Enzymatic Polymerization of (*R*)-3-Hydroxyalkanoates by a Bacterial Polymerase

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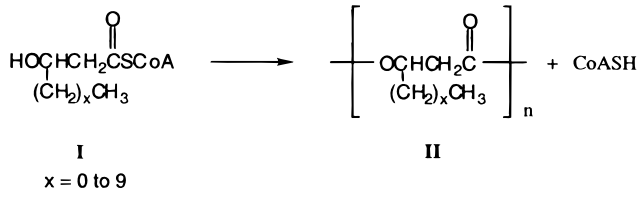
Introduction. Many species of bacteria produce poly-3-hydroxyalkanoates **II** (PHAs) as intracellular, reserve polymers by the polymerization of the coenzyme A (CoA) thioester derivatives of 3-hydroxyalkanoic acids **I**, as shown in the following reaction:[1−3]



I **II**

x = 0 to 9

The polymerization reaction is initiated and catalyzed within the cell by an enzyme that is referred to as a PHA synthase or polymerase. The polymerase of the bacterium *A. eutrophus*, which was recently renamed *Ralstonia eutropha*, has been cloned, overexpressed in *E. coli*, and purified to homogeneity.[4] *A. eutrophus* has been used for the industrial production of poly-(*R*)-3-hydroxybutyrate (PHB) and copolymers poly-(*R*)-3-hydroxybutyrate-*co*-3-(*R*)-hydroxyvalerate (PHBV). The polymerase gene of this bacterium has also been used in transgenic organisms engineered for the production of PHAs.[3]

In this report, we describe the *in vitro* synthesis of the homopolymers, PHB and poly-(*R*)-3-hydroxyvalerate (PHV), and of the random and block copolymers containing these two types of units, HB and HV, using the purified, recombinant polymerase from *A. eutrophus*. All polymerization reactions were carried out in aqueous solutions by reacting the polymerase with the appropriate monomer(s), **I**. The monomer for the synthesis of PHB and the copolymers was (*R*)-3-hydroxybutyryl-CoA (HBCoA, $x = 0$ in **I**), which was prepared by a combined chemical and enzymatic process, as previously described,[5] while the monomer for PHV and the copolymers, (*R*)-3-hydroxyvaleryl-CoA (HVCoA, $x = 1$ in **I**), was prepared chemically as described in the Supporting Information.

Results. Polymerization reactions were performed without stirring at room temperature in aqueous solutions containing potassium phosphate buffer (200 mM, pH 7−7.4), as described in detail in the Supporting Information. For the homopolymerization of HBCoA, either 3 or 6 mL of a 32 mM aqueous solution of the monomer was added to 1.5 mL of a 1.6×10^{-9} M aqueous solution of the polymerase for a monomer-to-enzyme molar ratio of either 2×10^4 or 4×10^4, respectively, to prepare polymers A and B of Table 1, respectively. For the former, 15.6 mg or 95% yield of the polymer was obtained after centrifugation, washing, and drying. The rates of the polymerization reactions of both HBCoA and HVCoA and of the copolymerization reaction of these two monomers, using equivalent reaction conditions, were estimated from conversion−time plots, as shown in Figure 1. Monomer conversions were determined by removing small aliquots (5 μL) of the reaction mixtures at various periods of time, quenching the reactions in 5% trichloroacetic acid solution, and measuring the amounts of CoASH released using Ellman's reagent.[6] The polymerization of HBCoA went to complete conversion within 30 min, but the copolymerization reaction required approximately 2 h to reach 100% conversion, and for the polymerization of HVCoA the conversion was only about 90% in that time period. Recent studies in our laboratory have shown that CoASH is a competitive inhibitor for this enzyme,[7] so the lower conversion of HVCoA is believed to be due to inhibition of the slower polymerization reaction of HCCoA at high conversions.

Rates of Polymerization. As shown in the rate plots in Figure 1, the polymerization reactions began after short induction periods of approximately 3−5 min. After the induction period, the slopes of the linear portions of these plots indicated that the rates of polymerization of the two monomers were significantly different. Approximate propagation reaction rates for each were calculated from these slopes by assuming that each enzyme molecule that has a molecular mass of 64 kDa[4] initiated one polymer chain (as discussed below). The rates so calculated indicate that each molecule of the polymerase converted approximately 40 molecules of HBCoA and 20 molecules of HVCoA per second into PHB and PHV, respectively, under the reaction conditions used.

The polymerization rate observed for HBCoA is considerably higher than the values calculated previously for the production of PHB inside the cell by *A. eutrophus*, which was approximately two molecules of HBCoA per polymerase per second.[9] However, as noted above, the conditions for the present *in vitro* reactions were most likely quite different from those of the intracellular reactions in that the polymerization reactions *in vivo* probably occur at monomer concentrations that are far below saturation, in contrast to the concentrations used in the present *in vitro* systems. In the present studies, the polymerase should be operating near its maximum velocity because the monomers were used at concentrations (12.8 mM)that were much above their estimated K_m values. In recent *in vitro* investigations we estimated that the K_m value for this polymerase was 0.243 mM for HBCoA.[6]

The results for the copolymerization reaction of an equimolar mixture of the two monomers are shown in Figure 1. The diad and triad contents of the copolymer,

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Table 1. Molecular Weight of PHB as a Function of HBCoA Monomer-to-Polymerase Ratio for the in Vitro Polymerization Reaction

		GPC molecular weights[a]			calculated M_n	
polymer	monomer-to-polymerase, mole ratio	M_n	M_w	M_w/M_n	absolute[b]	theoretical[c]
A	2×10^4	2.95×10^6	5.95×10^6	2.01	2.07×10^6	1.72×10^6
B	4×10^4	4.21×10^6	7.41×10^6	1.76	2.95×10^6	3.44×10^6

[a] As measured by GPC relative to polystyrene standards. [b] Based on estimate that absolute M_n = 0.7 GPC M_n. [c] Theoretical value calculated from the monomer-to-polymerase ratio for a "living polymer" system.

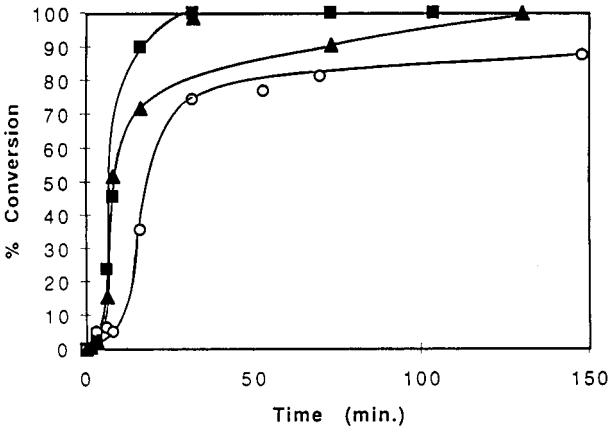

Figure 1. Monomer conversion vs reaction time for the homopolymerization and copolymerization of HBCoA and HVCoA monomers: (■) HBCoA alone (HBCoA 0.193 mmol, 0.3 mg of polymerase, total volume 15 mL); (○) HVCoA alone (HVCoA 0.093 mmol, 0.15 mg of polymerase, total volume 7.5 mL); (▲) equimolar mixture of HBCoA and HVCoA (HBCoA 0.093 mmol, HVCoA 0.093 mmol, 0.3 mg of polymerase, total volume 15 mL).

as determined by ^{13}C NMR spectroscopy, indicated that the copolymers contained higher amounts of both HV triads and HB triads than expected for a random copolymer of the same composition based on Bernoullian statistics (see Supporting Information). These results are consistent with the formation of a somewhat blocky copolymer structure as would be expected for this type of high conversion "batch copolymerization reaction" involving two monomers of unequal reactivity. In contrast, the copolymer produced *in vivo* by *A. eutrophus*, which also contained approximately equal amounts of HB and HV units, was shown by ^{13}C NMR analysis to be essentially random.[8]

Molecular Weights of Polymers. The homopolymerization of HBCoA was carried out at two different molar ratios of monomer to enzyme by the *in vitro* reaction method described above. In this case, bovine serum albumin and a detergent (Hecameg) were added to stabilize the polymer colloid formed during the reaction, and the reaction was buffered to pH 8.[7] The number-average, M_n, and weight-average, M_w, molecular weights of the PHB samples obtained, as determined by gel permeation chromatography, GPC, relative to polystyrene standards, are given in Table 1. Studies by Doi and co-workers on PHB, which compared the values of M_w as determined by GPC to those obtained by solution light scattering, which gives an absolute value of M_w, showed that the latter is 0.7 times that of the former relative to polystyrene standards.[10] The absolute values of M_n of the two samples of PHB in Table 1, as calculated from this relationship and from the M_w/M_n ratio, are also given in the table. Additionally, Table 1 provides the theoretical values of M_n calculated from the monomer-to-polymerase molar ratio if the *in vitro* polymerization reactions are "living polymerization" systems in which each enzyme molecule initiates (and catalyzes) the formation of one polymer chain and the reaction proceeds without chain transfer or termination.[11]

The absolute M_n results for the two PHB samples in Table 1 indicate that in each case the molecular weight of the polymer obtained was closely related to the monomer-to-polymerase ratio in a manner expected for the formation of a "living polymer."[11] In such a case, at complete conversion of the monomer and the initiator (the polymerase), the M_n of the resulting polymer is exactly determined only by the mole ratio of monomer to initiator,[11] and higher molecular weights are obtained at higher monomer/initiator ratios, as was observed in these *in vitro* polymerization reactions.

Generally, the molecular weight distribution of the products of living polymerization reactions are very narrow, but the somewhat broad distributions obtained here, as indicated by the M_w/M_n ratios in Table 1, are still consistent with a living polymerization if the rate of initiation is considerably slower than the rate of propagation. The observed induction periods in Figure 1 show that the initiation process is, indeed, much slower than the propagation process, so a broadening of the distribution was to be expected.

Sequential Copolymerization Reactions. The fairly close correlation between the absolute M_n value and the theoretical M_n value, as shown in Table 1, is a strong indication of the formation of living polymers in these *in vitro* systems. To further investigate this hypothesis, a sequential polymerization reaction was carried out in which HBCoA was first polymerized by the polymerase, and after complete reaction of this monomer, HVCoA was added and polymerized to completion. A plot of conversion vs time for each of the two monomers, which were reacted sequentially, is shown in Figure 2.

As indicated by the rate plots in Figure 2, the enzyme apparently remained highly active for a long period of time (at least 30 min) after the polymerization of HBCoA because the polymerization reaction of HVCoA began immediately and very rapidly on the subsequent addition of this monomer, with no detectable induction period. An approximately equimolar ratio of the two monomers was used for this sequential copolymerization reaction, and the total monomer-to-polymerase ratio was 10 000 to 1, so the calculated M_n value of the diblock copolymer expected for this system (taking into account that the conversion of HVCoA to form the second block was only 85%) was 0.88×10^6. The observed value for the copolymer obtained, based on GPC analysis, was 0.82×10^6 with an M_w/M_n ratio of 1.6.

The conversion factor relation of the M_w value (as measured by GPC) to the absolute value of M_w for the HB/HV copolymers is not known, but the results indicated that the product had a molecular weight very close to the sum of the expected molecular weights for a copolymer containing the expected amounts of the HB and HV monomers in single polymer chains, which in

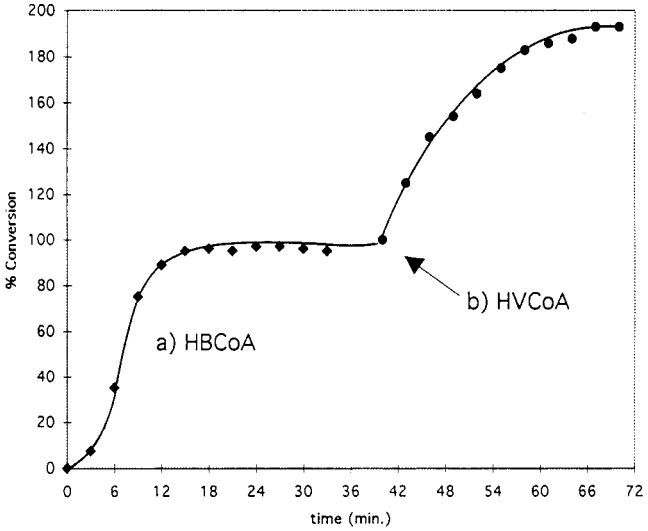

Figure 2. Monomer conversion vs time for the sequential copolymerization of (a) HBCoA (HBCoA 0.0365 mmol, 0.467 mg of polymerase, total volume 14.6 mL) and (b) HVCoA (HVCoA 0.0365 mmol, total volume 15.0 mL).

this case should be a block copolymer. In support of this proposal, the ^{13}C NMR analysis of the product of this sequential polymerization reaction, which contained approximately 54% HB units and 46% HV units, contained peaks only for HB−HB and HV−HV homodiads, and no peaks were present for heterodiads of HB−HV. Similarly, DSC analysis gave thermograms that contained only melting endotherms for PHB and PHV and none for P(HB/HV) copolymers. Therefore, both the molecular weight and sequence distribution results strongly support the proposal that the sequential copolymerization process resulted in the formation of a block copolymer of HB and HV units and that these polymerization reactions have "living polymer" characteristics.

Summary and Conclusions. The results above suggest that these *in vitro* polymerization reactions are chain growth, living polymerization reactions, in which each molecule of the polymerase initiates and catalyzes the formation of one molecule of the polymer, and at complete conversion, the polymers formed have a predictable number average molecular weight. It is important to note that a similar qualitative relationship between the molecular weight of the polymer formed and the amount of polymerase, as was observed here, was also found for the *in vivo* synthesis of PHB in a recombinant strain of *E. coli* in which the polymerase activity could be controlled.[12] If so, then PHA production in some recombinant bacteria may also occur by polymerization processes in which there are no chain transfer or termination reactions, unlike those in natural PHA-producing bacteria.[13]

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Supporting Information Available: The procedures for the synthesis of HVCoA, PHB, PHV, and PHBV, as well as ^{1}H NMR data for HBCoA and HVCoA and ^{1}H NMR and ^{13}C NMR data of the polymers. This material is available free of charge via the Internet at http://pubs.acs.org.

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