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Thiolysis of Poly(3-hydroxybutyrate) with Polyhydroxyalkanoate Synthase from *Ralstonia eutropha*

Keiichi Uchino and Terumi Saito*
Laboratory of Molecular Microbiology, Department of Biological Sciences, Faculty of Science, Kanagawa University, 2946 Tsuchiya, Hiratsuka, Kanagawa 259-1293

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Poly(3-hydroxybutyrate) (PHB) is synthesized from 3-hydroxybutyryl-CoA by polyhydroxyalkanoate synthase and hydrolyzed by PHB depolymerase. In this study, we focused on the reverse reaction of polyhydroxyalkanoate synthase, and propose the possibility that PHB can be degraded through a novel process, that is thiolysis of PHB with CoASH. Polyhydroxyalkanoate synthase of *Ralstonia eutropha* was incubated with ^{14}C-labeled PHB and CoASH. The reaction mixture was fractionated by HPLC and then analyzed with a scintillation counter. The analysis revealed 3-hydroxybutyryl-CoA to be a product of the reaction. When NADP$^+$ and acetoacetyl-CoA reductase were added to the reaction mixture, an increase in absorbance at 340 nm was observed. Native PHB inclusion bodies from *R. eutropha* also showed thiolytic activity. This is the first indication that polyhydroxyalkanoate synthase catalyzes both the synthesis and degradation of PHB, and that native PHB inclusion bodies has thiolytic activity.

Key words: bacteria, catalysis, CoA, PHA synthase, thiolysis.

Abbreviations: 3HB, D(–)-3-hydroxybutyrate; 3HBCoA, 3-hydroxybutyryl-CoA; PHA, polyhydroxyalkanoate; PHB, poly(3-hydroxybutyrate).

Polyhydroxyalkanoates (PHAs), bacterial polyesters, comprise a group of storage materials used as sources of carbon and energy (*1*). PHAs are accumulated in a variety of bacteria, and appear under a phase contrast microscope as discrete inclusion bodies. PHAs are synthesized by PHA synthases (*2*, *3*), and mobilized by intracellular PHA depolymerases (*4*).

One of the most abundant PHAs is poly (3-hydroxybutylate) (PHB), a homopolymer of D(–)-3-hydroxybutyrate (3HB). The synthesis of PHB has been studied extensively in PHB-producing bacteria, such as *Ralstonia eutropha* strain H16, which can accumulate PHB up to almost 90% of the cell dry weight under suitable conditions. PHB is synthesized from acetyl-CoA through a reaction catalyzed by three enzymes in *R. eutropha* (*5*). The first step in the pathway is the synthesis of acetoacetyl-CoA from acetyl-CoA by β-ketothiolase [EC 2.3.1.16] encoded by *phaA*. Acetoacetyl-CoA is reduced to D(–)-3-hydroxybutyryl-CoA (3HBCoA) with NADPH by an NADP-dependent acetoacetyl-CoA reductase [EC 1.1.1.36] encoded by *phaB*. Finally, PHA synthase (EC not assigned) encoded by *phaC* synthesizes PHB from 3HBCoA:

$$n\text{3HBCoA} \rightarrow (\text{3HB})_n + n\text{CoASH} \qquad \text{(a)}$$

PHB-producing bacteria have several intracellular PHA depolymerases to mobilize the accumulated intracellular PHA inclusion bodies. In *R. eutropha* H16, the genes for several intracellular PHB depolymerases have been cloned (*6*, *7*), and some properties of their products have been reported. All known PHB depolymerases hydrolyze PHB to 3HB or 3HB-oligomers, which are finally hydrolyzed to 3HB by 3HB-oligomer hydrolase:

$$(\text{3HB})_n + (n-1)\,\text{H}_2\text{O} \rightarrow n\text{3HB} \qquad \text{(b)}$$

Reaction (b) is an ordinary hydrolytic reaction, but it is also uneconomical. ATP is required to activate 3HB; activated 3HB, 3HBCoA, can enter the main metabolic route. Doi *et al.* suggested that the synthesis and degradation of PHB occur simultaneously (*8*), but how the cell prevents this futile cycling remains unclear (*9*).

In contrast to reaction (b), glycogen, fatty acids, and inorganic polyphosphate are degraded more economically: CoA derivatives of fatty acids are thiolyzed and glycogen is phosphorolyzed. The phosphorolysis of glycogen comprises:

$$\text{glycogen}\ (n\ \text{residues}) + \text{P}_i \rightarrow \text{glycogen}\ (n-1\ \text{residues}) + \text{glucose-1-phosphate} \qquad \text{(c)}$$

Glucose-1-phosphate in reaction (c) can enter the main route of glucose metabolism through glucose-6-phosphate immediately. In reaction (c), the energy in the glycosyl bond is preserved in the phosphoester bond of glucose-1-phosphate. In the case of polyphosphate, the synthetase catalyzes the reverse reaction of polyphosphate synthesis (*10*).

If PHA synthase catalyzes the reverse reaction of PHB synthesis shown as reaction (a), the resulting product, 3HBCoA, would be metabolized more easily without activation, and this would represent a way of preventing the loss of energy during simultaneous cycling for synthesis and degradation. In this study, we examined whether PHA synthase degrades PHB with CoASH and whether thiolytic activity is observed in native PHB inclusion bodies prepared from *R. eutropha* or *Escherichia coli*.

*To whom correspondence should be addressed. Tel: +81-463-59-2361, Fax: +81-463-58-9684, E-mail: 43saito-bio@kanagawa-u.ac.jp

MATERIALS AND METHODS

Bacterial Strains and Plasmids—*R. eutropha* H16 (ATCC17699) was used in this study for the preparation of PHB and assaying of thiolysis. We also used *E. coli* strains JM109 (Takara, Kyoto, Japan) and BLR (DE3)/pLysS (Novagen, Madison, Wisc.) for gene cloning, overexpression, and PHB accumulation. The genes for PHA synthesis, *i.e.*, the *phaCAB* operon of *R. eutropha* H16, were inserted into pUC19 (Takara) or pET23b (Novagen) to yield pAE100 and pET100, respectively. pAE100 was used as a template for amplification of the *phaB* or *phaC* gene and pET100 was used to produce native PHB inclusion bodies in *E. coli*.

Growth Conditions—All *E. coli* strains were grown aerobically in Luria-Bertani (LB) medium or on solid LB agar (1.5%, w/v) plates at 37°C if not otherwise specified. *R. eutropha* H16 was cultivated aerobically at 30°C in a nutrient-rich medium (*6*) or a minimum salt medium (*2*, *11*). For *E. coli* strains, typical concentrations of antibiotics used were as follows: ampicillin, 50 μg/ml; chloramphenicol, 34 μg/ml; and tetracycline, 12.5 μg/ml.

For the production of PHB, *R. eutropha* was grown in a nutrient-rich medium for 48 h, and then transferred to minimum salt medium containing 2% fructose or 1% 3HB as described previously (*12*). To prepare ^{14}C-labeled PHB, *R. eutropha* was grown in 100 ml of nutrient-rich medium for 48 h, and then transferred to 100 ml of minimum salt medium containing 1% 3HB and [3-^{14}C]-3HB (total, 440 kBq). *E. coli* harboring pET100 was grown in LB medium at 37°C, and then glucose and isopropyl-β-D-thiogalactopyranoside were added to 2% and 0.1 mM, respectively, when the absorbance at 600 nm reached 0.4–0.6. After incubation overnight, native PHB inclusion bodies were prepared as described below.

Preparation of Native PHB Inclusion Bodies—Native PHB inclusion bodies were obtained from PHB-rich cells of *R. eutropha* or *E. coli*. Cells were harvested by centrifugation at 5,000 × *g* for 10 min and then suspended in 5 volumes (v/w) of 50 mM Tris-HCl (pH 7.5). Cells were disrupted on ice by sonication at 70 W for 10 min twice for *R. eutropha*, and 30 W for 3 min twice for *E. coli*, and then centrifuged at 10,000 × *g* for 10 min. After being washed with 50 mM Tris-HCl (pH 7.5), the precipitates were resuspended in five volumes (v/w) of the same buffer containing 5% (v/v) glycerol. Native PHB inclusion bodies were prepared from sonicated cells centrifuged at 60,000 × *g* for 4 h in a sucrose density gradient (1 to 2 M, total 10 ml or 30 ml) as described previously (*7*).

Chemicals—CoASH and $NADP^+$ were obtained from Oriental Yeast (Tokyo, Japan). Acetoacetyl-CoA was synthesized chemically from CoASH and diketene (*13*). 3HBCoA was prepared enzymatically from acetoacetyl-CoA by the method of Ploux *et al.* (*14*) with some modifications: To promote the synthesis of 3HBCoA, $NADP^+$ was reduced using glucose-6-phosphate and glucose-6-phosphate dehydrogenase (Wako Pure Chemical Industries, Tokyo, Japan). The 3HB-oligomers were prepared as described previously (*15*). [3-^{14}C]-3HB was purchased from DuPont/NEN (Boston, Mass.). PHB was isolated from *R. eutropha* and purified by hypochlorite treatment. Purified PHB is semicrystalline or denatured PHB. Artificial amorphous PHB was prepared from denatured PHB (*7*). Other chemicals of reagent grade were obtained commercially.

Plasmid Construction—Standard methods were used for the preparation and manipulation of DNA (*16*). All restriction enzymes, DNA ligase, and T4 DNA polymerase were purchased from Takara. Based on the *phaC* sequence of *R. eutropha* (*2*, accession number: J05003), a pair of primers was designed: 5′-TTTTTTTTCATATGGCGACCGGCAAAGGC-3′ and 5′-TTTTTTTTCTCGAGTGCCTTGGCTTTGACGTATCG-3′. The PCR products were digested with *Nde*I and *Xho*I, and then inserted into the corresponding sites of pET23b (Novagen). The resultant plasmid was named pETphaC. pETphaB was constructed as follows. *R. eutropha phaB* (*17*, accession number: J04987) was amplified by PCR with a pair of primers: 5′-ATGCTAGCACTCAGCGCATTGCGTATGTGACCGGCGGC-3′ and 5′-TTCTCGAGCCCATATGCAGGCCGCCGTTGAGCG-3′. The PCR products were ligated into pUC19 (Takara), digested with *Nhe*I–*Xho*I, and then inserted into the corresponding sites of pET23b.

Overexpression of phaC and Purification of PHA synthase—*E. coli* BLR (DE3)/pLysS transformed with pETphaC was cultured in the presence of ampicillin, chloramphenicol, and tetracycline. The culture was incubated in LB medium at 30°C until the absorbance at 600 nm reached 0.4–0.6, and then was incubated at 18°C overnight in the presence of 0.1 mM isopropyl-β-D-thiogalactopyranoside. Cells were harvested and resuspended in 20 mM sodium phosphate (pH 7.5) containing 5% (v/v) glycerol. The cell suspension was sonicated and centrifuged at 15,000 × *g* for 20 min. PHA synthase was purified from the supernatant with HiTrap Chelating HP (Amersham Biosciences, Tokyo, Japan) according to the instructions of the manufacturer. The final preparation used in this study showed apparent homogeneity on a SDS–polyacrylamide gel. The control *E. coli* extract comprised the supernatant of the sonicated BLR (DE3)/pLysS that had been transformed with pET23b.

Overexpression of phaB and Purification of Acetoacetyl-CoA Reductase—*E. coli* BLR (DE3)/pLysS transformed with pETphaB was cultured in the presence of ampicillin, chloramphenicol, and tetracycline. The culture was incubated in LB medium at 37°C until the absorbance at 600 nm reached 0.4–0.6, and then was incubated at 27°C overnight in the presence of 0.1 mM isopropyl-β-D-thiogalactopyranoside. Cells were harvested and resuspended in 20 mM Tris-HCl (pH 8.0) containing 5 mM $MgCl_2$, 1 mM DTT, and 20% (v/v) glycerol. The cell suspension was sonicated and centrifuged at 15,000 × *g* for 20 min. Acetoacetyl-CoA reductase was purified from the supernatant on a blue-Sepharose (Amersham Biosciences) column pre-equilibrated with 10 mM Tris-HCl (pH 8.0) buffer. The active fractions eluted with a linear gradient of NaCl (0 to 0.5 M) were collected and stored at –20°C in 50% (v/v) glycerol.

Purification of β-Ketothiolase—*R. eutropha* H16 was cultivated aerobically at 30°C in nutrient-rich medium for 2 days. Cells were harvested and then sonicated. The supernatant of the cell extract was used for the purification. β-Ketothiolase was purified through four chromatography steps: Toyopearl DEAE-650M (Tosoh, Tokyo, Japan), blue-Sepharose, Mono-Q, and then Sephacryl S-200 (Amersham Biosciences). The activity of neither

PHA synthase nor acetoacetyl-CoA reductase was observed.

Enzyme Assays—PHA synthase was assayed in both directions. (a) Synthesis of PHB. PHA synthase activity was measured spectrophotometrically using Ellman's reagent as described by Gerngross *et al.* (*18*). The reaction mixture (200 μl) comprised 20 mM sodium phosphate (pH 7.2), 0.5 mM 3HBCoA, and enzyme. At defined time points, the reaction was stopped with 5% (w/v) trichloroacetic acid, followed by centrifugation. The supernatant was neutralized, and the absorbance at 412 nm was measured after the addition of 5,5′-dithio-bis(2-nitrobenzoic acid) (1 mM, final concentration). (b) Thiolysis of PHB. For purified PHA synthase, the assay mixture (200 μl) comprised 50 mM Tris-HCl (pH 8.5), 0.4 mg/ml artificial amorphous PHB, 1 mM CoASH, 1 mM $NADP^+$, 1 mM DTT, 0.05 U of acetoacetyl-CoA reductase, and enzyme. For native PHB inclusion bodies, the assay mixture was essentially the same except that the enzyme and substrate PHB were replaced with the inclusion bodies. The reaction was initiated by adding the enzyme at 30°C. At defined time points, the reaction mixture was cooled to 0°C and then centrifuged at 15,000 × *g* for 10 min. After centrifugation, the absorbance of the supernatant was measured at 340 nm. One unit of PHA synthase catalyzes the formation of 1 μmol of 3HBCoA per min. The concentration of 3HBCoA was estimated from the absorbance in the same assay mixture without PHA synthase using a known concentration of 3HBCoA as a standard.

Acetoacetyl-CoA reductase and β-ketothiolase were assayed as described previously (*19*, *20*), and 1 unit of the enzymes catalyzes the formation of 1 μmol of 3HBCoA or cleavage of acetoacetyl-CoA per min, respectively.

Identification of 3HBCoA, Acetoacetyl-CoA, or Acetyl-CoA on HPLC—The CoA derivatives were analyzed with a HPLC system (Hitachi, Tokyo, Japan) equipped with an L-6200 pump, a D-2500 integrator, a L-4200 detector operated at 254 nm, and a Shodex RSpak RP18-415 column (4.6ϕ × 150 mm, Showa Denko, Tokyo, Japan). Buffer A comprised 0.2 M sodium phosphate, pH 4.5, and buffer B 0.2 M sodium phosphate, pH 4.5, containing 20% (v/v) of acetonitrile. The buffers were filtered through a 0.2-μm OMNIPORE type JH filter (Millipore, Bedford, Mass.) by which dissolved air was removed. The column was equilibrated with 5% buffer B and 20 μl of the sample was injected. The initial conditions were maintained for 5 min after which the composition was changed to 30% buffer B at 30 min using a linear gradient at 1 ml/min.

Other Methods—Protein concentrations were determined by the method of Lowry *et al.* (*21*) or Bradford (*22*) with reagents obtained from Bio-Rad Laboratories (Hercules, Calif.). SDS-PAGE was performed by the procedure of Laemmli (*23*).

RESULTS

Identification of the Reverse Reaction of PHA Synthase Expressed in E. coli—Whether PHA synthase degrades PHB to 3HBCoA was examined by HPLC. PHA synthase was incubated with artificial amorphous PHB and CoASH, and then the reaction mixture was analyzed by HPLC (Fig. 1A, a). Compared with the reaction mixture without

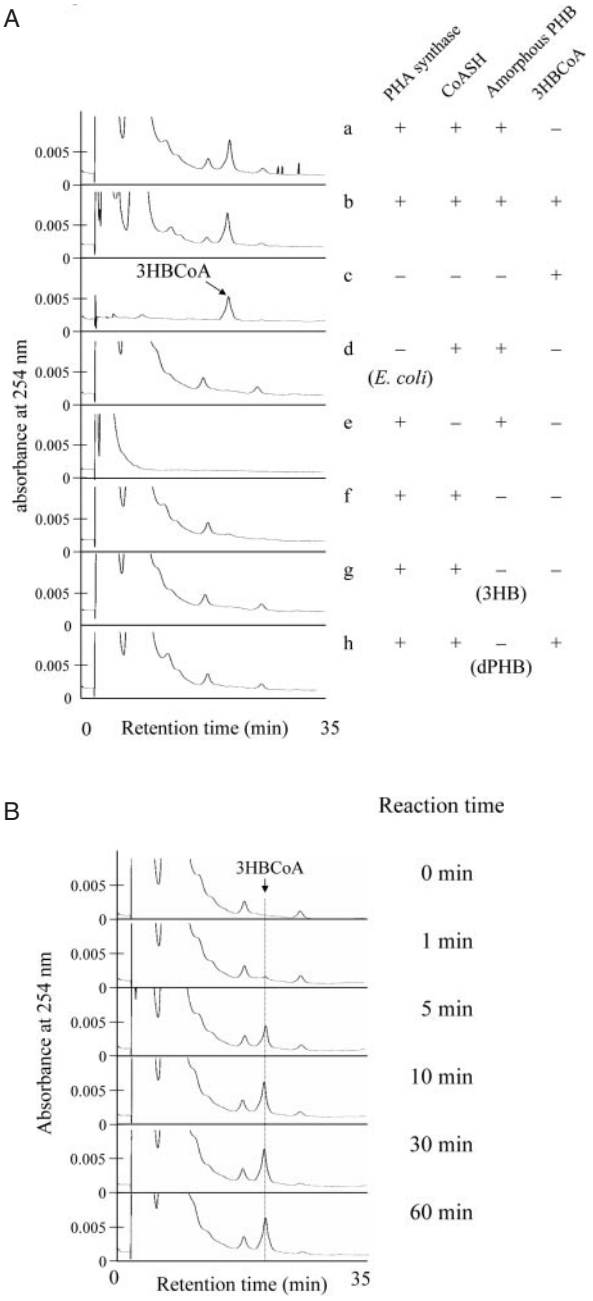

Fig. 1. **HPLC-based analysis of the products of thiolysis.** (A) The reaction mixtures obtained under various conditions were analyzed by HPLC. In the top column (a), the assay mixture (100 μl) comprised 50 mM Tris-HCl (pH 8.5), 0.4 mg/ml artificial amorphous PHB, 1 mM CoASH, 1 mM DTT, and 10 μl of PHA synthase (1 mU). The next column (b) shows co-chromatography of the assay mixture with 10 μM standard 3HBCoA (1:1 mixture). Standard 3HBCoA (10 μM) is shown in (c). The last four columns show assay mixtures with a component changed: (d) The *E. coli* extract was added instead of PHA synthase; (e) without CoASH; (f) without PHB; (g) 5 mM 3HB was used instead of PHB; (h), 0.4 mg/ml denatured PHB was used instead of artificial amorphous PHB. These assay mixtures were incubated at 30°C for 30 min and then 20 μl of 60% trichloroacetic acid was added. (B) Time course of the reaction. The assay mixture described above (350 μl) was incubated. An aliquot (50 μl) was transferred to a new tube at defined time points and the reaction was stopped with 10 μl of 60% trichloroacetic acid. The reaction mixture was centrifuged and then the supernatant was analyzed by HPLC.

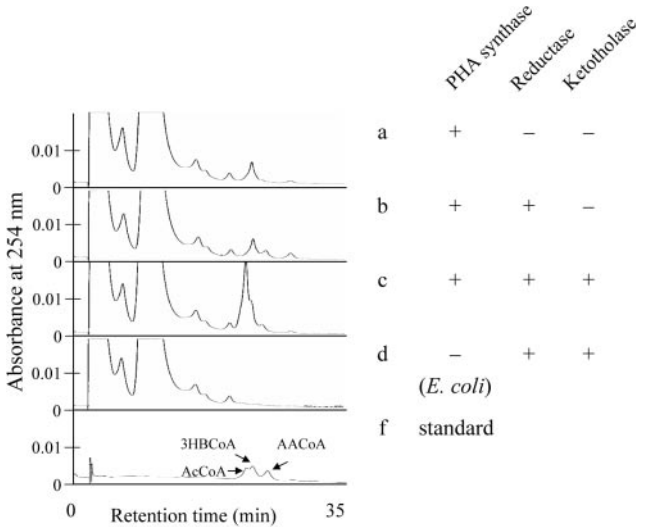

Fig. 2. **HPLC-based analysis of the products of thiolysis with acetoacetyl-CoA reductase and β-ketothiolase.** The assay mixture (total 100 μl) comprised 50 mM Tris-HCl (pH 8.5), 0.4 mg/ml artificial amorphous PHB, 1 mM CoASH, and 1 mM DTT. The enzymes were 2 mU PHA synthase (PHA synthase), 1 mM $NADP^+$ and 50 mU acetoacetyl-CoA reductase (reductase), and 15 mU β-ketothiolase (ketothiolase), and were added to the assay mixture when indicated by "+." In column (e), the assay mixture contained the *E. coli* extract instead of PHA synthase. The assay mixture was incubated at 30°C for 30 min. The reaction was stopped with 20 μl of 60% trichloroacetic acid. The reaction mixture was centrifuged and then the supernatant was analyzed by HPLC. The last column shows standard acetyl-CoA (AcCoA), acetoacetyl-CoA (AACoA), and 3HBCoA (10 μM each).

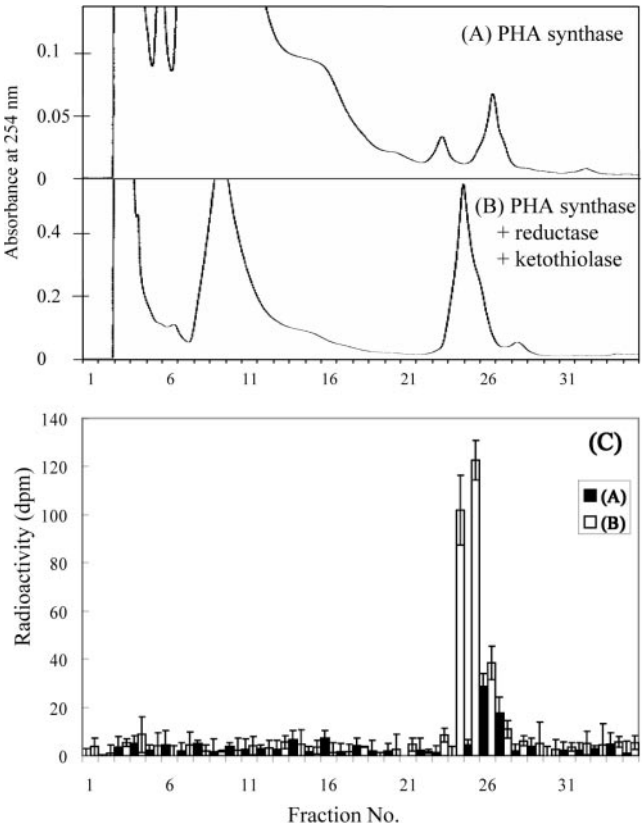

Fig. 3. **HPLC-based fractionation and radioactivity counting.** The assay mixture (1 ml) comprised 50 mM Tris-HCl (pH 8.5), 0.4 mg/ml ^{14}C-labeled artificial amorphous PHB, 1 mM CoASH, 1 mM DTT, and 12 mU PHA synthase (A). In addition, 0.5 U acetoacetyl-CoA reductase, 1 mM $NADP^+$, and 0.15 U β-ketothiolase were added in (B). The mixture was incubated for 30 min, 100 μl of 60% trichloroacetic acid was added, and then the mixture was centrifuged. The supernatant was concentrated to 100 μl and then fractionated by HPLC (fraction/min). Radioactivity was analyzed with a scintillation counter (C). Three independent experiments were performed and the means and SE are shown. Filled bars, dpm for (A); blank bars, dpm for (B).

PHA synthase (Fig. 1A, d), a peak with a retention time of about 21 min appeared in Fig. 1Aa. The peak showed the same retention time as the peak of 3HBCoA (Fig. 1A, c) and 3HBCoA in a 1:1 mixture of the reaction mixture with 10 μM standard 3HBCoA (Fig. 1A, b). The peak did not appear without CoASH or PHB, with 5 mM 3HB instead of PHB, or with denatured PHB instead of amorphous PHB (Fig. 1A, e–h, respectively). The peak of 3HBCoA became bigger as the reaction progressed (Fig. 1B). The reaction had almost reached a state of equilibrium at 10 min when the area of the peak was bigger than that of 10 μM standard 3HBCoA. Figure 2 shows the thiolysis of PHB in the presence of acetoacetyl-CoA reductase and β-ketothiolase. These enzymes further converted 3HBCoA into acetyl-CoA *via* acetoacetyl-CoA. The concentration of 3HBCoA in Fig. 2a at 10 min was about 12 μM. When acetoacetyl-CoA reductase and $NADP^+$ were added to the reaction mixture, the concentrations of 3HBCoA and acetoacetyl-CoA were about 10 and 2 μM, respectively, at 10 min (Fig. 2b). When β-ketothiolase was further added to the reaction mixture, acetyl-CoA was recognized as the main peak and its concentration was about 70 μM at 10 min (Fig. 2c).

To ensure 3HBCoA was generated from PHB, ^{14}C-labeled artificial amorphous PHB (110,000 dpm/mg of PHB) was prepared and used as a substrate for assaying the thiolysis. The reaction mixture comprising the synthase, ^{14}C-artificial amorphous PHB, and CoASH was incubated for 30 min, concentrated 10-fold, and then fractionated by HPLC (Fig. 3A). The radioactivity in the fractions was measured (Fig. 3C). The fractions comprising the peak of 3HBCoA in Fig. 3A exhibited a low but significant level of radioactivity (46 ± 12 dpm, sum of fraction numbers 26 and 27, Fig 3C). No radioactivity was detected when the control *E. coli* extract was used. Radioactivity was readily detected (280 ± 36 dpm, sum of fraction numbers 23–27, Fig. 3C) when acetoacetyl-CoA reductase, $NADP^+$, and β-ketothiolase were added to the reaction mixture. The concentration of acetyl-CoA reached about 0.3 mM at 30 min (Fig. 3B).

The reverse reaction was also revealed spectrophotometrically. Because the reaction was detected as an increase in the absorbance at 340 nm, $NADP^+$ was reduced by acetoacetyl-CoA reductase concomitantly with oxidation of 3HBCoA. The reaction mixture for thiolysis (see "MATERIALS AND METHODS") was incubated and centrifuged, and the increase in absorbance at 340 nm in the supernatant was measured. The absorbance at 340 nm increased in the presence of CoASH, and depended on the amount of purified PHA synthase and the reaction time (Fig. 4, opened circles). PHA synthase, CoASH, artificial amorphous PHB,

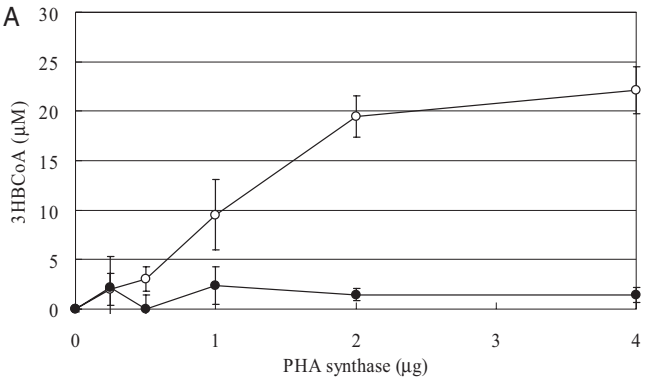

Fig. 4. **Dose and time-dependence of the thiolysis by PHA synthase.** The assay mixture (200 μl) comprised 50 mM Tris-HCl (pH 8.5), 1 mM CoASH, 1 mM $NADP^+$, 1 mM DTT, 0.05 U of acetoacetyl-CoA reductase, PHA synthase, and 0.25 mg of artificial amorphous PHB (open circles) or denatured PHB (filled circles). The amount of enzyme added was 0 to 4 μg in (A) and 2 μg in (B). The reaction was initiated by adding the enzyme at 30°C. The reaction mixture was cooled to 0°C at 10 min in (A) and at a defined time in (B), and then centrifuged at 15,000 × *g* for 10 min. After centrifugation, the optical density at 340 nm of the supernatant was measured.

Table 1. **Localization of thiolytic activity in PHB-rich cells of *R. eutropha*.**[a]

Sample[b]	Activity (nmol/min/mg of protein)	PHB (μg)[c]
Sonicated cell extract	0.73 ± 0.078	62
Supernatant	0.03 ± 0.02	Not detected
Precipitate	0.82 ± 0.19	100
Native PHB inclusion bodies	0.42 ± 0.13	34

[a]Thiolytic activity was assayed as described under "MATERIALS AND METHODS." [b]All samples were prepared from cells accumulating PHB. Once harvested, cells were sonicated, and then centrifuged to give a supernatant and a precipitate. Alternatively, sonicated cells were centrifuged in a sucrose-density gradient to prepare native PHB inclusion bodies. [c]Amount of PHB in each reaction mixture (total 200 μl).

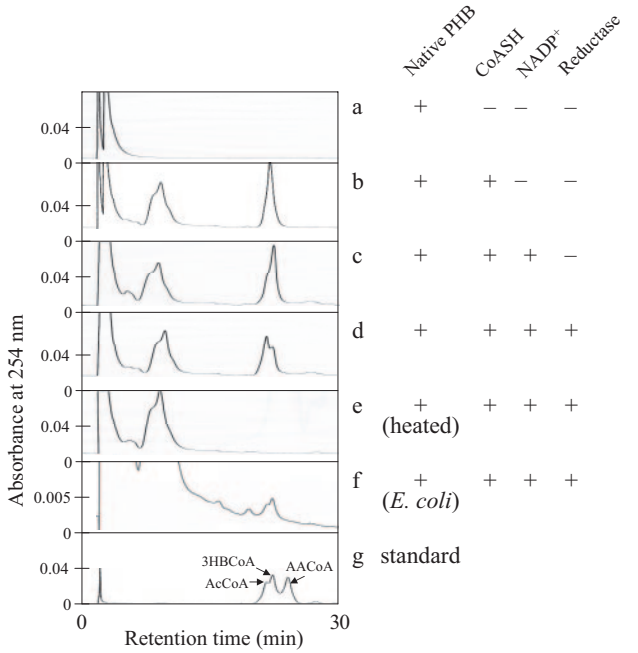

Fig. 5. **HPLC-based analysis of the products of thiolysis**. The assay mixture (total 100 μl) comprised 50 mM Tris-HCl (pH 8.5) and 1 mM DTT. Where indicated by "+," 1 mM CoASH, 10 μl of native PHB inclusion bodies (native PHB, 82 μg of protein, 15 μg of PHB), 1 mM $NADP^+$, and 0.03 U acetoacetyl-CoA reductase (reductase) were contained in the assay mixture. Native PHB inclusion bodies were treated with heat (e) or native PHB inclusion bodies from *E. coli* were used (f). The assay mixture was incubated for 30 min, and the reaction was stopped with 20 μl of 60% trichloroacetic acid. The assay mixture was centrifuged and then the supernatant was analyzed by HPLC. The vertical axis in (f) indicates a value eight times smaller than the others. The last column shows standard acetyl-CoA (AcCoA), acetoacetyl-CoA (AACoA), and 3HBCoA (100 μM each).

$NADP^+$, and the reductase were necessary for the increase in absorbance. The absence of any one of them resulted in no increase in absorbance. Denatured PHB could not be used as a substrate (Fig. 4, filled circles). Neither 1 mM 3HB-pentamer nor 5 mM 3HB served as a substrate (data not shown).

Thiolysis of Native PHB Inclusion Bodies Prepared from R. eutropha—R. eutropha was examined to ensure that PHA synthase catalyzes the thiolysis of PHB in PHB inclusion bodies. A sonicate of PHB-rich cells (10 μl) containing 62 μg of PHB and 89 μg of protein was incubated at 30°C for 10 min in the presence of CoASH, $NADP^+$, and acetoacetyl-CoA reductase (total 200 μl). The increase in absorbance was 0.158 ± 0.017 in three independent experiments. This corresponds to about 60 μM 3HBCoA, based on a calibration curve obtained with known concentrations of 3HBCoA in the same reaction buffer.

Sonicated *R. eutropha* cells were then centrifuged to separate the soluble fraction from the precipitate containing native PHB inclusion bodies, and the thiolytic activity was also assayed spectrophotometrically. The precipitate showed an increase in absorption at 340 nm but the supernatant did not (Table 1). The native PHB inclusion bodies prepared by ultracentrifugation confirmed the result described above (Table 1, last column).

HPLC analysis of the reaction products also revealed the formation of 3HBCoA from the native PHB inclusion bodies of *R. eutropha* in the presence of CoASH (Fig. 5). The native PHB inclusion bodies and CoASH were

incubated for 30 min, and then analyzed by HPLC. In the presence of CoASH, 3HBCoA was detected (Fig. 5b). The peak area corresponded to about 0.2 mM 3HBCoA. Whether or not the reaction mixture contained $NADP^+$, a peak of 3HBCoA was detected (Fig. 5, b and c). When CoASH was omitted from the reaction mixture, 3HBCoA was not detected (Fig. 5a). When CoASH, $NADP^+$, and acetoacetyl-CoA reductase were added, the peaks corresponding to 3HBCoA and acetyl-CoA decreased and increased, respectively (Fig. 5d). Treatment of native PHB inclusion bodies with heat abolished the peaks of these CoA derivatives (Fig. 5e). The addition of 3HB to the reaction mixture caused almost no increase in 3HBCoA. When the reaction mixture was incubated with CoASH and $NADP^+$ overnight, most of the 3HBCoA disappeared and the peak corresponding to acetyl-CoA increased considerably (data not shown).

Native PHB Inclusion Bodies from E. coli—The thiolytic activity of native PHB inclusion bodies containing 78 μg of protein prepared from *E. coli* harboring pET100 was also examined. In the same assay system containing $NADP^+$ and the reductase, native PHB inclusion bodies of *E. coli* exhibited an increase in absorption at 340 nm. The increase in absorbance was 0.043 ± 0.005 for 10 min, corresponding to 10 μM 3HBCoA. The HPLC results show the appearance of 3HBCoA and acetyl-CoA in native PHB inclusion bodies from *E. coli* (Fig. 5f).

DISCUSSION

A novel form of degradation, thiolysis, of PHB was investigated, and the data presented above showed that PHA synthase degraded PHB into 3HBCoA through thiolysis *in vitro* and *in situ* (inclusion bodies). As far as we know, this is the first indication of the reverse reaction of PHA synthase and of the thiolysis of native PHB inclusion bodies, this maybe providing a new metabolic route.

HPLC-based analysis of the reaction mixture identified 3HBCoA as the reaction product of the reverse reaction of PHA synthesis (Figs. 1 and 3). Since the *E. coli* extract without PHA synthase did not exhibit thiolytic activity, PHA synthase alone accounts for the activity. 3HB could not be used as a substrate, excluding the involvement of 3HB thiokinase or CoA transferase that might be present in the *E. coli* extract. β-Ketothiolase and acetoacetyl-CoA reductase made the thiolytic activity readily detectable, although the product was acetyl-CoA (Fig. 2). Also, the appearance of 3HBCoA was deduced spectrophotometrically during the reaction (Fig. 4), where the time dependency and substrate specificity were consistent with the results of HPLC.

When ^{14}C-labeled PHB was used as a substrate for the thiolysis of PHB, the reaction product was ^{14}C-labeled 3HBCoA or acetyl-CoA in the absence or presence of acetoacetyl-CoA reductase, $NADP^+$, and β-ketothiolase, respectively (Fig. 3). If PHB was converted into 3HBCoA and then acetyl-CoA completely, the specific radioactivity should amount to *ca.* 5,500 dpm/μmol of acetyl-CoA and 11,000 dpm/μmol of 3HBCoA. Based on the concentrations of acetyl-CoA and 3HBCoA used for the HPLC, the radioactivity is calculated to be 300 dpm and 50 dpm for acetyl-CoA and 3HBCoA, respectively. The radioactive counts found in the experiments, 280 ± 36 and 46 ± 12 dpm, are roughly consistent with these values, indicating strongly that the two CoA derivatives were derived from PHB. The fact that no other radioactive peak was found also confirms the thiolytic cleavage of PHB.

Although it is quite natural for enzymes to catalyze a reverse reaction, there had been no indication of the reverse reaction of PHB synthesis before. It is believed that PHB synthesis is essentially irreversible because PHB is a water-insoluble polymer. Since the produced PHB is eliminated from the reaction system, the equilibrium will change to produce the polymer. But the reverse reaction actually occurred, as described in this report. The reason why it occurred may be the amorphous nature of PHB used as a substrate, since semicrystalline PHB did not act as a substrate for the reverse reaction (Fig. 1A, h).

It was suggested that only PHA synthase catalyzed the thiolysis of PHB in native PHB inclusion bodies of *E. coli* because the *E. coli* cell extract without PHA synthase did not exhibit thiolytic activity (Fig. 1). Thiolytic activity was also observed in *R. eutropha*. PHA synthase is localized to PHA inclusion bodies, and thiolytic activity was also found in PHA inclusion bodies (Table 1). In *R. eutropha*, however, it is unclear whether other enzymes are involved in the thiolysis of PHB. The involvement of 3HB thiokinase or CoA-transferase was excluded on the addition of 3HB, whereas it is possible that PHB depolymerases catalyze the thiolysis of PHB.

Acetoacetyl-CoA reductase and β-ketothiolase promoted the thiolysis of PHB *in vitro* (Fig. 2) and may also do so *in vivo*. In native PHB inclusion bodies, the specific activity was decreased compared with that in the cell extract (Table 1). This was possibly because of elimination of most of the β-ketothiolase and acetoacetyl-CoA reductase. These enzymes are usually soluble (*24*). But it is assumed that some acetoacetyl-CoA reductases and β-ketothiolases are included in PHB inclusion bodies. Recently, a kind of β-ketothiolase, BktB, was found in PHB inclusion bodies (*25*). In the reaction involving native PHB inclusion bodies, acetyl-CoA was detected without the addition of β-ketothiolase (Fig. 5d) and in the reaction mixture incubated overnight, acetyl-CoA was recognized in the reaction products without the addition of acetoacetyl-CoA reductase. β-Ketothiolase and acetoacetyl-CoA reductase are involved in both the synthesis and the thiolysis of PHB *in vitro*.

It remains unclear whether PHA synthase catalyzes both the synthesis and degradation in living cells. The enzyme itself can react in the reverse direction, but there has been no report that PHB is degraded in *E. coli* cells harboring PHA synthetic genes or in PHA-accumulating bacteria from which PHA depolymerase genes have been deleted (*26*). If PHA synthase catalyzes the mobilization of PHB, no PHB depolymerase is required. The precise roles of intracellular PHB depolymerases and thiolysis of PHB with PHA synthase *in vivo* need to be researched further. The PHA synthase of *R. eutropha* belongs to one of three classes and the thiolytic activity of the other types of PHA synthase must be examined.

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