# In Vitro Analysis of the Chain Termination Reaction in the Synthesis of Poly-(*R*)-*β*-hydroxybutyrate by the Class III Synthase from *Allochromatium vinosum*

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*Allochromatium vinosum* polyhydroxyalkanoate synthase catalyzes formation of poly-(*R*)-3-hydroxybutyrate (PHB) from (*R*)-3-hydroxybutyryl-coenzyme A (HB−CoA). (*R*)-3-Hydroxybutyryl-*N*-acetylcysteamine (HB−NAC) is an alternative substrate for this synthase in vitro, with a turnover 1% that of HB−CoA. With HB−NAC, the molecular weight ($M_w$) of PHB produced at substrate-to-enzyme ratios of 1500−15 000 is ∼75 kDa. $^1$H NMR shows that PHB produced has hydroxybutyrate at the alcohol end and *N*-acetylcysteamine (NAC) at the carboxylate end of the polymer. Exogenous NAC has no effect on the $M_w$ of PHB produced with HB−CoA or HB−NAC in vitro, whereas PHB from a polymerization reaction with both HB−CoA and HB−NAC has intermediate $M_w$. These results can be accommodated by two models. In the first, NAC liberated at the active site during polymerization acts as a chain transfer agent. In the second, there is a noncovalent polymer intermediate covalently linked to NAC, which can dissociate from the active site.

## Introduction

Polyhydroxyalkanoic acids (PHAs) are polyesters produced by a wide range of microorganisms as intracellular storage materials when limited for a nutrient other than carbon.[1,2] Over 125 different constituents have been identified in PHAs produced under a variety of growth conditions in vivo.[1,3] These biodegradable materials exhibit a range of properties from elastomers to thermoplastics.[4] In the past decade efforts have focused on generation of PHAs in a biorenewable source such as bacteria or plants.[5] More recently efforts have focused on identifying a specialty niche for these materials in tissue engineering or coatings and paints.[6−8] Understanding the chemistry and biology of PHA production in vitro and in vivo is an essential step in making these materials in a useful form, including controlling the molecular weight and polydispersity of the polymers.

PHA synthase catalyzes the formation of insoluble PHA from soluble (*R*)-3-hydroxyacyl-CoA substrates, and the product accumulates in the cytoplasm of cells in the form of insoluble inclusions or granules.[2] Four classes of PHA synthase have been identified, their classification being based on their subunit composition and substrate specificity.[9,10] Classes I and III synthases, share a substrate preference for HB−CoA and 3-hydroxyvaleryl-CoA, but the class I synthase consists of a single ∼65 kDa subunit (PhaC), whereas the class III synthase is made of two subunits, each ∼40 kDa (PhaC and PhaE, referred to subsequently as PhaEC).[9,11] The mechanism of elongation (Scheme 1) in the prototypical class I synthase $PhaC_{We}$ from *Wautersia eutropha* (formerly *Ralstonia eutropha*[12]) and class III synthase $PhaEC_{Av}$ from *Allochromatium vinosum* (formerly *Chromatium vinosum*[13]), both involve a covalent intermediate **2** where the synthase is acylated at an active site cysteine residue[14−16] and have been proposed to involve a noncovalent intermediate **1**.[11] Little is known about the mechanism of termination.

It has long been known that chain termination and reinitiation occurs in vivo in wildtype *Wautersia eutropha*,[17] and we have recently performed a quantitative analysis showing that ∼350 PHB molecules are synthesized per synthase when cells are cultured to produce high amounts of PHB.[18] The literature contains only one model for the mechanism of termination (Scheme 2), in which a nucleophilic "chain transfer agent" attacks at the active site thioester of **2**, thereby releasing PHB and terminating elongation.[17] Activated water,[17,19] CoA[20,21] and hydroxybutyrate[22] have been proposed to act as agents to terminate the elongation reaction via chain transfer in vivo.

Supporting evidence for this model has come from the observation that addition of alcohols (poly(ethylene glycol),[23] glycerol,[22] 1,3-propanediol[22] and others) to *W. eutropha* grown under PHB producing conditions results in PHB with a lower molecular weight ($M_w$) compared to PHB from fermentations lacking these compounds. Furthermore, the polymers produced in the presence of these alcohols have

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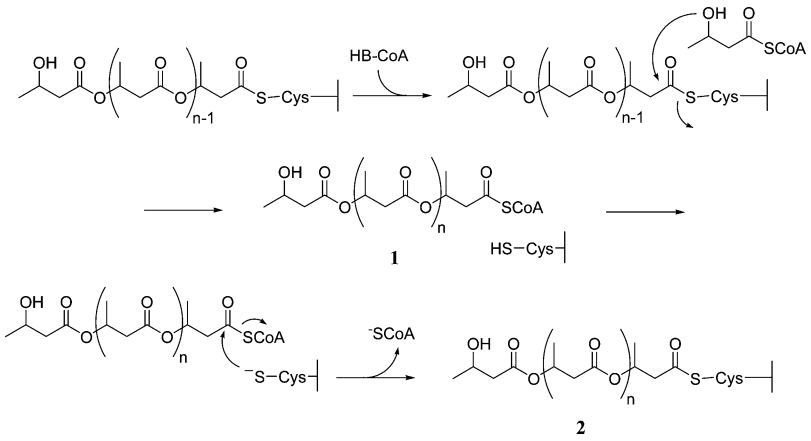
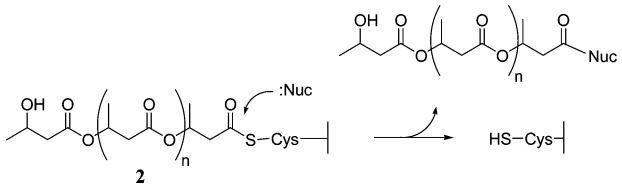
**Scheme 1**




**Scheme 2**




been isolated and characterized by NMR and shown to be attached covalently to the carboxy terminus of the PHB chain.[22,24,25] These alcohols are proposed to act as more efficient chain transfer agents than the candidates for termination in vivo under normal growth conditions (e.g., water, hydroxybutyrate[17,22]). An increased rate of termination by chain transfer accounts for the observed decrease in $M_w$.

In contrast to the observations in the wild-type organism, when the *W. eutropha* class I synthase is heterologously expressed in *Escherichia coli*, the $M_w$ of PHB depends on the $PhaC_{We}$ activity.[26] In addition, enzyme concentration influences molecular weight in vitro.[27] These data suggest that this synthase is not capable of carrying out chain termination by itself and that the chain transfer model needs to be reevaluated.

The class III synthase, $PhaEC_{Av}$, has not been studied in vivo. In vitro, however, the $M_w$ of PHB produced from $PhaEC_{Av}$ has been reported to be independent of substrate-to-enzyme ratio, suggesting that this enzyme is capable of performing chain termination[11,20,28] and may be a good system in which to study the chain termination reaction. In the present paper, we report the use of a substrate analogue (*R*)-3-hydroxybutyryl-*N*-acetylcysteamine (HB−NAC), in which the CoA moiety has been replaced by *N*-acetylcysteamine (NAC). As we recently reported,[29] this analogue is a substrate for both class I and III synthases with turnover numbers 1/5000 and 1/100, respectively, of those observed with HB−CoA. Initial studies with $PhaEC_{Av}$ and HB−NAC on the termination process in vitro are presented.

## Materials and Methods

**Materials**. $PhaEC_{Av}$ was isolated as previously described[16] and had a specific activity of 140 $\mu$mol/min/mg. HB−CoA and HB−NAC were prepared as described by Yuan et al.[29] The concentration of the former was determined using $\epsilon_{260\ nm} = 16.4\ cm^{-1}\ mM^{-1}$ (pH 7) and the latter using $\epsilon_{234\ nm} = 5.33\ cm^{-1}\ mM^{-1}$ (pH 7).

**In Vitro PHB Production from HB−NAC or HB−CoA and $PhaEC_{Av}$.** All reactions were carried out at 30 °C in 25 mM sodium phosphate (pH 7.8), 22 mM NaCl with variable concentrations of HB−NAC or HB−CoA (ranges are listed in table legends). The reaction was initiated by the addition of 1.0 $\mu$M $PhaEC_{Av}$. The substrate consumption was monitored using Ellman's reagent and the discontinuous assay method.[16] Under the assay conditions the synthase was shown to retain enzymatic activity after 16 h (data not shown).

**Preparation of PHB from in Vitro Reactions for $M_w$ Analysis.** When the reaction reached completion (6 h for HB−NAC, 10 min for HB−CoA), Proteinase K was added to remove $PhaEC_{Av}$ (40 U per mg $PhaEC_{Av}$) and the reactions were incubated at 37 °C for 16 h. Samples were frozen at −80 °C and lyophilized. The residue was extracted with 2 mL of $CHCl_3$ by refluxing for 16 h. $CHCl_3$ was removed with $N_2$, and the residue was redissolved in 2,2,2-trifluoroethanol (TFE) (Aldrich, St. Louis, MO) to give a concentration of 0.25 mg/mL for HB−CoA samples or 1.0 mg/mL for HB−NAC samples. Samples were then filtered through a 0.2 $\mu$m PTFE membrane (Pall, East Hills, NY) and used for gel permeation chromatography (GPC).

**Preparation of PHB from in Vitro Reactions for NMR**. When the reaction was complete (6 h), the sample was centrifuged (5000 × g, 30 min) and the pellet was dried in vacuo. The pellet (6 mg PHB) was dissolved in 700 $\mu$L of $CDCl_3$ (Cambridge Isotope Laboratories, Inc., Andover MA) by refluxing for 2 h, cooled, and analyzed by NMR spectroscopy. After the first analysis by NMR, the sample was repurified twice by precipitation with 3 volumes of cold methanol (−20 °C). The precipitant was collected by centrifugation (5000 × g, 30 min) and dissolved in $CDCl_3$ (reflux 2 h). This repurified sample was then reanalyzed by NMR spectroscopy.

**Molecular Weight Determination of PHB**. Coupled multi-angle light scattering (LS) and GPC were used to determine the $M_w$ of PHB. LS was performed using a DAWN-EOS ($\lambda$ = 690 nm) multiangle laser photometer (Wyatt Technology Corporation, Santa Barbara, CA). GPC was carried out on a Knauer HPLC (Berlin, Germany) attached to PLgel 10 $\mu$m Mixed-B (for in vitro PHB samples) or 20 $\mu$m Mixed-A (for PHB from *E. coli*) column (Polymer

Laboratories, Amherst MA). A Wyatt Optilab DSP differential refractometer was used for quantitation of PHB (d*n*/d*c* = 0.144). TFE was used as a mobile phase at a flow rate of 1.0 mL/min and 100 μL were injected. Narrow molecular weight poly(methyl-methacrylate) (PMMA) (18.2 kDa) (cat. no. 602, Scientific Polymer Products, Ontario, NY) was used to normalize LS detector and other PMMA standards in the range of 20−2000 kDa were used to confirm calibration (cat. no. STD-4) (d*n*/d*c* = 0.172). The calculation of molecular weight from light scattering was performed with ASTRA 4.9 software (Wyatt Technology) employing the Zimm formalism.

**NMR End Group Analysis of PHB.** PHB (6 mg) was isolated as described above from a polymerization reaction containing 7.5 mM HB−NAC and 1.0 μm $PhaEC_{Av}$ and analyzed by NMR spectroscopy. $^1$H NMR spectra were recorded at room temperature on a VARIAN Inova-500 spectrometer (Varian, Palo Alto, CA). Chemical shifts were determined relative to $CHCl_3$ ($\delta = 7.27$ ppm): $\delta = 1.23$ (d, 3H, $J = 6.4$ Hz, C*H*$_3$CHOH), 1.28 (d, 3H, $J = 6.1$ Hz, OCH(C*H*$_3$)CH$_2$), 1.98 (s, 3H, NHCOC*H*$_3$), 2.62 and 2.48 (dd, $J_1 = 7.3$ Hz, $J_2 = 15.7$ Hz, dd, $J_1 = 5.7$ Hz, $J_2 = 15.7$ Hz, 2H, OCH(CH$_3$)C*H*$_2$), 2.81, (dd, 2H, $J_1 = 8.0$ Hz, $J_2 = 15.7$ Hz, OCH(CH$_3$)C*H*$_2$COS), 3.05 (td, 2H, $J_1 = 1.2$ Hz, $J_2 = 6.3$ Hz, C*H*$_2$NH), 3.43 (qd, 2H, $J_1 = 2.5$ Hz, $J_2 = 6.3$ Hz, SC*H*$_2$-CH$_2$), 4.19 (m, 1H, CH$_3$C*H*OHCH$_2$), 5.26 (sex, 1H, $J = 6.1$ Hz, OC*H*(CH$_3$)CH$_2$), 6.16 (m, 1H, N*H*).

**Production of PHB in Recombinant *Escherichia coli*.** PHB was produced in *E. coli* DH5α (New England Biolabs, Beverly, MA) harboring the plasmid pAeT41[30] (*phaCAB*$_{We}$ operon in pUC18 (Invitrogen, Carlsbad, CA)) or pJOE7 which is identical to pAeT41, except the *phaC*$_{We}$ ORF has been precisely replaced by the *phaEC*$_{Av}$ ORFs. pJOE7 was constructed by ligating the 2.2 kb BamHI/PstI fragment of pGY53[31] into the 5.3 kb BamHI/PstI fragment from pAeT41. For PHB production, a fresh transformant was picked into 5 mL of LB ampicillin (100 μg/mL) and grown at 37 °C for 6 h. This preculture (1 mL) was used to inoculate duplicate cultures of 100 mL of LB amp + 2% glucose (w/v) either with or without 1% (w/v) 1,3-propanediol in 500 mL baffled flasks. Cultures were grown 24 h at 37 °C with shaking at 140 rpm (Infors CH-4103 incubator, Bottmingen, Switzerland).

**Isolation of PHB from *E. coli*.** At 24 h, 10 mL of each culture (described above) were pelleted (5000 × g, 5 min) in a test tube, resuspended in 10 mL cold (4 °C) distilled water, pelleted again (5000 × g, 5 min), and resuspended in ~0.5 mL of residual supernatant. Samples were then frozen at −80 °C and lyophilized. Samples were extracted in their original test tubes by refluxing with 5 mL of $CHCl_3$ for 4 days with stirring, with solvent added as needed to replace solvent lost to evaporation. Samples were then brought up to 5 mL and transferred to a fresh vial where they were dried under $N_2$ and then redissolved in 5 mL of TFE. Samples were diluted to 0.25 mg/mL and filtered through a 0.2 μm PTFE membrane (Pall, East Hills, NY). $M_w$ analysis was carried out as described above.

**Table 1.** $M_w$ of PHB from HB−NAC

| S/E[a] | $M_w$ (Da)[b] |
|---|---|
| 1500 | $5.84 \pm 0.12 \times 10^4$ |
| 3000 | $7.40 \pm 0.01 \times 10^4$ |
| 6000 | $7.97 \pm 0.04 \times 10^4$ |
| 12 000 | $7.36 \pm 0.09 \times 10^4$ |
| 15 000[c] | $7.34 \times 10^4$ |

[a] All reactions carried out with 1 μM $PhaEC_{Av}$ and the appropriate concentration of HB−NAC. S/E = molar ratio of HB−NAC to $PhaEC_{Av}$ dimer. The PHB synthase has been shown to be active as a dimer of two $PhaEC_{Av}$.[14] [b] Results are from duplicate reactions, except at S/E = 15 000. [c] Sample used for NMR analysis.

## Results

**$PhaEC_{Av}$ Produces PHB of low $M_w$ from HB−NAC.** In vitro reactions were carried out varying the molar ratio of substrate to enzyme (S/E) from 1500 to 15 000 (Table 1). PHB was extracted and $M_w$ was characterized by coupled gel permeation chromatography and multi-angle light scattering (GPC/LS). At all molar ratios of S/E above 1500 the $M_w$ was ~75 kDa. Since all the HB−NAC was consumed and incorporated into PHB, termination or transfer must be occurring ~20 times during the course of substrate consumption at S/E = 15 000.

**$^1$H NMR Shows that PHB−NAC Is Produced from HB−NAC.** Since the polymer was small (<1000 monomer units), previous results[32] suggested that the polymer ends could be resolved by $^1$H NMR. At the hydroxy terminus, we expected to see a multiplet at $\delta = 4.19$ for the indicated proton (CH$_3$C***H***OHCH$_2$), and comparison of its integration to the main chain protons could be used to determine $M_n$. We observed the expected multiplet at $\delta = 4.19$ (Figure 1c) and integration of this peak relative to the internal PHB peaks (Figure 1b, $\delta = 1.28$, 2.48 and 2.62, 5.26) indicated a degree of polymerization of ~700, or $M_n$ of $\sim 6.1 \times 10^4$ Da, which agrees well with our observed $M_w$ of $7.34 \times 10^4$ Da.

In addition, we observed a set of resonances close to, but not identical to, the NAC moiety of HB−NAC (Figure 1c, $\delta = 1.98$, 3.05, 3.43, 6.16). These resonances were present in stoichiometric proportions to the peak at $\delta = 4.19$. After repurification of the sample and repetition of the experiment failed to alter the peak ratios, we concluded that we had synthesized PHB covalently modified with NAC (PHB−NAC). We ruled out contamination with HB−NAC by spiking the sample with HB−NAC, which produced resonances that did not overlap with those assigned to PHB−NAC (data not shown).

**Exogenous NAC Does Not Promote Chain Termination in Vitro.** Since we had synthesized PHB−NAC instead of PHB, we reasoned that NAC in the thiolate form may be a more efficient chain transfer agent (nucleophile) than water and thereby promote premature termination of the elongation reaction. To test this model, we carried out in vitro polymerizations using both HB−CoA and HB−NAC substrates with and without added NAC. Little or no change was observed in the $M_w$'s of the PHB derived from either substrate in the presence of NAC (Table 2 reactions A vs B and D vs E). This indicates that exogenous NAC does not act as a chain transfer agent.

**1,3-Propanediol Does Not Promote Chain Termination in Vitro.** Madden et al. reported that adding 1 g/L 1,3-

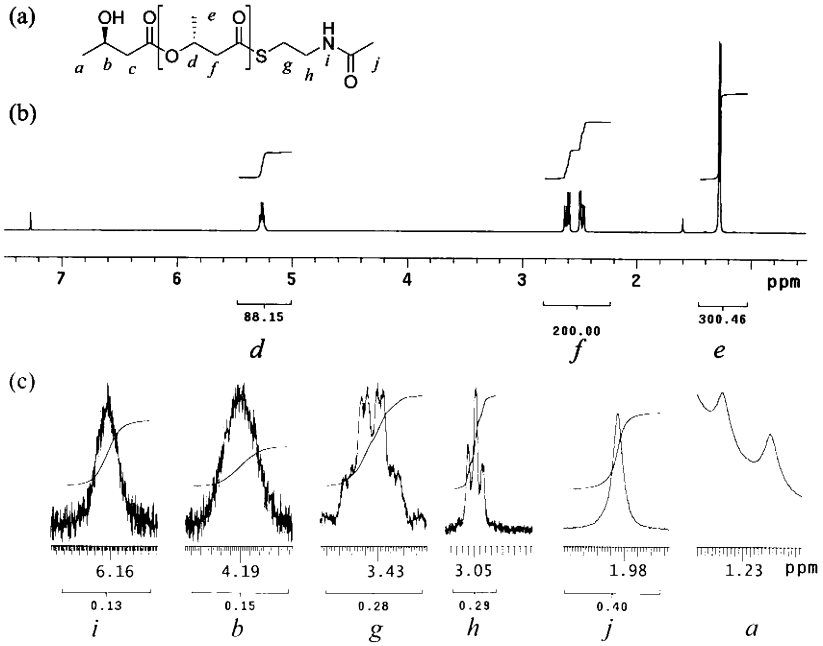



**Figure 1.** (a) Structure of PHB-*N*-AC with protons labeled. (b) $^1$H NMR spectrum of PHB−NAC. Numbers beneath scale indicate relative integrated areas of each peak. (c) Blowup of regions containing resonances for endgroup protons a, j, h, g, b, and i ($\delta = 1.23$ ($CH_3CHOH$), 1.98 ($NHCOCH_3$), 3.05 ($CH_2NH$), 3.43 ($SCH_2CH_2$), 4.19 ($CH_3CHOHCH_2$), and 6.16 (N*H*), respectively). Numbers beneath scale indicate relative integrated areas of each peak.

**Table 2.** PHB from Mixed Polymerizations

| reaction[a] | substrate | addition | $M_w$ (Da) |
|---|---|---|---|
| A | 6 mM HB−NAC | none | $6.87 \pm 0.13 \times 10^4$ |
| B | 6 mM HB−NAC | 10 mM NAC | $6.44 \pm 0.10 \times 10^4$ |
| C | 6 mM HB−NAC | 10 mM 1,3-propanediol | $6.85 \pm 0.07 \times 10^4$ |
| D | 7 mM HB−CoA | none | $1.71 \pm 0.06 \times 10^6$ |
| E | 7 mM HB−CoA | 10 mM NAC | $1.69 \pm 0.02 \times 10^6$ |
| F | 7 mM HB−CoA | 10 mM 1,3-propanediol | $1.76 \pm 0.04 \times 10^6$ |
| G | 7 mM HB−CoA + 7 mM HB−NAC | none | $4.31 \pm 0.40 \times 10^5$ |

[a] All reactions carried out with 1 $\mu$M $PhaEC_{Av}$. Results are from duplicate reactions.

propanediol to fermentations of *Wautersia eutropha* produced a ~50% reduction in PHB $M_w$.[22] We performed in vitro polymerizations using HB−NAC or HB−CoA in the presence of 1,3 propanediol. As with exogenous NAC, little or no effect on $M_w$ was observed (Table 2, reaction A vs C and D vs F).

None of the experiments in the literature showing the production of reduced $M_w$ PHB in vivo from fermentations in the presence of exogenous alcohols had been carried out with an organism containing a class III synthase. Therefore, we performed the following control experiment. PHB was produced in recombinant *Escherichia coli* harboring the plasmid pAeT41, which contains the *phaCAB* operon from *W. eutropha* (encoding class I PHA synthase, $\beta$-ketothiolase and reductase, respectively), or the plasmid pJOE7, which is identical to pAeT41 except the $phaC_{We}$ ORF is precisely replaced by the cotranscribed ORFs for $phaE_{Av}$ and $phaC_{Av}$. Cells were cultured in media with and without 1,3-propanediol and PHB was extracted and characterized by GPC/LS. PHB produced in the presence of propanediol had a much lower $M_w$ in each strain (Table 3). To our knowledge, this constitutes the first observation of this phenomenon in an organism that does not naturally produce PHB. We also performed an analogous experiment in wildtype *W. eutropha* and in a *W. eutropha* strain in which the class I synthase $PhaC_{We}$ was replaced by $PhaEC_{Av}$ and saw similar results (data not shown).

**Table 3.** $M_w$ of PHB from *E. coli* DH5α[a]

| | $M_w$ (Da) | |
|---|---|---|
| 1,3-propanediol | pAeT41[b] | pJOE7[c] |
| none | $3.83 \pm 0.10 \times 10^6$ | $1.72 \pm 0.04 \times 10^6$ |
| 1% | $6.04 \pm 0.66 \times 10^5$ | $1.22 \pm 0.04 \times 10^5$ |

[a] Cultivated in LB with 2% glucose and 100 $\mu$g/mL ampicillin. [b] Encodes $phaC_{We}$, $phaA_{We}$, and $phaB_{We}$. [c] Encodes $phaEC_{Av}$, $phaA_{We}$, and $phaB_{We}$.

**HB−NAC Promotes Premature Chain Termination during Heteropolymerization of HB−CoA and HB−NAC.** Finally, we carried out a heteropolymerization reaction using both HB−CoA and HB−NAC (Table 2, reaction G). The rate of thiolate release was biphasic in these reactions, with a rapid phase followed by a slow phase (data not shown). The $M_w$ of PHB produced in this reaction was 430 kDa and showed a bimodal distribution (Figure 2). The elution volume of the later eluting peak was close to the elution volume of the peak of PHB from a HB−NAC homopolymerization reaction, indicating a similar $M_w$. In contrast, the earlier eluting peak eluted between the elution volume of PHB produced from HB−CoA and HB−NAC

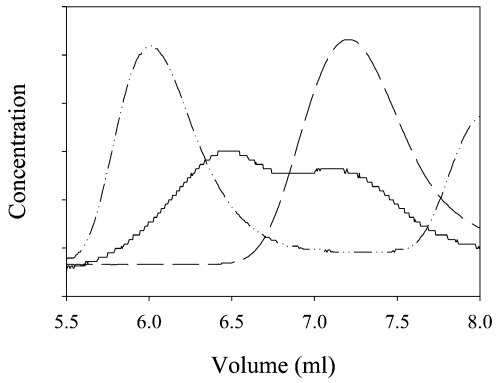



**Figure 2.** GPC of PHB from in vitro reactions of HB−CoA (-·· ·-), HB−NAC (− −), and HB−CoA and HB−NAC (—). Concentration axis of each sample was normalized to produce similar sized peaks.

homopolymerizations. The intermediate $M_w$ of the earlier peak is likely due to some incorporation of HB−NAC monomers that are able to promote premature termination during the fast phase of predominantly HB−CoA polymerization. The result of this mixed polymerization shows that only when delivered to the active site during polymerization is NAC (or HB−NAC) able to terminate the growing PHB chain.

## Discussion

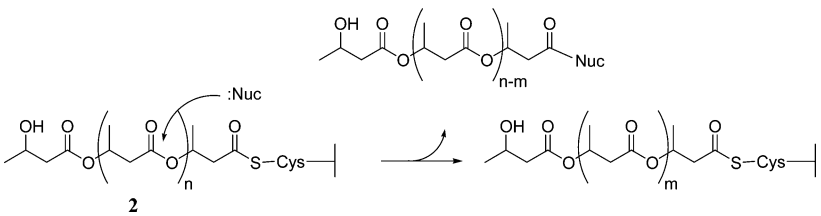
The mechanism of PHA polymerization has been shown to involve covalent catalysis.[14,16] According to these models, a dimer of PHA synthase catalyzes both the initiation and elongation of PHB chains. Here we provide evidence that the class III PHA synthase carries out a chain termination reaction and propose models for how this might occur.

In this report, we show that low $M_w$ PHB is produced from HB−NAC (Table 1). As reviewed in the Introduction, the prevailing model of chain termination involves transfer of the elongating PHB to a nucleophile (water,[17] hydroxybutyrate,[22] or CoA[20]) in the active site by attack at the thioester of **2**, regenerating unmodified enzyme (Scheme 2). We show that each PHB chain synthesized from HB−NAC ends in a NAC moiety (Figure 1), so if chain transfer is occurring, it must be NAC that is the chain transfer agent. However, increasing the concentration of exogenous NAC or adding NAC to HB−CoA polymerizations has little or no effect on $M_w$ (Table 2). $M_w$ is, however, altered in heteropolymerization reactions of HB−NAC and HB−CoA, and two populations of PHB chains are produced (Table 2, Figure 2). The early eluting population has an intermediate $M_w$ relative to PHB produced in homopolymerization reactions of each substrate. We believe that this intermediate $M_w$ population is produced during the early phase of polymerization, when all of the HB−CoA is consumed and that incorporation of some HB−NAC derived monomers during this phase increases the rate of chain termination.

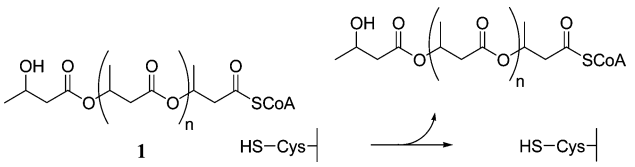
HB−CoA and HB−NAC are both substrates for the synthase and consequently can function as competitive inhibitors of one another. The initial rates of substrate incorporation will be governed by the formula $v_0 = v_{max}[S]/(K_m' + [S])$, where $K_m' = (K_m \times (1 + [I]/K_i)$ and $v_{max} = k_{cat}[E]$. Given a $K_m$ and $k_{cat}$ of 0.13 mM and 3920 min $^{-1}$ for HB−CoA and 8.6 mM and 39 min$^{-1}$ for HB−NAC and [PhaEC$_{Av}$ dimer] = 0.5 μM, $v_0$ for each substrate will be 1960 μM/min for HB−CoA and 0.29 μM/min for HB−NAC, a 6700-fold difference. As the concentration of HB−CoA declines, the rate of HB−NAC consumption will increase and the rate of HB−CoA consumption will decrease slightly so that that the average difference in $v_t$ throughout the course of HB−CoA consumption will be ∼3500-fold. The $M_w$ of the PHB fraction produced during this fast phase was not determined by LS since it overlaps with the later eluting fraction, but based on elution volume it can be estimated to be around 400 kDa, which corresponds to a degree of polymerization of 5000 or less depending on the sample's polydispersity. If we are correct that HB−NAC incorporation is responsible for the observed premature termination, then it is extraordinarily efficient as a terminator in this reaction. Yet, on its own, HB−NAC will produce polymer that contains an average of 700 monomers. This implies there is a change in the system during polymerization of HB−NAC that allows termination to occur only after several hundred monomers have been incorporated. The existence of this change from a highly processive to a less processive state is also suggested by the fact that reports of PHB polydispersity in the literature are typically low. Understanding this process is a problem itself that we are currently investigating and is distinct from determining the mechanisms of termination, which we address in this study.

Scheme 2 shows the prevailing model for the mechanism of chain termination. We recently reported observations consistent with an alternative mechanism of termination by chain transfer,[28] distinct in that after chain transfer, the enzyme is left covalently linked to an HB oligomer at the active site cysteine. Performing SDS−PAGE and autoradiography on in vitro reactions of PhaEC$_{Av}$ and [$^{14}$C]-HB−CoA at S/E = 45, we saw the time-dependent formation of a small uniformly labeled species well *after* the polymerization reaction had finished. This species appeared to be generated from a larger labeled species. This suggested a model similar to that of the polyketide synthases,[33] in which chain transfer occurs, but at an internal ester bond within **2** rather than at the thioester linkage itself (Scheme 3). Hydrolysis would thereby release most of the PHB chain but leave behind enzyme primed with a short HB oligomer.

**Scheme 3**



**Scheme 4**




If chain termination is in fact occurring by nucleophilic attack of NAC in the thiolate form at either the active site thioester or an internal PHB ester, it only occurs when NAC is released in the active site during polymerization of HB−NAC. Exogenous NAC, added to the reaction, is unable to promote termination. NAC exiting the active site after donating its HB moiety to the growing PHB chain is either in a favorable position to attack the active site thioester or it passes through an exit channel that is not solvent accessible and can attack the PHB chain during exit. However, both of these explanations are at odds with the observation that molecules as large as PEG-1000 can influence PHB $M_w$ in vivo.[23]

A third model to explain our results is based on a polymerization model that includes a noncovalent intermediate **1** (Scheme 4). In this model, substrate binds at the active site, and the hydroxyl is activated for nucleophilic attack on the PHB chain bound to the active site cysteine, transferring the chain to the noncovalently bound substrate. Either chain elongation continues as PHB is transferred back to the active site cysteine (Scheme 1) or termination occurs as **1** is released from the active site (Scheme 4). In this model, the $K_d$ of **1** affects the partitioning between the elongation and termination reactions, thereby influencing $M_w$. Chain length would be independent of substrate-to-enzyme ratio and independent of exogenous thiolate concentration in the reaction.

Presumably, PHB−CoA (**1**) would have a lower $K_d$ than PHB−NAC (identical to **1**, but CoA replaced by NAC), similar to the difference in $K_m$ of the respective substrates (HB−CoA $K_m = 0.14$ mM; HB−NAC $K_m = 8.6$ mM). The differences in $K_d$ would account for the $M_w$'s observed in our work. In this model, every chain produced from HB−NAC would be modified with NAC as we showed. Additionally, reactions with mixed polymerizations would be expected to produce intermediate chain lengths, since addition of HB−NAC to a long, HB−CoA derived PHB chain would produce a noncovalent intermediate with a much higher $K_d$ and early chain termination.

None of the models discussed explain the observation that propanediol does not increase the rate of chain termination in vitro although it readily causes production of lower $M_w$ PHB from the class III synthase in *E. coli* (Tables 2 & 3). Since we were able to produce this $M_w$ reduction in *E. coli*, we are able to rule out the possibility that propanediol reduced $M_w$ in *W. eutropha* by activating a PHB depolymerase. Based on our in vitro findings, we believe that alcohols are not in fact involved in a chain termination reaction in vivo. Instead, we propose that the alcohols examined become associated with the PHB granule and undergo a transesterification reaction with PHB during the long time scale of fermentation, hydrolyzing the PHB chain. This would be consistent with observations of much higher polydispersities in alcohol supplemented media than are typically observed for PHB from enzymatic polymerization.

Currently, this disparity between in vivo and in vitro remains unexplained and the mechanism of termination is unclear. We are carrying out further experiments to distinguish among the models proposed. We believe that by probing this process in vitro we are gaining insights that may be obscured in in vivo experimentation and that the mechanism for chain termination will ultimately prove to be quite different from the model in the literature.

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