Polyhydroxybutyrate Synthase: Evidence for Covalent Catalysis

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Received April 4, 1996

Polyhydroxybutyrate (PHB) synthase from *Alcaligenes eutrophus* catalyzes the conversion of 3(*R*)-hydroxybutyryl-CoA (HBCoA) to a polyester of greater than 10^6 Da.[1] The polyesters have properties of thermoplastics and are biodegradable,[2] and hence efforts to express the protein in plants to provide a cheap, renewable source of polymer have attracted recent attention.[3,4] While the genes for many PHA synthases have been available for some time, only recently have soluble forms of synthases from *A. eutrophus*[5] and *Chromatium vinosum*[6] been overexpressed and purified to near homogeneity. The ability to isolate milligram quantities of the *A. eutrophus* synthase has greatly facilitated efforts to study various mechanistic aspects of the polymerization, and studies presented herein provide the first direct evidence for covalent catalysis in this process.

Our previous studies revealed that PHB synthase exists as an equilibrium mixture of monomeric and dimeric forms and that the rate of polymer formation, based on extraction of [^{3}H]-polyester into chloroform starting from [^{3}H]-HBCoA, experienced a long and variable lag phase dependent on the protein concentration.[5] We postulated at that time that this lag phase could be the result of the requirement of the dimeric form of the protein for activity and/or a priming process that could involve CoA esters of oligomeric 3(*R*)-hydroxybutyric acid. These postulates are not mutually exclusive or all inclusive. Gerngross et al.[7] recently suggested that a physical step, involving granule formation, could be slow and therefore responsible for the lag phase.

To investigate the early steps in polymer formation, we synthesized a number of oligomeric CoA analogs **1**, **2**, **3**, and **4** (Figure 1). These analogs have been examined for their ability to induce dimerization of the synthase, to enhance the rate of polymer formation, and to alleviate the lag phase in this process. The results are summarized in Table 1 and Figure 2. When **1** or **2** is preincubated with synthase[8] and the reaction mixture

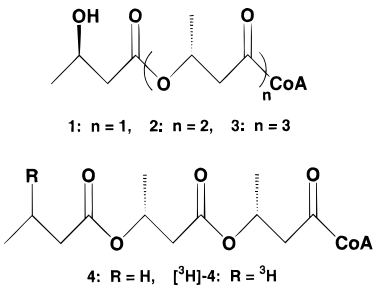

Figure 1. Structures of the oligomeric CoA analogs.

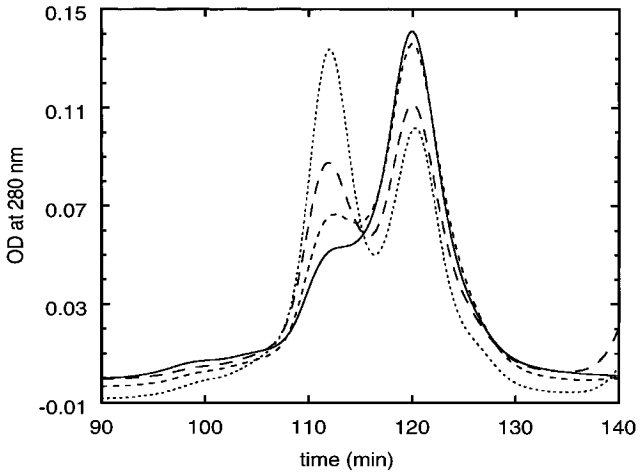

Figure 2. Size exclusion chromatography profiles of PHB synthase preincubated with (− − −) **1**, (•••) **2**, or (- - -) **3** or (—) without any CoA analog.

Table 1. Ability of Oligomeric CoA Analogs (**1**−**3**) To Affect the Lag Phase and Turnover of PHB Synthase

	dimeric enzyme		monomeric enzyme	
enzyme incubated with	specific activity (U/mg)	lag phase[a] (min)	specific activity (U/mg)	lag phase (min)
1	88	3.5	45	7.5
2	131	1.2	37	8.5
3	49	5.0	35	9.0
	44	6.0	39	8.5

[a] Lag phase is defined as an *X*-intercept of a line fitted to the linear part of the time course of the enzymatic reaction.

examined by size-exclusion chromatography, the ratio of the dimeric to the monomeric form of the enzyme increases relative to wild type synthase (wt-synthase) in the absence of a CoA analog (Figure 2). The effect is most pronounced in the case of trimeric "primer" **2**. Furthermore, determination of the specific activity of the monomeric and dimeric forms of protein[9] indicates that in each case, the dimeric form is substantially more active than the monomeric form which is identical to the synthase when no primer has been added. The effect again is most pronounced in the case of **2** (Table 1). Finally, the lag phase in each case was also examined and found to be reduced from 6.5 min in the case of wt-synthase to 1.2 min in the case of synthase preincubated with **2**. Interestingly, compound **4**,

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(8) The reaction mixture contained in a final volume of 200 μL: PHB synthase (2.0 mg/mL, 31 μM), **1**, **2**, **3**, or **4** (1 mM), Tris-HCl (45 mM including 4.5% glycerol and 0.045% Hecameg (6-*O*-(*N*-heptylcarbamoyl)-methyl α-D-glucopyranoside), pH 8.5). It was incubated for 15 min at 20 °C and then loaded onto two Superose 12 HR 10/30 (Pharmacia) columns connected in sequence equilibrated in 50 mM Tris-HCl (pH 7.5) containing 150 mM NaCl and 0.05% Hecameg at 4 °C. The synthase was eluted with the same buffer at a flow rate of 0.2 mL/min at 4 °C. The dimer eluted at 22.4 mL and the monomer at 24.0 mL. The molecular weight was determined on the basis of a calibration curve prepared using the following molecular weight standards: thyroglobulin (669 kDa), apoferritin (443 kDa), β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (64 kDa), and carbonic anhydrase (29 kDa).

(9) The assay for CoA release is based on a modification of the procedure of Ellman.[17] The reaction mixture contained in a final volume of 400 μL: 150 mM phosphate (pH 7.0), 1 mM HBCoA, 27 nM PHB synthase (0.7 μg enzyme, 0.2% glycerol, and 0.002% Hecameg, the latter two introduced with enzyme). It was incubated at 20 °C, and aliquots (40 μL) were removed at timed intervals and quenched by addition to 100 μL of ice cold 10% trichloroacetic acid. This mixture (125 μL) was added to 675 μL of a freshly prepared solution of 15 mM 5,5′-dithiobis(2-nitrobenzoic acid) in 500 mM phosphate (pH 7.5). The absorbance at 412 nm (ϵ = 13.7 mM^{-1} cm^{-1}) was measured after incubation of this mixture for 5 min at room temperature.

S0002-7863(96)01108-0 CCC: $12.00

Table 2. MALDI TOF MS and Edman Degradation Analysis of Radiolabeled Peptides Generated from PHB Synthase Labeled with [^{3}H]-**4** and Treated with Trypsin

m/z of $[M + H]^+$ ion[a]			N-terminal	determd by
found	calcd	peptide	sequence[b]	Edman degr.
3232.0[c,d]	3232.74	306−335	DISGQ	DISGQ
2488.6[d]	2488.53	313−335	INVLG	INVLG
2675.8[c]	2674.70	313−337	INVLG	INVLG

[a] Includes 242.27 Da, i.e., **4** minus the mass of HSCoA. [b] From DNA sequence.[18] [c] Found in fraction 59. [d] Found in fraction 61.

also trimeric, has an effect on synthase dimer formation, lag phase, and specific activity similar to that of **2**, while increasing the chain length to a tetramer (**3**) has very little effect on synthase activity (Table 1) and the monomer/dimer equilibrium relative to the wt-synthase (not shown).

To investigate the mechanism by which trimeric CoA analogs (**2** or **4**) might reduce the lag phase, increase the specific activity, and increase the amount of dimeric synthase, [^{3}H]-**4** was synthesized and incubated with PHB synthase. Studies using size-exclusion chromatography similar to that described in Figure 2 indicated that only the dimeric form of the protein contained radiolabel with a stoichiometry of approximately 1 equiv of radiolabel per protein dimer. To determine if the label is covalently bound to the protein, the [^{3}H]-labeled synthase was digested with trypsin.[10] The digest was separated using a Vydac C4 column, and fractions of the eluent were analyzed by scintillation counting.[11] Fractions 59 and 61 accounted for 85% of the radiolabel injected and were further analyzed by MALDI TOF mass spectrometry[12] which indicated the presence of three peptides (see Table 2). From their molecular weights and the results of five steps of Edman degradation[13] on the two fractions, it was deduced that they are peptides 306−335, 313−335, and 313−337, each with a 242 Da adduct corresponding to the molecular weight of **4** minus the CoA moiety. Because all three contain only one cysteine and carry the radioactivity, the label must reside on C319. This residue is the only conserved cysteine in all synthases sequenced to date,[14] and our previous site-directed mutagenesis studies have revealed that it is essential for catalysis.[5]

To investigate if this [^{3}H]-labeled synthase can function as a precursor to [^{3}H]-labeled PHB, the acylated synthase was incubated with HBCoA and the polyester isolated and analyzed for radioactivity.[15] Under these conditions, 52% of the labeled material was incorporated into a polymer of molecular weight similar to that observed in a control with wt-synthase in the absence of **4**.[16] These results establish that the "primed" protein can function as an intermediate in polymer formation.

In summary, acylation of C319 causes a shift of the monomeric form of the synthase to its dimeric form, and this shift is accompanied by a substantial increase in its specific activity and a substantial decrease in the lag phase of polymer formation. How this process relates to the *in vivo* priming process remains to be established. However, these studies provide the first direct evidence for the importance of C319 and of covalent catalysis in the synthase-catalyzed reaction.

Acknowledgment. We are grateful to Professor ChoKyun Rha, Dr. JooHo Choi, and Scott Hogan for helpful discussions of PHB characterization by GPC and to Wei Wu for running two-dimensional NMR spectra of CoA analogs. This work was supported by NIH grants GM 49171 (to A.J.S. and J.S.) and GM05472 (to K.B.) and by an NSERC Postdoctoral Fellowship to J.W.

JA961108A

(10) PHB synthase (1.7 mg, 0.11 mM) was incubated with [^{3}H]-**4** (1 mM, 23.3 mCi/mmol) in 50 mM Tris-HCl (pH 8.5) containing 0.5% glycerol and 0.05% Hecameg for 15 min at 20 °C in a final volume of 275 μL. The protein was then precipiated by the addition of 300 μL acetonitrile, pelleted by centrifugation, and the supernatant removed. The protein pellet was washed three times with 1 mL of acetonitrile/water (ratio 5/1) being careful to resuspend the protein in each wash. The protein pellet was then resuspended in 50 mM ammonium bicarbonate (pH 7.8) and digested with trypsin (0.2 mg) for 3.5 h at 37 °C after which time a second portion of trypsin (0.2 mg) was added, and the mixture was incubated for an additional 17 h.

(11) The entire mixture[10] was loaded on a Vydac C4 reversed phase column (220/4.6 mmD) equilibrated in 0.1% trifluoroacetic acid (TFA) and 0.1% Hecameg at 40 °C. The peptides were eluted with a linear gradient from 0 to 90% acetonitrile in 0.1% TFA and 0.1% Hecameg at a flow rate of 1 mL/min over a period of 90 min. Consecutive 1 mL fractions were collected automatically and analyzed by scintillation counting.

(12) MALDI TOF mass spectra were obtained on a PerSeptive Biosystems Voyager Elite spectrometer operated in the linear mode. To 1 μL of the 1 mL fraction, 10 μL of matrix (sinapinic acid) solution was added, and 1 μL of this mixture applied on the sample probe.

(13) Automated Edman sequencing was carried out by the MIT Biopolymers Laboratory using an Applied Biosystems Model 477A Protein Sequencer with on-line Model 120PTH Amino Acid Analyzer.

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(15) The dimeric form of PHB synthase (36 μg, 0.47 μM) acylated with [^{3}H]-**4**[8] was incubated with HBCoA (1 mM) in 150 mM phosphate (pH 7.0) in a final volume of 1.2 mL. The mixture also contained 11.8 mM Tris, 35.6 mM NaCl, 1.2% glycerol, and 0.012% Hecameg which were introduced with the enzyme. After incubation for 30 min at 20 °C, proteinase K (50 μg, 0.33 mg/mL) in 150 mM phosphate was added to the reaction mixture, and it was incubated at 37 °C for 17 h. The reaction mixture was then lyophilized, and the polymer was extracted with trifluoroethanol (TFE) (3 × 1 mL). Extracts were combined, and the solvent was evaporated to about 100 μL under a stream of air. This sample was loaded on a Shodex K807L GPC column equilibrated in TFE (1 mL/min) at 35 °C for at least 12 h and eluted with TFE at 1 mL/min. Fractions (0.5 mL) were collected and, after evaporation of the solvent, analyzed for radioactivity using scintillation counting. Radioactive polymer eluted between 8.0 and 11.5 mL TFE and contained 52% of recovered radioactivity. The remaining radioactivity eluted as small molecules between 12 and 13 mL of TFE.

(16) In the control experiment, PHB synthase (36 μg, 0.47 μM) which was never exposed to **4** was incubated with [^{3}H]-HBCoA (1 mM, 13.5 mCi/mmol) under conditions identical to those described above[15] and submitted to an identical workup procedure. Radioactive polymer eluted between 8.0 and 11.5 mL TFE.

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