polymer terminus via a chain transfer reaction in which the growing P(3HB) chain is esterified to the hydroxy compound.[16,17] It is also found that the P(3HB) produced during exponential growth phase of *Ralstonia eutropha* (reclassified from *Alcaligenes eutrophus*) contains only carboxy chain ends; evidence of secondary hydroxyl is found only in polymers produced in the polymer accumulation phase. So P(3HB) produced by fermentation always contains an excess of carboxy groups in relation to the secondary hydroxyls. When the fermentation is carried out in the presence of diol, the number-average molecular weight, M_n, of the P(3HB) measured by end-group analysis of carboxy, and hydroxyalkyl-derivatized carboxy chain ends, is generally in excellent agreement with the true M_n measured by GPC.[17] On the other hand, M_n calculated on the basis of secondary hydroxyls is considerably higher due to the fact that not all the chains contain secondary hydroxyl end groups.[17] Measurement of secondary hydroxyl concentration underestimates the actual number of chain ends and hence incorrectly leads to high M_n.

This paper describes the synthesis of P(3HB) by fermentation using ^{13}C-labeled ethylene glycol, EG, in the fermentation medium. 1,2-^{13}C-EG is utilized in the fermentation to get better insight into the disposition of EG during the biosynthesis of P(3HB). Chain termination reaction by EG and participation of hydroxyethyl-terminated chains in further transesterification reactions are investigated by ^{13}C NMR.

Experimental Section

Materials. Unless specified, all reagents were purchased from Aldrich Chemicals, USA, and solvents were from VWR Scientific Products. The organic components of all fermentation media were from Difco, and the inorganic media components were purchased from Sigma.

Production of Hydroxy-Terminated PHA in Shake Flask Culture. *R. eutropha* strain NCIMB 40529 was grown in 250 mL shake flasks containing 50 mL of LB medium (LB medium contains 10 g L^{-1} of tryptone (Difco), 5 g L^{-1} of yeast extract (Difco), and 5 g L^{-1} of NaCl), plus glucose (2% w/v) and 1,2-^{13}C-EG (3% w/v). The culture flasks were incubated at 30 °C for 4 days in a shaker (New Brunswick) orbiting at 250 rpm to obtain hydroxyethyl-terminated P(3HB).

PHA Analysis. ^{13}C NMR spectra suitable for integration were acquired on a 400 MHz Varian Unity NMR spectrometer operating at a ^{13}C frequency of 100.574 MHz, using a 90° pulse, 16 s delay, and gated decoupling.

Three statistical models were calculated for quantitatively approximating the experimental peak intensity distributions. The first was calculated with the assumption that the ^{13}C nuclei originating from 1,2-^{13}C-EG were atomically randomized in the polymer. They were therefore indistinguishable from naturally occurring ^{13}C nuclei (0.01 abundance), and each carbon position had an equal chance of being occupied by a ^{13}C nucleus (Table 1, column 6). The second model allowed integral ^{13}C−^{13}C pairs (from 1,2-^{13}C-EG) to randomly occupy the C2−C1, C3−C2, and C4−C3 positions, as well as naturally occurring ^{13}C nuclei to randomly occupy single atomic positions (Table 1, column 7). The probability of block substitution was determined by a least-squares fit of the calculated peak intensity distribution to the experimental data and was found to be 0.0044 (i.e., $<^1/_2$ the natural abundance of ^{13}C nuclei). The third model differed from the second by restricting ^{13}C−^{13}C pairs to C2−C1 and C4−C3 positions (Table 1, column 8).

Results and Discussion

Microbial synthesis of P(3HB) by *R. eutropha* in the presence of diols has previously been described. In an effort to obtain hydroxyethyl-terminated P(3HB), fermentation was carried out using *R. eutropha* in the presence of 3% w/v EG in the culture media. Glucose was used as the carbon source. The purpose of using 1,2-^{13}C-EG in the fermentation was to determine, by ^{13}C NMR, whether EG participates only in a chain termination reaction and whether hydroxyethyl-terminated P(3HB) participates further in chain-transfer reactions.

The ^{13}C NMR spectrum (Figure 1) of the polymer produced from the fermentation using ^{13}C-labeled EG in the feed is dominated by the four peaks of P(3HB). In addition, the two doublets of an AB pattern, centered at 66.8 and 60.8 ppm, are attributed to the two inequivalent ^{13}C nuclei of EG acylated on the carboxyl end of the P(3HB) chain. No evidence is seen of a peak expected at 63 ppm for the equivalent ^{13}C nuclei of EG

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10.1021/ma000387q CCC: $19.00
Published on Web 08/01/2000

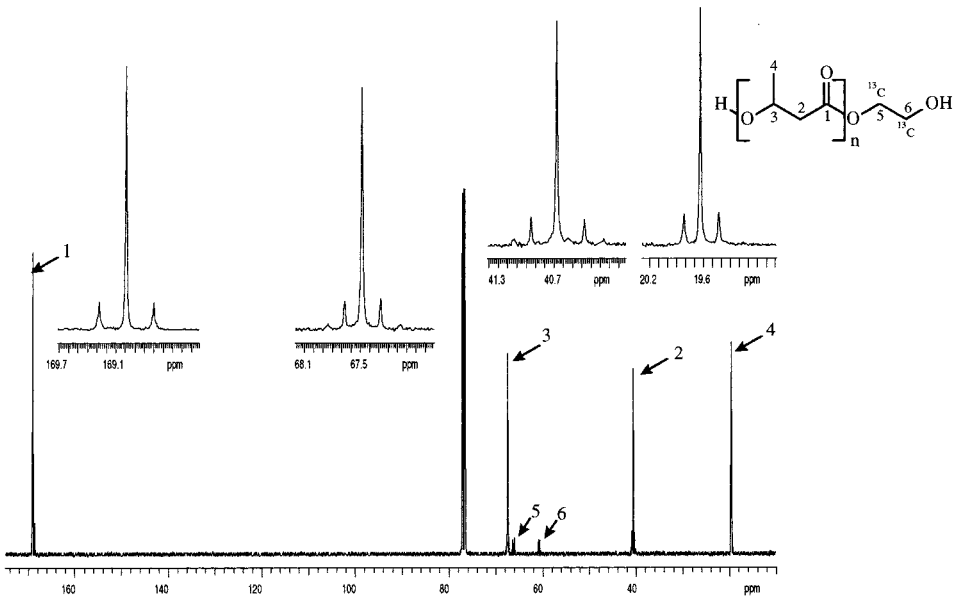

Figure 1. ^{13}C NMR spectrum of P(3HB) terminated by 1,2-^{13}C-ethylene glycol.

Table 1. ^{13}C NMR Analysis of P(3HB) Produced in the Presence of ^{13}C-EG

isotope sequence	atom sequence	chemical shift [ppm]	coupling constant [Hz]	normalized exptl intensities	calcd random single ^{13}C nuclei	calcd random EG in 1−2 and 3−4 positions	**calcd nonrandom EG in 1−2, 3−4 positions**
-12-**13**	C2-**C1**	169.09		**0.18**	0.19	0.178	**0.178**
-13-**13**	C2-**C1**	169.088	58.3	**0.07**	0.06	0.071	**0.071**
13-12-	**C4**-C3	19.735		**0.16**	0.19	0.178	**0.178**
13−13-	**C4**-C3	19.726	39.3	**0.07**	0.06	0.071	**0.071**
-12-**13**-12	C3-**C2**-C1	40.772		**0.17**	0.15	0.178	**0.178**
-12-**13**-13	C3-**C2**-C1	40.761	58.0	**0.06**	0.04	0.071	**0.071**
-13-**13**-12	C3-**C2**-C1			**0.00**	0.04	0.002	**0.002**
-13-**13**-13	C3-**C2**-C1	40.745	58.0, 38.7	**0.03**	0.01	0.000	**0.030**
12-**13**-12-	C4-**C3**-C2	67.582		**0.17**	0.15	0.178	**0.178**
13-**13**-12-	C4-**C3**-C2	67.575	38.9	**0.05**	0.04	0.071	**0.071**
12-**13**-13-	C4-**C3**-C2			**0.00**	0.04	0.002	**0.002**
13-**13**-13-	C4-**C3**-C2	67.559	38.9, 38.9	**0.03**	0.01	0.000	**0.030**

that is acylated on both ends. This observation rules out further reaction of the hydroxyl end of acylated EG.

A striking feature of the spectrum is that while all peaks corresponding to the carbons of the polymer repeat unit are observed, they are all flanked by lower intensity peaks of equal intensity to each other. Expansions of the carboxy (C1), methylene (C2), methine (C3), and methyl (C4) peaks are also shown in Figure 1. The presence of spinning sidebands is ruled out since the frequency difference between the peaks is variable throughout the spectrum (∼18−28 Hz), whereas the spectrum was acquired at constant spinning (20 Hz). Moreover, the patterns are not consistent with incomplete proton decoupling from protons. This is further evidenced by the presence of these peaks for the carbonyl group which has no protons and the presence of multiple peaks surrounding the methine carbon which has only one proton.

The low-amplitude peaks flanking the major peaks are ^{13}C satellite peaks due to *J*-coupled neighboring ^{13}C nuclei. In natural abundance ^{13}C NMR spectra such peaks are frequently buried in the noise since their amplitude is approximately 1/200 the amplitude of the main (uncoupled) peak. In the present situation the pool of available ^{13}C nuclei has been significantly increased over the natural level, judging by the ratio of satellite to center peak height (approximately 1/7); the source is the 1,2-^{13}C-EG added to the growth medium.

The ^{13}C NMR spectrum provides qualitative as well as quantitative clues to the mechanism by which the ^{13}C nuclei of EG are incorporated in P(3HB). Nuclei coming from 1,2-^{13}C-EG are shown in bold-type in the table and in further discussion. The *J*-coupling constants and the chemical shifts are shown in Table 1. The magnitudes of the coupling constants are as predicted:[19] $J \sim 39$ Hz between alkyl nuclei and $J \sim 58$ Hz between alkyl and carboxyl nuclei. The spectrum of the ^{13}C nuclei, C1 (carboxy) and C4 (methyl), consists of a singlet, C2[^{12}C]−**C1[^{13}C]** and **C4[^{13}C]**−C3[^{12}C], respectively, and a doublet, C2[^{13}C]−**C1[^{13}C] and C4[^{13}C]**−C3[^{13}C], respectively. The doublet coupling constants are ^1J(C2−C1) ∼ 58 and ^1J(C4−C3) ∼ 39 Hz. The spectrum of C3 (methine) consists of a singlet, C4[^{12}C]−**C3[^{13}C]**, a doublet, C4[^{13}C]−**C3[^{13}C]**, with ^1J(C4−C3) ∼ 39 Hz, and a triplet, C4[^{13}C]−**C3[^{13}C]**−C2[^{13}C], with ^1J(C4−C3) ∼ ^1J(C3−C2) ∼ 39 Hz. The spectrum of C2 (methylene) consists of a singlet, **C2[^{13}C]**−C1[^{12}C], a doublet, **C2[^{13}C]**−C1[^{13}C], with ^1J(C2−C1) ∼ 58 Hz, and an AB-type spectrum, C3[^{13}C]−**C2[^{13}C]**−C1[^{13}C] with ^1J(C2−C1) ∼ 58 Hz, ^1J(C3−C2) ∼ 39 Hz. In view of the large difference between the two coupling constants, one expects a separate doublet, C3[^{13}C]−**C2[^{13}C]**, with a 39 Hz coupling constant, to be well resolved from the observed **C2[^{13}C]**−C1[^{13}C] doublet. The crucially important observation is, however, that this doublet is missing from the C2 signal altogether.

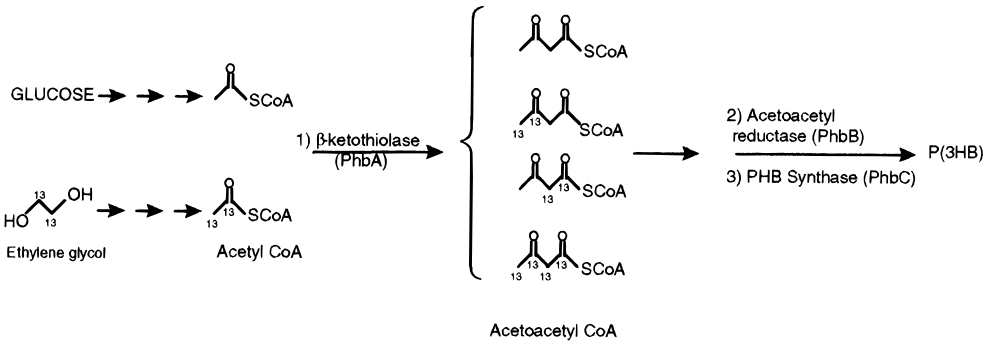

Figure 2. Schematic showing the biosynthesis of p(3HB) in *Ralstonia eutropha* using glucose and doubly labeled ethylene glycol as the carbon sources.

If the ^{13}C nuclei originating from ^{13}C-labeled EG were atomically randomized in the polymer, the intensity of the doublet C3[^{13}C]−**C2[^{13}C]** would be equal to the intensity of the doublet **C2[^{13}C]**−C1[^{13}C]. The intensities calculated for random distribution of ^{13}C nuclei agreed well with the observed ^{13}C singlet and $^{13}C-^{13}C$ doublet intensities, but not with the C3[^{13}C]−**C2[^{13}C]**−C1[^{12}C] and C4[^{12}C]−**C3[^{13}C]**−C2[^{13}C] intensities (Table 1). These observations suggested that ^{13}C-labeled EG was incorporated as entity C2−C1 and C4−C3 and rarely, if at all, as C3−C2 (in which case the C3−C2 population would be considerably larger than zero). Indeed, the surprisingly high intensity of the C3[^{13}C]−**C2[^{13}C]**−C1[^{13}C] and C4[^{13}C]−**C3[^{13}C]**−C2[^{13}C] intensities indicate that synthesis of 3HB units consisting of two ^{13}C-labeled EG molecules is favored. The first attempt to understand the spectrum quantitatively is by describing it as a superposition of two spectra: one, the common ^{13}C satellite spectrum of randomized natural abundance ^{13}C nuclei and the other, the spectrum of randomized $^{13}C-^{13}C$ blocks as C2−C1 and C4−C3. The predicted (best fit) intensities are shown in column 7 of Table 1. The intensities, including the near-zero intensity of the C3−C2 doublet, were correctly predicted, except those of C3[^{13}C]−**C2[^{13}C]**−C1[^{12}C] and C4[^{12}C]−**C3[^{13}C]**−C2[^{13}C] (an order of magnitude low). This indicated that the $^{13}C-^{13}C$ blocks are not incorporated randomly but preferably in pairs in positions C2−C1 and C4−C3. In view of this, the lack of detection of C3−C2 doublets may seem counterintuitive, but one should note that the creation of a large number of C3-[^{13}C]−C2[^{13}C] dyads from ^{13}C-labeled EG pairs does not give rise to C4[^{12}C]−**C3**[^{13}C]−C2[^{13}C] and C3[**^{13}C**]−C2-[^{13}C]−C1[^{12}C] triads.

In conclusion, the ^{13}C NMR spectrum of P(3HB) prepared using ^{13}C-labeled EG clearly demonstrates that EG acts as chain terminator; no evidence of EG acylated at both ends was observed. It is found that EG is metabolized by *R. eutropha* and used as carbon source for the production of P(3HB) inspite of being toxic at higher levels in the culture medium.

In *R. eutropha*, biosynthesis of P(3HB) from glucose and most other substrates involves a series of enzyme-catalyzed reactions (Figure 2). Two molecules of acetyl-CoA are condensed to form acetoacetyl-CoA, which is then reduced to [R]-3-hydroxybutryl-CoA, the substrate for the polymerizing enzyme PHB synthase. The biochemical pathway involved in EG metabolism has not been established in this organism, but the most likely first step is the oxidation of this substrate to glycollate. Acetyl-CoA could be produced from glycollate via a pathway postulated to occur in a *Pseudomonas sp.*[20] Further experiments would be required to confirm this hypothesis. ^{13}C NMR analysis of P(3HB) produced from 1,2-^{13}C-EG (Figure 1) shows that the labeled carbons are incorporated in pairs, as C2−C1 and C4−C3 constituents of 3HB monomers. This is consistent with the condensation of molecules of labeled and unlabeled acetyl-CoA from EG and glucose, respectively, in the biosynthesis of P(3HB) (Figure 2).

Acknowledgment. The authors acknowledge useful and thought-provoking discussions with Henry Valentin.

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MA000387Q