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Extracellular polymerization of 3-hydroxyalkanoate monomers with the polymerase of *Alcaligenes eutrophus*

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Abstract

Previous investigations on the role of the polymerase in the synthesis of poly-3-hydroxybutyrate (PHB) are reviewed, and the results from earlier in vitro studies on the activity and selectivity of the polymerase of *Alcaligenes eutrophus* are discussed. In the present study the effect of glycerol on stabilizing the polymerase after purification and on eliminating the lag phase in in vitro polymerization reactions of 3-hydroxybutyl CoA (HBCoA), and 3-hydroxyvaleryl CoA (HVCoA) are described. K_M values were determined for the activity of the polymerase with both HBCoA and HVCoA, and the rates of propagation for both monomers were estimated. With a racemic mixture of HBCoA, the enzyme polymerized only the [*R*] monomer.

Keywords: Extracellular polymerization; 3-Hydroxyalkanoate monomers; Polymerase; *Alcaligenes eutrophus*

1. Introduction

Poly-3-hydroxybutyrate (PHB) is produced in *Alcaligenes eutrophus* from acetyl CoA (AcCoA) in three steps, each of which is catalyzed by an appropriate enzyme, as follows [1,2]: (1) the dimerization of AcCoA to a four-carbon intermediate, acetoacetyl CoA (AcAcCoA), by a Claisen condensation-type reaction catalyzed by a β-ketothiolase; (2) the hydrogenation of AcAcCoA to the actual monomer for the polymerization reaction, [*R*]-3-hydroxybutyryl CoA (HBCoA), catalyzed by a reductase; and (3) the chain growth polymerization of HBCoA to high molecular weight PHB, with the release of AcCoA, catalyzed by a synthase, which, in this case, serves as a polymerase. The activities and reaction mechanisms of the β-ketothiolase [2–5] and the reductase [5,6] were studied and quantified well before those of the polymerase primarily because of the difficulty in isolating the latter in sufficient amounts and purity in an active form [5,6]. However, as discussed below, the polymerase of *A. eutrophus* is now available in reasonable quantity, purity and activity to make such studies possible, and investigations are now proceeding rapidly on the kinetics and mechanism of the polymerization reaction of hydroxyalkanoyl CoA monomers with this bacterial polymerase.

2. Historical review

The role of a polymerase in the bacterial production of PHB from AcCoA was first inferred by Merrick and Doudoroff in 1961 [7] 35 years after the initial report on, and identification of, PHB as an inclusion body in a bacterium by Lemoigne [8,9]. Lemoigne carried out his pioneering investigations with *Bacillus megaterium*,

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while Merrick and Doudoroff investigated both that bacterium and *Rhodospunllum rubrum* in their initial studies. In their subsequent investigations in 1968, Merrick et al. [10] carried out kinetic studies on the polymerization of HBCoA with active granules from *B. megaterium*. In that report they refer specifically, apparently for the first time, to the role of a 'PHB synthetase' in catalyzing the polymerization of HBCoA, and they proposed that the active site of the polymerase contains a sulfhydryl (thiol) group [11]. That proposal was expanded much later (1987) into a more detailed mechanism of the reactions involved at the active site of the enzyme for the polymerization of HBCoA by Ballard et al. [12]. Their proposal was subsequently elaborated by Doi et al. [13,14].

Merrick and coworkers proposed that the active site in the PHB polymerase contains a thiol group from a cysteine unit, which is covalently bound as a thioester derivative to the growing polymer chain. Subsequently, it was proposed that a second thiol group is also involved in the active site and that it forms a thioester with the next, or incoming, monomer to be inserted into the polymer chain [12]. To date, only one of the thiol groups on a cysteine unit has been identified, Cys 319, but more recent studies indicate that, for the *A. eutrophus* polymerase, the second thiol group may be derived by the post-translational modification of a serine unit with phosphopanthetheine, possibly at Ser 260 [15]. This unit and the Cys 319 unit, although widely spaced in the protein, would provide the two thiol groups necessary for the Merrick-Ballard mechanism of the chain-growth polymerization reaction, but the protein would have to adopt a conformation that would bring these two units in close proximity to create the active site.

2.1. Characterization of polymerase activity

In their pioneering studies in 1961 on the active 'particles' (inclusion bodies) of both *B. megaterium* and *R. rubrum*, Merrick and Doudoroff [7] carefully separated the 'particles' from both types of cells so that the polymerase remained active and the polymerization reaction could be continued in vitro. The particles were reacted with C-14-labeled HBCoA, and the PHB of the particles became labeled with C-14 units, which verified the presence of polymerase activity on the particles, although the terms 'polymerase', 'synthetase' or 'synthase' were not used in that study [7]. That is, as noted above, only in their later report on the determination of the rate of polymerization of HBCoA with their active 'particles' (which are referred to in the more recent literature as 'granules'), did Merrick and coworkers specify that a 'synthetase' was responsible for the polymerization reaction [10,11]. In those studies they also attempted to, but could not, obtain an active polymerase from the granule in soluble form [10]. However, they were able to determine the effect of incubation time, protein concentration and pH on the rate of polymerization of HBCoA with the bound polymerase. In addition, they determined a value K_M for the granule-bound polymerase of *R. rubrum* with HBCoA of 9.25×10^{-5} M at pH 7.5 using the Lineweaver-Burk method, although in a subsequent study a different value for K_M of 3.12×10^{-4} M was found [11]. In the latter study more intensive efforts to obtain an active polymerase in soluble form were again unsuccessful, but instead they concluded that the presence of the granules was necessary to obtain an active polymerase [11]. They also showed that the polymerase was inactivated by sulfhydryl inhibitors.

A soluble polymerase was apparently first obtained in 1976, in the work of Tomita et al. on *Zooloea ramigera* [16,17]. They studied both the granule-bound and soluble forms of the polymerase of that bacterium. In their case the soluble enzyme was active, and a K_M of 5.3×10^{-5} M was obtained for the polymerase with HBCoA.

Following that investigation, in 1989, Haywood et al. reported on the isolation of a soluble polymerase from *A. eutrophus* in active form, and they determined the K_M values of the enzyme in both the granule-bound and soluble forms [18] with both HBCoA and [*R*]-3-hydroxyvaleryl CoA (HVCoA). The K_M for the granule-bound enzyme with HBCoA was 0.68×10^{-3}, while that for HVCoA was 1.63×10^{-3} M at pH 8.5. In terms of enzyme activity, HBCoA was estimated to be about 13 times more active than HVCoA. The soluble polymerase, which was unstable, had a K_M of 0.72×10^{-3} M for HBCoA.

Haywood et al. also investigated the activity of the enzyme for the polymerization of higher 3-hydroxyalkanoate monomers; i.e. those containing from six to ten carbon atoms. They found that only the four- and five-carbon monomers could be polymerized with the granule-bound polymerase [18]. In addition, they found that the polymerase was active only for [*R*]-HBCoA and not for the [*S*] isomer, which was also observed by Fukui et al. for the polymerase of *Z. ramigera* [16,17].

2.2. Isolation and activity of soluble synthase

The breakthrough in efforts to characterize the activity and selectivity of the polymerase of *A. eutrophus* came in the substantial production of that enzyme by a recombinant strain of *E. coli*, which contained the polymerase gene but not the genes for monomer production. The recombinant cells could be made to overexpress the enzyme, which, in the absence of HBCoA, was present in sufficiently high yields to be extracted and purified to a highly active, water soluble form [15,19]. In this manner, the polymerase of *A. eutrophus*

[15] and subsequently by the same route that of *Chromanum vinosium* [20], was isolated and characterized for composition and activity with HBCoA.

The soluble polymerase of *A. eutrophus*, which was isolated in active form by Sinskey et al., was found to have a molecular weight of approximately 64 000 Da [15]. Kinetic analysis of the polymerization reaction of HBCoA by this enzyme showed a lag phase, which increased in time of duration with decreasing polymerase concentration. This observation suggested that the initiation process of the polymerization reaction was much slower than the propagation (elongation) process with the enzyme catalyst, and they proposed that activation of the catalyst may require a 'priming process', which involved formation of a dimeric form of the enzyme, before polymerization could occur.

In a follow-up study, Sinskey et al. first reacted the polymerase with an oligomer of HB as a 'priming' step to eliminate the lag period, and they analyzed the reaction products by size exclusion chromatography [21]. Two peaks were present in the chromatogram, and were assigned to the monomeric and dimeric forms of the enzyme. On addition of HBCoA the dimeric form was found to be much more active than the monomeric form, which seemed to verify the suggestion that the dimer was the active species. However, there may be an alternative explanation for that observation, which is that the 'dimer' species was formed by a physical association of the water-insoluble, oligomeric chains attached to two 'primed' enzyme molecules. Conversely, the monomeric form could be a fraction of the enzyme molecules that had not formed derivatives with the oligomers and, therefore, had not been 'primed', so their ability to initiate a polymerization reaction would be much lower.

A more detailed in vitro study of the polymerization process of HBCoA catalyzed by this polymerase in aqueous solution was subsequently carried out by Gerngross and Martin [22,23]. They followed monomer conversion quantitatively by a spectrophotometric method and also PHB formation by visually observing the amount of precipitated polymer, which they referred to as 'granules'. These 'granules' must be very different in composition and structure than the inclusion body 'granules' formed in vivo, which have organized protein coatings. The polymer so obtained had a weight average molecular weight, M_w, greater than 10^7 Da, and the M_w increased with decreasing polymerase concentration [22]. Their in vitro polymerization reaction also had a lag time, and the enzyme on the 'granules' was found to be much more active than the enzyme in solution. They attributed the higher activity of the former to the need for 'granule assembly', but as above and in a similar manner, an alternative possibility should be considered. That is, by the same process as above, it is likely that the enzyme in solution had not initiated a polymerization reaction while that associated with the 'granule' had and was covalently attached to a precipitated, growing polymer chain [22]. Therefore, because the initiation process was much slower (hence, the observed induction period) than the propagation process, their 'granule'-bound polymerase was much more active than the polymerase still in solution.

The soluble polymerase of *C. vinosium*, which was subsequently isolated by Steinbüchel et al. [20] has a considerably different structure than that of *A. eutrophus*. The *C. vinosium* polymerase contains two different proteins of 39 000 and 41 000 Da, which form a complex that catalyzes the polymerization of HBCoA with a K_M of 6.3×10^{-5} M. Their rate studies, however, indicated that the enzyme lost its activity during the polymerization reaction, and PHB yields of only 66–87% were obtained. In their case, the M_w of the polymer was in the range $1.0–2.1 \times 10^6$ Da, and it was independent of enzyme concentration. As a result, they concluded that each enzyme complex produced approximately 25 polymer chains and that some type of chain transfer reaction occurred during the polymerization.

3. Results

In the present study the polymerase of *A. eutrophus* was characterized for its activity with both HBCoA and HVCoA in aqueous solution. The polymerase used was obtained in the same manner from the same recombinant strain of *E. coli.* as that of Sinskey et al. [21].

3.1. Enzyme activity

The purified polymerase used in the present study was stored, after isolation from the cells, at −80°C, but the enzyme rapidly lost its activity, and after a daily freeze-thaw cycle for 3 days, the enzyme retained only about 10% of its initial activity. However, if a large amount of glycerol was added to the enzyme solution (50% by volume), the enzyme lost only about 20% of its activity in 3 days. Aliquots of these solutions were used for the polymerization reactions discussed below.

It was also observed that the purified polymerase, in the absence of any additives, also slowly lost its activity during the polymerization reaction so that when the initial polymerization reaction was nearly completed and the same amount was added again, the polymerization reaction continued at a much slower rate than initially. In contrast, the polymerase in the crude extract from the cells could catalyze several cycles of polymerization reactions without substantial loss of activity.

This observation suggests that some component was present in the crude extract that helped to stabilize the polymerases during the polymerization reaction, but

that component was removed during the purification process. Even so, when the crude extract was mixed with the buffered solution in the absence of HBCoA, the enzyme began to lose some of its activity within a few minutes, indicating that once polymerization began the enzyme involved in the reaction retains its activity. Also, the addition of BSA [21] to the reaction solution enabled the polymerase to retain its activity and to initiate at least three separate polymerization reactions without noticeable loss of activity, as shown in Fig. 1. As shown in this figure, the higher the concentration of BSA in the reaction mixture, the more the enzyme retained its activity.

3.2. Elimination of lag phase

It was observed in this study that the addition of glycerol to the polymerase solution eliminated the lag phase of polymerization reaction as well as helping to stabilize the enzyme for storage. The effect of the addition of 50% by volume of glycerol to the crude extract enzyme solution in eliminating the lag phase is shown in Fig. 2.

As discussed above, the presence of a lag phase in the in vitro polymerization reaction has been known for many years [20] but its cause is still unknown. Because a chain-growth polymerization reaction involves two different processes, initiation and propagation (elongation), it was recognized that the lag phase is a manifestation of the different rates of these processes, with a slower rate for initiation than for propagation. Consequently, a 'priming' reaction which accelerated the initiation process, could reduce or eliminate the lag phase [19,20]. This hypothesis, however, cannot explain our observation that addition of glycerol eliminates the lag phase because it is unlikely that glycerol functions as a reactive primer, although the enzyme still showed the lag phase when it was stored in a solution containing only 5% glycerol.

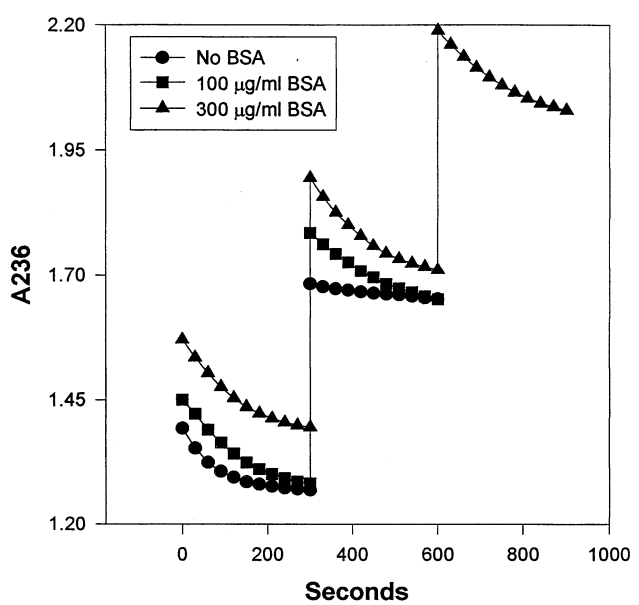

Fig. 1. Effect of addition of increasing amounts of BSA to the polymerization reaction solution on the activity of the polymerase over three cycles of polymerization reactions.

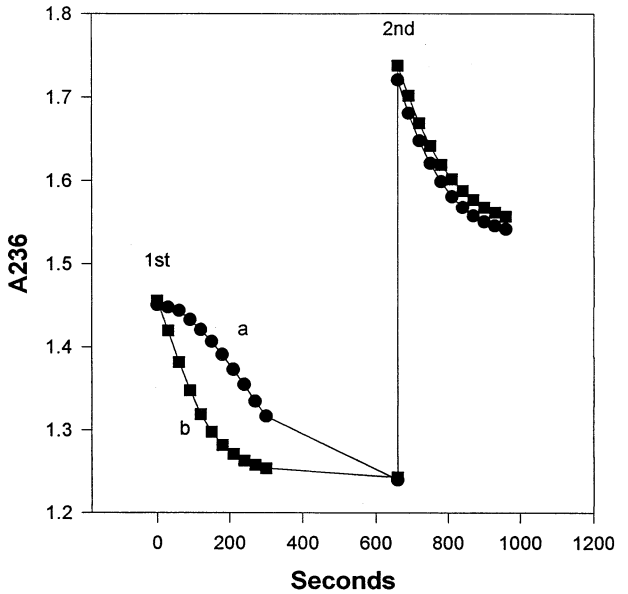

Fig. 2. Effect of addition of glycerol to the polymerization reaction solutions on the activity of the polymerase over two cycles of polymerization reactions: (a) crude extract solution containing polymerase in absence of glycerol; and (b) addition of 50% of glycerol by volume to crude extract solution.

Our results suggest that the observed lag phase is basically physical instead of chemical in nature. The addition of 'Hecameg' also reduces the lag phase [22,23] but it may do so in a different way. Nevertheless, the results with both glycerol and 'Hecameg' suggest that the monomeric form of the enzyme is the active form, not the dimeric form, especially in consideration of the hydrophobic character of the polymerase and the amphiphilic properties of these two reagents. Therefore, the observation that glycerol completely eliminated the lag phase indicates that the rate of the initiation reaction (not the initiation process) may not be greatly different than that of propagation reaction, so eliminating the lag phase should also eliminate the severe imbalance between the initiation and propagation processes.

It has been reported that the specific activity of the purified polymerase increased with increasing enzyme concentration, but we have found that by eliminating the lag phase, the specific activity of the polymerase was maintained at a constant level over a wide range of the enzyme concentrations.

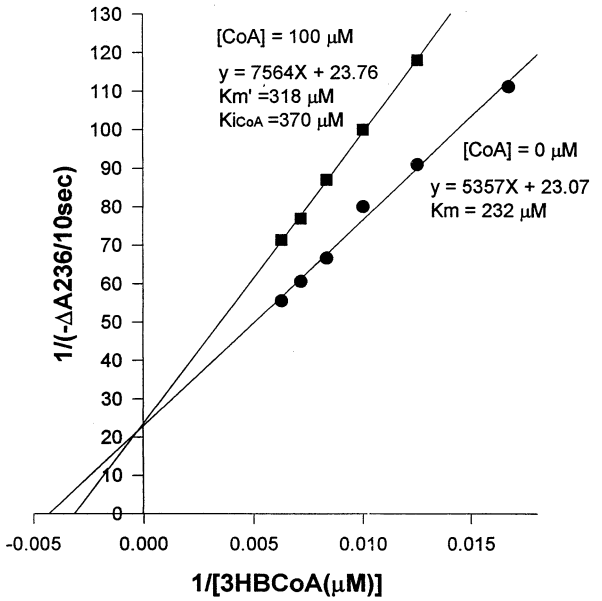

Fig. 3. Initial rate of polymerization for the first 10 s as a function of HBCoA concentration; upper curve is for reaction solution containing an additional amount of CoASH at the start.

3.3. K_M measurements

By eliminating the lag phase of the polymerization process it was possible to accurately estimate the K_M value directly by measuring monomer conversion from the decrease of absorption at 236 nm [15,22]. However, because CoASH is a competitive inhibitor for the enzyme [24], only data from the first 10–30 s of the reaction could be used to calculate the K_M values.

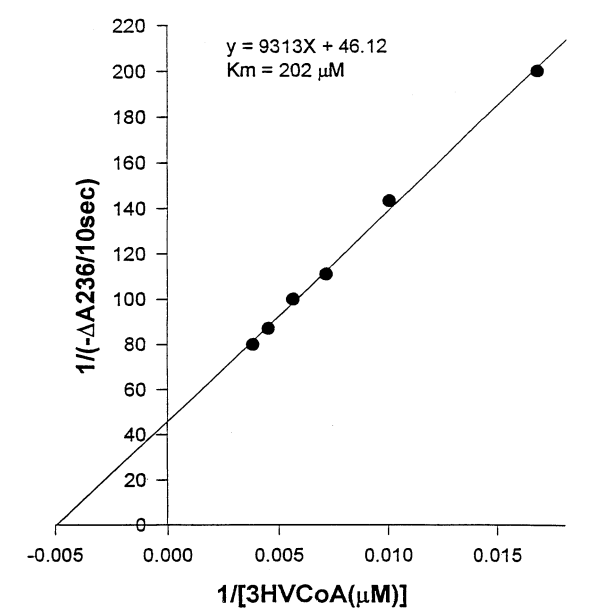

Fig. 4. Initial rate of polymerization for the first 10 s as a function of HVCoA concentration.

The plots of $1/v$ versus $1/S$ and the K_M values for HBCoA and HVCoA are given in Figs. 3 and 4, respectively. As indicated in these figures, the K_M values found for 3HBCoA and 3HVCoA were 2.32 and 2.02×10^4 M, respectively. These K_M values were also determined previously by using solutions that had gone through several cycles of polymerization and addition of fresh monomer until all of the polymerase present had initiated polymerization reactions. In that case, the K_M values obtained were 2.43 and 1.80×10^4 M, respectively, which are essentially identical to the other values. The present rate studies with glycerol present revealed that the polymerization reaction of 3HBCoA was approximately $3\frac{1}{2}$ times faster than that of 3HVCoA, so the smaller K_M value of 3HVCoA compared to that of 3HBCoA indicates that their relative rates of polymerization were a result of a much slower reaction rate for the 3HVCoA-enzyme complex. That is, the data for the initial rates of monomer conversion in Figs. 3 and 4 can be converted into absolute rates of propagation, and the rates so obtained for HBCoA and HVCoA are 25 and 7 mol of monomer/mol enzyme/s, respectively. It should be noted in relation to the discussion in Section 3.4, that these reaction mixtures contained only about 1% of glycerol by volume.

Fig. 3 also shows the effect of adding CoASH to the polymerization reaction at the start. As discussed in our previous report [24] and as shown in Fig. 3, CoASH is a competitive inhibitor for the polymerase with an inhibition constant of 3.70×10^{-4} M.

3.4. Selectivity of polymerase

In the present investigations the activity of the polymerase was also determined using a racemic mixture of HBCoA; that is, with an equimolar mixture of the [*R*]- and [*S*]-HBCoA monomers. The rate of polymerization of [*R*,*S*]-HBCoA was compared to that of pure [*R*]-HBA by following the decrease of adsorption of the monomer at 236 nm in the same manner as above [21–23]. Both the crude extracts from the cell containing the enzyme and the purified enzyme were again used in this study, and, for both, glycerol was added to the enzyme solution for storage and to avoid the lag period.

The observed rates for the polymerization of [*R*,*S*]-HBCoA at two different concentrations, and for [*R*]-HBCoA at one of those concentrations, showed that only the [*R*] monomer was polymerized by the soluble polymerase, as was previously observed for the granule-bound polymerase of *A. eutrophus* [18]. Furthermore, the presence of the [*S*] monomer did not reduce the rate of polymerization of [*R*]-HBCoA, so it does not act as a competitive inhibitor for the polymerase.

The racemic monomer was also reacted with the polymerase at two different molar ratios of

Table 1
Effect of glycerol addition on propagation rate in the polymerization of HBCoA

HBCoA:polymerase, mol	Glycerol, %[a]	Rate of propagation[b]
2×10^4:1	1	25
2×10^4:1	11	16
1×10^4:1	23	8

[a] Volume percent of glycerol in reaction solution.
[b] Rate in mol of [*R*]-HBCoA reacted/mol of polymerase/s.

monomer:enzyme, 1×10^4:1 and 2×10^4:1. The concentration of monomer was held constant in these two reactions, and because the amount of glycerol present in the polymerization reaction solution was based on the amount of monomer, the molar ratio of glycerol to enzyme decreased with the increase in the monomer:enzyme ratio. The observed initial rates of the polymerization reactions were very close for the two different monomer:polymerase ratios, but the rates of propagation calculated from the results obtained, in terms of moles of monomer reacted per mole of enzyme per second, were quite different as shown in Table 1. Included in this table is the rate with 1% glycerol from the K_M study on HBCoA described in the Section 3.3. The results in Table 1 show that addition of large amounts of glycerol to the reaction mixture caused a substantial decrease in the rate of propagation (elongation). The cause of this decrease is unknown at present, but it could be attributed either to the effect of glycerol in greatly increasing the viscosity of the reaction solution, and thereby reducing the monomer diffusion rate for these very fast reactions, or to some effect of glycerol on the activity of the enzyme itself.

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