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Simultaneous Accumulation and Degradation of Polyhydroxyalkanoates: Futile Cycle or Clever Regulation?

Qun Ren,*,†,‡ Guy de Roo,†,§ Katinka Ruth,‡ Bernard Witholt,|| Manfred Zinn,‡ and Linda Thöny-Meyer‡

Laboratory of Biomaterials, Swiss Federal Laboratories for Materials Testing and Research (Empa), CH-9014 St. Gallen, Switzerland, Synthon BV, P.O. Box 7071, 6503 GN Nijmegen, The Netherlands, and Institute of Molecular Systems Biology, Swiss Federal Institute of Technology, CH-8093 Zurich, Switzerland

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The regulation of medium-chain-length polyhydroxyalkanoates (mcl-PHA) metabolism in *Pseudomonas putida* GPo1 was studied by analysis of enzymes bound to PHA granules and enzymes involved in fatty acid oxidation. N-terminal sequencing of granule-bound enzymes revealed the presence of PHA polymerase (PhaC) and PHA depolymerase (PhaZ) and an acyl-CoA synthetase (ACS1), which recently was found to be associated with PHA granules by in vivo study. The *acs1* knockout mutant accumulated 30−50% less PHA than its parental strain, confirming the involvement of ACS1 in PHA metabolism. Isolated PHA granules showed both PhaC and PhaZ activities. PhaC activity was found to be sensitive to the ratio of [*R*-3-hydroxyacyl-CoA]/[CoA] in which free CoA was a mild competitive inhibitor. Fatty acid oxidation was regulated by the [acetyl-CoA]/[CoA] and [NADH]/[NAD] ratios, with high ratios resulting in accumulation and low ratios leading to rapid oxidation of 3-hydroxyacyl-CoA. These results suggest that PHA metabolism is likely to be controlled by the [acetyl-CoA]/[CoA] and [NADH]/[NAD] ratios. The physiological roles of simultaneous PHA accumulation and degradation are also discussed.

Introduction

Biodegradable and biocompatible polyesters such as poly(hydroxyalkanoates) (PHA) gained considerable attention during the last decades. Depending on the number of carbon atoms in the monomer units, PHA can be classified into three groups: short-chain-length (scl) PHA, which consist of monomers with 3−5 carbon atoms, medium-chain-length (mcl) PHA, consisting of monomers with 6−12 carbon atoms, and long-chain-length (lcl) PHA, consisting of monomers with 13 or more carbon atoms.[1,2]

One of the best-studied mcl-PHA producers is *Pseudomonas putida* GPo1.[3] This bacterium relies on the β-oxidation pathway to convert fatty acid intermediates into (*R*)-3-hydroxyacyl-coenzyme A ((*R*)-3-hydroxyacyl-CoA) thioesters, which are the precursors of the PHA synthesis.[4−7] It accumulates mcl-PHA under environmental conditions in which there is a surplus of fatty acids but a limitation for vital nutrients (N, P, or S) or O_2.[3,8] The accumulated polyesters can later be degraded when the limitation for N, S, P, or O_2 is released.[8]

In vivo, PHA are accumulated in intracellular granules that are covered by a surface layer composed of proteins and phospholipids.[9,10] In *Pseudomonas*, PHA polymerases and PHA depolymerase, which are the key enzymes of mcl-PHA synthesis and degradation, respectively, have been found to be associated with the granules.[11−13] In addition, other phasins such as PhaI and PhaF and proteins of unknown function seem to be embedded in the phospholipid layer surrounding the granules.[10,14,15] Recently, an acyl-CoA synthetase was shown to be located on PHA granules and to convert the PHA degradation products, hydroxycarboxylic acids, into CoA-linked forms.[16]

P. putida strains contain two PHA polymerases (PhaC1 and PhaC2); the substrate specificities of these enzymes differ slightly.[12] Moreover, it has been demonstrated that both of these polymerases are functional proteins which are able to catalyze PHA formation independent of each other; that is, one of the polymerase-encoding genes is sufficient to produce mcl-PHA in heterologous hosts.[12] Degradation of PHA to monomeric *R*-hydroxycarboxylic acids is catalyzed by the intracellular PHA depolymerase.[17] Released PHA monomers are assumed to be activated by acyl-CoA synthetase (ACS or FadD) to their CoA form that can enter the β-oxidation pathway as well as be channeled back to PHA synthesis.[16] Two acyl-CoA synthetases have been identified in *P. putida* GPo1 (ACS1 and ACS2) and *P. putida* U (FadD1 and FadD2), respectively.[16,18] The knockout mutant of FadD1 in *P. putida* U led to delayed growth on fatty acids.[18] The role of FadD2 has not yet been investigated in detail. It was suggested that FadD2 can replace the function of FadD1 when the activity of FadD1 is absent.[18] The cyclic nature of PHA metabolism has been shown, e.g., by radioactive labeling experiments.[19,20]

Currently, it is not clear how the physiological conditions influence mcl-PHA accumulation or degradation. Reporter-fusion studies of the PHA polymerase C1 promotor region (P_{C1}) of *P. putida* GPo1 revealed that the transcriptional activity is increased under nitrogen-limited growth conditions.[14] It was also suggested that the alternative sigma factor gene *rpoN* plays a role.[21,22] Mcl-PHA synthesis is therefore based at least partly on enhanced PHA polymerase production by activation of transcription via specific environmental signals. Additional targets for regulation could be the enzymes involved in fatty acid β-oxidation, which affect the synthesis and availability of PHA precursors.

* To whom correspondence should be addressed. Phone: 41-71-2747688. Fax: 41-71-2747788. E-mail: qun.ren@empa.ch.
† These authors contributed equally.
‡ Swiss Federal Laboratories for Materials Testing and Research.
§ Synthon BV.
|| Swiss Federal Institute of Technology.

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In the well-characterized β-oxidation pathway of *Escherichia coli*, fatty acids are activated with CoA via the action of an acyl-CoA synthetase encoded by *fadD*.[23,24] The activated fatty acids are oxidized by an acyl-CoA dehydrogenase and then processed by a protein complex containing five additional enzymatic activities (enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase, *cis*-Δ^3-*trans*-Δ^2-enoyl-CoA isomerase, 3-hydroxy-acyl-CoA epimerase, and 3-ketoacyl-CoA thiolase).[23,25,26] One set of intermediates of this β-oxidation cycle are the *R*-3-hydroxyacyl-CoAs, which are substrates for the PHA polymerase. The availability of these mcl-PHA precursors could vary considerably with different physiological states, depending on the activities of the β-oxidation enzymes.

Despite the progress made in understanding the functions of individual proteins involved in PHA metabolism, the molecular mechanisms with which a cell decides whether to synthesize or to degrade (mobilize) PHA are not known. Several reports have indicated that PHA synthesis and PHA degradation can happen simultaneously.[19,20] In this study, to better mimic the physiological environment of PhaC and PhaZ, we examined PHA granules as a whole system. We first analyzed the proteins attached to PHA granules and studied the activities of key enzymes involved in PHA metabolism. Then various physiological compounds were examined for their influence in PHA synthesis and degradation using isolated PHA granules. Our results suggest that PHA metabolism is likely to be controlled mainly by the [acetyl-CoA]/[CoA] and [NADH]/[NAD] ratios, which regulate fatty acid oxidation as well.

Materials and Methods

Biochemicals. All chemicals were purchased from Sigma-Aldrich (Buchs, Switzerland), *R/S*-3-hydroxycarboxylic acids were supplied by Larodan Lipids (Malmö, Sweden). *R*-3-hydroxycarboxylic acids were prepared via hydrolysis of mcl-PHA.[27] *R*-3-hydroxyoctanoyl-CoA was synthesized as described previously.[28] 2-Octenoyl-CoA was prepared from octanoyl-CoA using acyl-CoA oxidase (Aldrich, Switzerland); octanoyl-CoA (5 mM) was incubated in 50 mM 2-(*N*-morpholino)-ethanesulfonic acid (MES) buffer, 0.2% Triton X-100, 10 μM FAD, and 0.5 U/mL acyl-CoA oxidase for 1 h (30 °C). The reaction mixture was purified using a C18 RP preparative HPLC column after which the product was eluted with 70% methanol in 10 mM ammonium acetate buffer (pH 5.5). The eluate (2-octenoyl-CoA) was freeze-dried and stored at −20 °C. The concentrations of all prepared CoA esters were estimated by hydroxylamine treatment,[29] which causes the release of bound CoA. The concentration of free CoA before and after hydroxylamine treatment was determined by the Ellman method.[30]

DNA Manipulation and Generation of *acs1* Knockout Mutant. DNA manipulation and other molecular biology techniques were performed as described previously.[31] *E. coli* DH5α [32] was used as host for plasmid construction. The gene *acs1* was amplified from genomic DNA of *P. putida* GPo1 using the primers 5′-CGCATGCATGATCGAAAATTTTTGGAAGG-3′ and 5′-TAAGCTTTCAGGCGATCTTCTTCAA-3′, and applying a standard PCR with 30 cycles of melting (95 °C/30s), annealing (55 °C/30s), and elongation (72 °C/60s). The obtained 1.7 kb fragment was cloned into a pGEM-T Esay vector (Promega, Madison, WI). A kanamycine resistance gene including its promoter region was amplified from pUTmini-Tn5 Km (Biomedal, Sevilla, Spain) by standard PCR with 64 °C annealing temperature and with the following primers: 5′-GGGCCATGGGGTAAGGTTGGGAA-3′ and 5′-GGGCCATGGTCAGAAGAACTCGTCAA-3′. NcoI restriction sites (introduced at the beginning and the end of the fragment by the primers) were used to insert kanamycine gene into NcoI digested *acs1* at 872 bp position of the 1698 bp entire gene on pGEM-T Easy. The resulting insertion *acs1::km* was cloned into pUC18Not and subsequently into NotI digested suicide vector pUTmini-Tn5 Tc by the means of the PANC-1plus mini-Tn5 vector kit (Biomedal). The obtained plasmid pUTmini-Tn5 Tc *acs1::km* was transformed into *E. coli* S17-1, which was used as a vector donor in conjugation.[33] For biparental mating, *E. coli* S17-1 harboring pUTmini-Tn5 Tc *acs1::km* and wild type *P. putida* GPo1 (both suspended in 0.9% NaCl solution to $OD_{600} = 4.0$) were mixed at a ratio of 1:3 and incubated on Luria−Bertani (LB) agar plate at 30 °C for 3 h. Afterward, the material was diluted and plated onto selective plates. Serial dilutions were performed with cells being both tetracycline and kanamycine resistant (single crossover). Colonies exhibiting tetracycline sensitivity and kanamycine resistance were identified as double crossover mutants. The genotype was confirmed by PCR with previously mentioned primers.

Bacterial Strains and Cultivation Conditions. *P. putida* GPo1, *P. putida* GPo500 (PHA depolymerase mutant [6]), and *P. putida* KO15 (*acs1* knockout mutant of GPo1 generated in this study) were precultured in LB medium at 120 rotations per minute (rpm) and 30 °C and transferred with 1:100 dilution into E2 mineral medium[16] containing either 10 mM sodium citrate or 15 mM sodium octanoate as carbon source in shake flasks. *E. coli* cells were grown in LB medium at 37 °C without or with shaking (120 rpm). If necessary, the following concentrations of antibiotics were added: kanamycine 30 μg/mL, tetracycline 12.5 μg/mL, and ampicillin 150 μg/mL.

PHA Granule Isolation and Analysis of Granule-Associated Proteins. To isolate PHA granules, *P. putida* cells were grown in 0.2NE2 medium (mineral medium containing 20% of the total nitrogen of E2 medium) supplemented with 15 mM sodium octanoate. Granules were isolated from the midexponential growth cells by density centrifugation as reported.[28] Samples of purified granules were mixed 1:1 (v/v) with SDS loading buffer,[31] and the bound proteins were separated on SDS-polyacrylamide gels as described.[34] Protein amounts were estimated by densitometric scanning of SDS-polyacrylamide gels using a Multimage Light Cabinet (Alpha Innovation Corp.) with chemiluminescence and visible light imaging. Known concentrations of BSA were used as standard. For N-terminal sequencing, proteins were electroblotted directly from a SDS-polyacrylamide gel onto a polyvinylidene difluoride membrane. The amino-terminal sequences were determined by Edman degradation using a G 1000 A automated protein sequencer (Hewlett-Packard).

Partial Purification of Fatty Acid Oxidation Complex. The fatty acid oxidation complex of *P. putida* GPo1 was partially purified using a method developed by Imamura et al.[25] After growth on octanoate, spheroplasting of the cells, lysis by cold acetone treatment, spinning down cell debris, ammonium sulfate precipitation of supernatant proteins, heating at 50 °C and dialysis, the protein extract was loaded on a trimethylaminoethyl (TMAE)-Sepharose column (3.5 × 15 cm) and eluted with a linear KCl gradient (0−0.5 M) in sodium phosphate buffer. The active fractions were pooled and stored at −20 °C in the presence of 20% sucrose.

Enzyme Assays. *PHA Polymerase Assay.* PHA polymerase activity (including both PhaC1 and PhaC2) was analyzed by following the release of CoA using 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB).[30] A typical mixture (300 μL) contained 0.5 mM *R*-3-hydroxyoctanoyl-CoA, 1 mg/mL PHA granules, 1 mg/mL BSA, and 0.5 mM $MgCl_2$ in 100 mM Tris-HCl, pH 8. Activity was measured spectrophotometrically as previously described.[28] The obtained data were further confirmed by another assay: measuring the depletion of *R*-3-hydroxyoctanoyl-CoA using HPLC (Labomatic liquid chromatography system, Allschwil, Switzerland).[35] One unit is defined as 1 μmol *R*-3-hydroxyoctanoyl-CoA consumption or accumulation per minute.

PHA Depolymerase Assay. PHA depolymerase activity was analyzed by following the release of 3-hydroxycarboxylic acids by gas chromatography (GC, Fisons, U.S.A.). A typical mixture (2 mL) contained 1 mg/mL PHA granules and 0.5 mM $MgCl_2$ in 100 mM Tris-HCl, pH 8. Aliquots (250 μL) were taken at timed intervals and the reaction was stopped by the addition of 250 μL ice-cold ethanol. After pelleting of the PHA granules by centrifugation (20000 rpm, 30 min), 400 μL of

the supernatant was transferred to a pyrex tube and subsequently lyophilized. The lyophilized samples containing the released 3-hydroxycarboxylic acids were methanolyzed by addition of 1 mL of chloroform and 1 mL of acidified methanol (containing 15% H_2SO_4), followed by heating in an oil bath (100 °C, 2.5 h). Addition of 1 mL of H_2O and subsequent thorough shaking resulted in the separation of two phases. The upper phase (methanol, H_2O, and H_2SO_4) was discarded. The lower phase (containing the 3-hydroxy acid methylesters) was dried over Na_2SO_4 and analyzed by GC.

Fatty Acid Oxidation Assay. During purification, the activity of the multienzyme complex was followed by measuring the increase in absorbance at 550 nm due to the formation of the formazan dye.[25] The activity of the partially purified enzyme complex was assayed by measuring the depletion of *R*-3-hydroxyoctanoyl-CoA and the concomitant increase of hexanoyl-CoA by HPLC. A typical assay mixture contained 0.5 mM *R*-3-hydroxyoctanoyl-CoA, 0.5 mg/mL BSA, 250 mM NaCl, and 0.125% Triton X-100 in 100 mM Tris-HCl, pH 9. Because equal molars of CoA and NAD are consumed in one β-oxidation cycle, same molar ratios of acetyl-CoA to CoA and NAD and NADH were added into the assay mixture. Acetyl-CoA and CoA were added in different ratios in which the total CoA pool (CoA + acetyl-CoA) was 1 mM. Similarly, NAD and NADH were added in different ratios in which the total NAD pool (NAD + NADH pool) was 1 mM. Aliquots (60 μL) were taken at timed intervals followed by immediate addition of 60 μL of TCA (5%) to terminate the reaction. After centrifugation (20000 rpm, 30 min), the supernatant was analyzed by HPLC using a LiChrospher 100 reverse phase column (Merck) with an ammonium acetate/acetonitrile elution gradient as described.[35] Free CoA, acetyl-CoA, 2-octenoyl-CoA, 3-hydroxyoctanoyl-CoA, and hexanoyl-CoA eluted at 1.2, 1.5, 8.0, 8.3, and 8.6 min, respectively. The formation of 3-hydroxyoctanoyl-CoA using 2-octenoyl-CoA as a substrate was determined using a GC-based assay similar to that described for PHA depolymerases, under similar assay conditions. Aliquots (200 μL) were taken at timed intervals followed by immediate addition of 1 M NaOH (200 μL) to terminate the reaction and stimulate hydrolysis of the CoA-esters. After centrifugation of the precipitated proteins at 14000 rmp, the supernatant (350 μL) was transferred to a pyrex tube and subsequently lyophilized. The lyophilized samples containing the hydrolyzed CoA esters were methanolyzed and analyzed by GC as described above for PHA depolymerases.

Determination of Octanoate and 3-Hydroxyoctanoate in the Medium. The residual amount of octanoate in bacterial cultures was determined by GC analysis. Samples (10 mL) of a growing cell culture were centrifuged (8000 rpm, 20 min). The supernatant was lyophilyzed and subsequently methanolyzed by a procedure similar to that described for the PHA depolymerase assay above. The excretion of 3-hydroxyoctanoate in the medium was followed using the same approach.

GC and LC-MS Analysis. Identification of the 3-hydroxycarboxylic acid methylesters was done by GC (Fisons, USA) using a 25 m ZB-1 capillary column (Brechbühler AG, Switzerland). The molecular mass of the synthesized *R*-3-hydroxyoctanoyl-CoA was confirmed by liquid chromatography−mass spectroscopy (LC-MS) using negative spray ionization (Agilent 1100 series). The MS settings were as follows: atmospheric pressure chemical ionization mode, negative ionization; fragmentor voltage, 50 V; gas temperature, 350 °C; vaporizer temperature, 375 °C; drying gas (N_2) flow rate, 4 L per min; nebulizer pressure, 0.023 N per m^2; capillary voltage, 2000 V; corona current, 6 μA.

Determination of PHA Content in Biomass. To determine the PHA content of bacteria, cells were grown in E2 minimal medium containing 15 mM sodium octanoate and assayed for the presence and composition of PHA by GC.[36,37] All experiments were carried out three times. For different cultivations, the absolute values regarding cell dry weights and intracellular PHA contents had variations due to sample taken at different time points; however, PHA synthesis and degradation exhibited similar pattern relative to cell growth in these three experiments. In this study, only the data obtained from one experiment was presented.

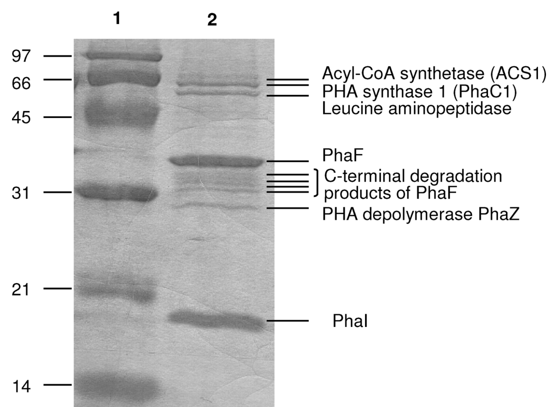

Figure 1. Proteins attached to isolated PHA granules of *P. putida* GPo1. Lanes: (1) molecular weight markers (kD); (2) isolated PHA granules (1 mg) harvested at the midexponential growth on octanoate. N-terminal sequencing identified an acyl-CoA synthetase (64 kD), PHA polymerase C1 (60 kD), leucine aminopeptidase (53 kD), PHA depolymerase (29 kD), and C-terminal degradation products of PhaF (32−40 kD). PhaF (40 kD) and PhaI (18 kD) are known granule associated proteins.[14]

Results and Discussion

Identification of Proteins Associated with Isolated PHA Granules. *P. putida* GPo1 accumulates mcl-PHA as distinct granules during growth on fatty acids. Upon isolation of these granules by density centrifugation and subsequent SDS-PAGE analysis, a typical protein pattern is observed as shown in Figure 1. The two predominant bands of 40 and 18 kD have previously been identified as the phasins PhaF and PhaI, respectively.[14] In this study, several other unknown protein bands were identified by N-terminal sequencing.

The upper protein band at 64 kD has a N-terminal sequence (MIEKFWKDKYRAGITAEI) that shows a 89% identity to a recently identified acyl-CoA synthetase (ACS1) from *P. putida* GPo1 (MIENFWKDKYPAGITAEI).[16] This enzyme could play a role in the synthesis of acyl-CoA intermediates for PHA synthesis or in activating the monomers released by the PHA depolymerase during degradation of the polymer. The protein band at 60 kD is the previously described PHA polymerase C1 (PhaC1), as confirmed by its N-terminal sequence (MNKNNDELQRQASENTLGLNPVIGVPVI). Surprisingly, PHA polymerase C2 (~62 kD) was not found on the PHA granules, which indicates that *phaC2* was not expressed to detectable levels under the conditions of these experiments. The protein band at 53 kD has an N-terminus (MEFVVKSVAAASVKTA) that is 100% identical to that of a leucine aminopeptidase of *P. putida* sp.[38] The role of this peptidase in PHA metabolism is not clear. It could be involved in the turnover of granule-associated proteins. In addition, the amino acid sequence of the 53 kD protein shows up to 71% similarity to PepA of *E. coli* and PhpA of *P. aeruginosa*, both of which are DNA binding proteins.[39−41] Therefore, the aminopeptidase may have a role in transcriptional regulation similar to that of phasin PhaF.[14,42] The 32−40 kD protein bands all have the same N-terminal sequence, similar to that of PhaF (i.e., AGKKNSEKEGSSVGGIEKY). It is likely that these are C-terminal degradation products of PhaF. The 29 kD protein band corresponds to the PHA depolymerase (PhaZ) with a N-terminal sequence of MPQPYIFRTVELDNQ.

The concomitant presence of a PHA polymerase, a PHA depolymerase and an acyl-CoA synthetase on the granule would result in a futile cycle if all enzymes were active at the same time. To investigate whether it is the case, each enzyme was

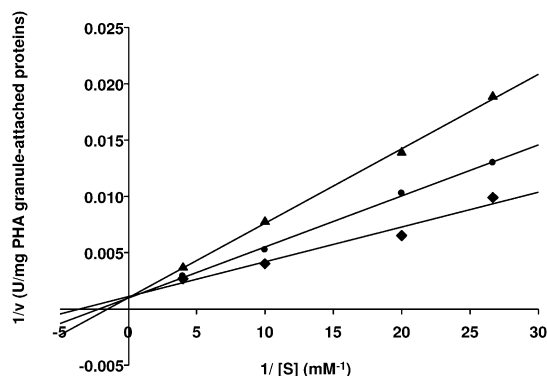

Figure 2. Double reciprocal plot of the initial reaction rate (*v*) of granule-bound PhaC vs *R*-3-hydroxyoctanoyl-CoA concentration ([*S*]). Assay conditions: 100 mM Tris-HCl pH 8, 1 mg/mL BSA, 0.5 mM $MgCl_2$, 0.0375−0.25 mM *R*-3-hydroxyoctanoyl-CoA, 1 mg/mL PHA granules with 0 mM CoA (-♦-), 0.5 mM CoA (-●-), or 1 mM CoA (-▲-). Initial PhaC activity was measured by measuring *R*-3-hydroxyoctanoyl-CoA depletion by HPLC. Data are averages of three determinations.

characterized in more detail and the effect of various possible physiological reagents on the activity was tested.

PHA Polymerase (PhaC). The previously reported method was used for the activity assay of the PHA granule-associated polymerase.[28] With this assay, the interference of thioesterases, esterases, or other enzymes could be ruled out.[28] In this study, even though PhaC2 could not be detected on SDS-gel, its activity may still present on the PHA granules. Thus, we used total protein activities instead of PhaC specific activities. The incorporation of *R*-3-hydroxyoctanoyl-CoA into PHA by granule-bound PhaC follows Michaelis−Menten kinetics, as depicted in the Lineweaver−Burk plot for different concentrations of free CoA (Figure 2).

In the absence of free CoA, the K_m for *R*-3-hydroxyoctanoyl-CoA and V_{max} for incorporation of the substrate into PHA were 277 μM and 9.2 U/mg total granule-bound proteins, respectively. Addition of CoA to the assay mixture resulted in a similar V_{max}, but a shift toward a higher K_m (Figure 2): K_m was increased to 476 and 625 μM when 0.5 and 1 mM CoA were applied, respectively. This phenomenon can be explained by inhibition of activities of PHA polymerase by free CoA.

The concentration of free CoA in the cytoplasm of aerobic bacteria is typically between 0.2−1.0 nmol per mg cell dry weight,[43] equivalent to intracellular concentrations of 0.05−0.25 mM (a factor of approximately 4 mL cell volume per g dry weight was calculated on the basis of measurements of the cell size). Thus, the in vivo inhibition of PHA synthesis by free CoA is not likely. Regulation and modulation of PHA polymerase activity by free CoA is therefore negligible.

Other physiological compounds including NAD(P), NAD(P)H, ATP, AMP, ADP, and acetyl-phosphate were tested for their effect on granule-bound PhaC activity. Concentrations up to 1 mM of any of these compounds had only a minor influence on PHA polymerase activity (Table 1).

PHA Depolymerase (PhaZ). Granule-bound PhaZ activities were measured by incubation of PHA granules from *P. putida* GPo1 and *P. putida* GPo500 in 100 mM Tris-HCl buffer pH 8 at 30 °C and following the release of 3-hydroxycarboxylic acids in the supernatant using GC analysis (Figure 3A). It was observed that the released 3-hydroxycarboxylic acids from GPo1 granules was increased from 0.24 to 0.85 μmol per mg PHA granules in 60 min, whereas GPo500 released less than 0.1 μmol 3-hydroxycarboxylic acids per mg PHA granules during the same period. LC-MS analysis confirmed that the released 3-hydroxycarboxylic acids were in both monomeric and dimeric form. Recently, it has been reported that the purified PHA depolymerase released both monomers and dimers from purified PHA granules.[17] In this study, similar results were obtained, confirming that PHA depolymerase is an *endo*/*exo*-acting enzyme.

Table 1. Effect of Various Cofactors and Metabolites on the Activity of Granule-Bound PHA Polymerase

physiological compound	% activity
none[a]	100
NAD (1 mM)	96
NADH (1 mM)	90
NADP (1 mM)	96
NADPH (1 mM)	95
AMP (1 mM)	93
ADP (1 mM)	93
ATP (1 mM)	94
acetyl-phosphate (1 mM)	98

[a] The standard assay mixture (300 μL) contained 0.5 mM *R*-3-hydroxyoctanoyl-CoA, 1 mg/mL BSA, 0.5 mM $MgCl_2$, and 1 mg/mL PHA granules in 100 mM Tris-HCl, pH 8. Initial activity of the first 5 min was measured spectrophotometrically (A412nm) by following release of CoA using DTNB.

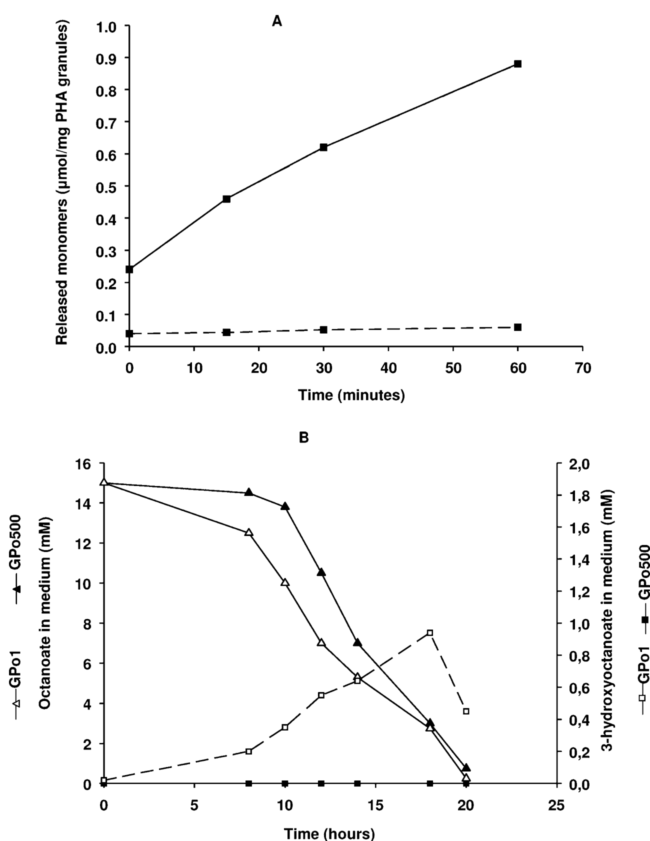

Figure 3. Activity of PHA depolymerase of *P. putida*. Panel A: Release of 3-hydroxycarboxylic acids by granule-bound PhaZ. Assay conditions: 100 mM Tris-HCl pH 8, 0.5 mM $MgCl_2$ and PHA granules (1 mg/mL) from either *P. putida* GPo1 (solid lines) or *P. putida* GPo500 (dashed lines). Samples (250 μL) were taken at timed intervals and analyzed for the amount of released 3-hydroxycarboxylic acids and related to the amount of residual PHA. Panel B: Octanoate uptake and 3-hydroxyoctanoate excretion by *P. putida* GPo1 and GPo500. Culture medium was analyzed for residual octanoate (triangles) and excretion of 3-hydroxyoctanoate (squares) during growth of either *P. putida* GPo1 (open symbols) or *P. putida* GPo500 (closed symbols). Culture medium samples were lyophilized and methanolyzed prior to analysis by GC.

In contrast to PHA polymerases, the PHA depolymerase does not appear to be controlled by physiological reagents such as CoA, acetyl-CoA, and octanoyl-CoA. Similar to PhaC, the activity of PhaZ was not influenced by up to 1 mM of acetyl-phosphate, NAD(P), NAD(P)H, AMP, ADP, or ATP (data not shown). It is possible that PhaZ is less dependent on fluctuations in the environment as compared to PHA polymerases because it does not share its substrate with other enzymes. Nevertheless, the degradation rate decreased about 1.5−2-fold during the experiment (60 min), suggesting that there may be some enzyme inactivation or product inhibition.

As the analyzed PHA granules were isolated in the mid-exponential growth phase when cells just started to synthesize PHA,[12] it was interesting to observe that PHA depolymerase was already active. Previously, it has been reported that a PhaZ negative mutant of *P. putida* U accumulated only about half the amount of the PHA found in the wild-type strain U during the exponential and stationary phases of growth.[44] This was surprising because one expects a higher PHA content when the degradation of the synthesized polymer cannot occur, such as in a PhaZ negative mutant. Apparently, the PhaZ activity is needed for the efficient production of PHA. Our data obtained in this study show simultaneous activities of PhaC and PhaZ in the PHA accumulation phase.

Further evidence for simultaneous accumulation and degradation is shown in Figure 3B in which the consumption of octanoate by *P. putida* GPo1 and GPo500 was followed by GC-analysis. Concomitant with the uptake of octanoate from the medium, 3-hydroxyoctanoate (and a minor amount of 3-hydroxyhexanoate) was excreted in the medium up to a concentration of 1 mM. When the octanoate in the medium is depleted, the excreted 3-hydroxyoctanoate was consumed and used for further metabolism. This excretion of 3-hydroxyoctanoate did not occur in *P. putida* GPo500, indicating that the excretion is dependent on PhaZ activity.

Acyl-CoA Synthetase. N-terminal sequencing revealed a 64 kD protein band as an acyl-CoA synthetase (ACS1), which was recently found to localize on PHA granules by in vivo microscopic studies and to catalyze the formation of 3-hydroxyacyl-CoA from 3-hydroxycarboxylic acids.[16] Studies on the substrate specificity of the granule-bound ACS1 revealed that ACS1 has a high affinity for medium and long chain fatty acids and 3-hydroxycarboxylic acids.[16] In this work, we further investigated the function of ACS1 by knocking out the *acs1* gene of *P. putida* GPo1.

The *acs1* mutant KO15 was first tested for its growth behavior. When grown on mineral medium supplemented with citrate as the sole carbon source, KO15 exhibited the same growth characteristics and maximal optical densities as the parental strain GPo1 (Figure 4A). This was expected because utilization of citrate does not require the enzymes involved in fatty acid degradation such as acyl-CoA synthetase. Furthermore, in GPo1 citrate cannot initiate PHA accumulation, and thus, the function of acyl-CoA synthetase in PHA metabolism is not needed. When octanoate was used as the sole carbon source, KO15 showed a significantly delayed growth compared to GPo1 (Figure 4B). Once adapted after the initial lag phase, growth rates and final cell densities were similar to the parental strain. This indicates that fatty acid uptake or assimilation might be affected in the knockout mutant and some alternative genes or enzymes have to be activated first.

The mutant was then analyzed for PHA synthesis and degradation. When grown on octanoate in a shake flask, the patterns of PHA accumulation and degradation were similar in

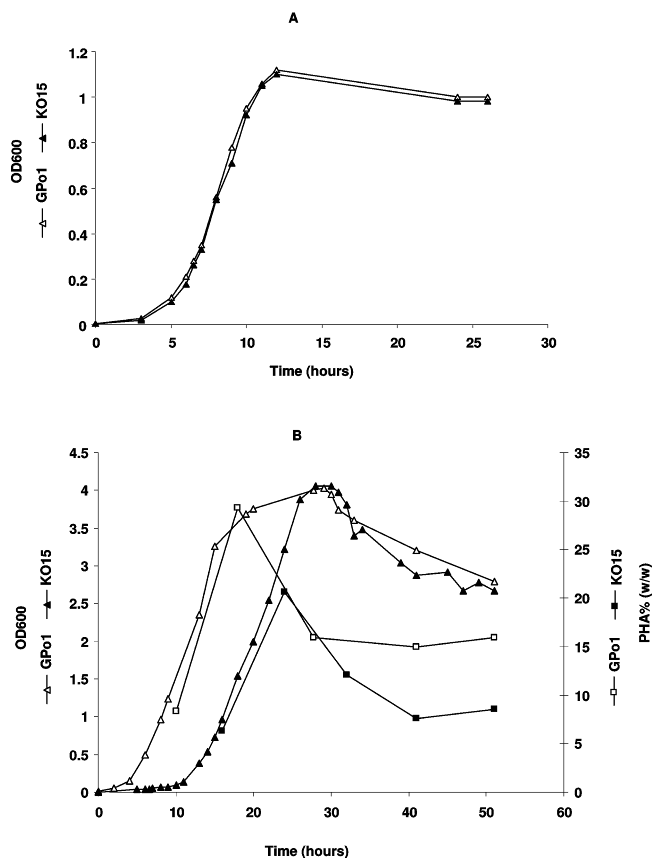

Figure 4. Comparison of *acs1* mutant KO15 and the parental strain GPo1. Panel A: GPo1 (open symbols) and KO15 (closed symbols) were cultivated in mineral medium with 10 mM citrate. Panel B: GPo1 (open symbols) and KO15 (closed symbols) were cultivated in mineral medium with 15 mM octanoate. Growth (triangles) and PHA synthesis (squares) were followed for 52 h. PHA content is displayed in weight % (wt %) of total cell dry mass.

both KO15 and GPo1, with increasing PHA content during the growth phase, followed by a decrease of PHA concentration in stationary cells (Figure 4B). However, KO15 accumulated only 50−70% of the PHA found in GPo1. This could be caused by the following: PHA accumulation and degradation might be a simultaneous process. In an intact cycle, part of the precursors for PHA synthesis, that is, 3-hydroxyacyl-CoA might result from previously depolymerized PHA. In KO15 CoA activation of 3-hydroxycarboxylic acids is hampered, thus, reutilization of monomers to form PHA is not possible or slowed down. It is also possible that the acyl-CoA synthetase is needed for optimal PHA production, as speculated for PhaZ.[44] Another explanation for the reduced PHA content in KO15 might be related to the activity of PHA polymerases: the results obtained in this study and reported previously suggest that free CoA inhibits PHA polymerase activity.[45] If *acs1* disruption leads to higher intracellular CoA levels, PHA polymerases might be less active, resulting in lower PHA accumulation.

It was also found that PHA accumulation in KO15 dropped after ∼24 h, similar to GPo1 after ∼18 h, and after ∼40 h, a slight increase of PHA was observed in both GPo1 and KO15. The decrease of PHA content in KO15 could be caused by a second acyl-CoA synthetase or some other pathway/proteins that can regenerate PHA precursors. The slight increase of PHA after 40 h could be caused by different reasons. One possibility is that cell lysis took place at this physiological stage; cells with PHA may survive better than those without or less PHA, leading to less cell lysis. Thus, the measured PHA content relative to

the total cell dry weight may increase. This slight increase could also be caused by the measurement error with GC, because the increases in both wild type (from 15 to 16% PHA) and mutant strain (from 7.6 to 8.5% PHA) were not significant.

Previously, two CoA synthetases have been discovered: ACS1 and ACS2 in *P. putida* GPo1[16] and FadD1 and FadD2 *P. putida* U.[18] When *fadD1* was knocked out growth on fatty acids had a long lag phase, similar to what has been observed for *acs1*-negative mutant KO15 of *P. putida* GPo1 in this study, leading to the assumption that *fadD2* might become induced when *fadD1* is inactivated.[18] However, in contrast to KO15, the *fadD1*-negative mutant of *P. putida* U synthesized similar amounts of PHA as its parental strain when grown on fatty acids.[18] The reason for the difference in PHA accumulation is not clear. It might be that the homologues, which can partially replace the functions of ACS1 and FadD1, have different substrate specificities.

Analysis of the *acs1* mutant further confirmed the involvement of the ACS1 enzyme in PHA metabolism. A putative role of this granule-bound acyl-CoA synthetase could be to activate 3-hydroxycarboxylic acid monomers, produced by degradation of PHA, to their CoA-form. The CoA-form monomers could then enter the fatty acid oxidation cycle or be reincorporated into PHA. Obviously, the activity of ACS1 is influenced by CoA concentrations because CoA is the substrate of ACS.

Fatty Acid Oxidation Complex. As PHA synthesis depends on the availability of *R*-3-hydroxyacyl-CoA precursors,[46,47] the influence of different physiological compounds of the central metabolism on fatty acid oxidation was investigated. The fatty acid oxidation complex of *P. putida* GPo1 was partially purified to remove the PHA polymerases and thioesterases, which were found to interfere with the β-oxidation assay. The partially purified β-oxidation complex showed to cleave *R*-3-hydroxyoctanoyl-CoA into stoichiometric amounts of hexanoyl-CoA and acetyl-CoA. This indicated that the oxidation complex contained active epimerase, 3-hydroxyacyl-CoA dehydrogenase, and 3-ketoacyl thiolase, and was essentially free of PHA polymerases and thioesterases. The 3-hydroxyacyl-CoA dehydrogenase and 3-ketoacyl thiolase require NAD and CoA, respectively, for activity, providing possible means for control and regulation of fatty acid oxidation. This was investigated by following the oxidation of *R*-3-hydroxyoctanoyl-CoA using different ratios of [acetyl-CoA]/[CoA] and [NADH]/[NAD] in the assay mixture (Figure 5).

Figure 5 clearly demonstrates the reduced consumption of *R*-3-hydroxyacyl-CoA when the ratios [acetyl-CoA]/[CoA] and [NADH]/[NAD] are increased from 0.1 to 1. Moreover, when 2-octenoyl-CoA was used as a substrate, it was found that 3-hydroxyoctanoyl-CoA was formed. The increased accumulation of 3-hydroxyoctanoyl-CoA is a result of increased ratios of [acetyl-CoA]/[CoA] and [NADH]/[NAD] (Figure 5). The accumulation of 3-hydroxyoctanoyl-CoA is likely the result of the higher activity of enoyl-CoA hydratase as compared to the reduced activities of 3-hydroxyacyl-CoA dehydrogenase and 3-ketoacyl thiolase which are dependent on the limiting amounts of NAD and CoA, respectively.

Considering the results described above, it appears that the ratios [acetyl-CoA]/[CoA] and [NADH]/[NAD] have considerable influence on fatty acid oxidation. The intracellular levels of 3-hydroxyacyl-CoA may be regulated by the ratios of [acetyl-CoA]/[CoA] and [NADH]/[NAD] such that high ratios result in accumulation while low ratios result in consumption of *R*-3-hydroxyoctanoyl-CoA.

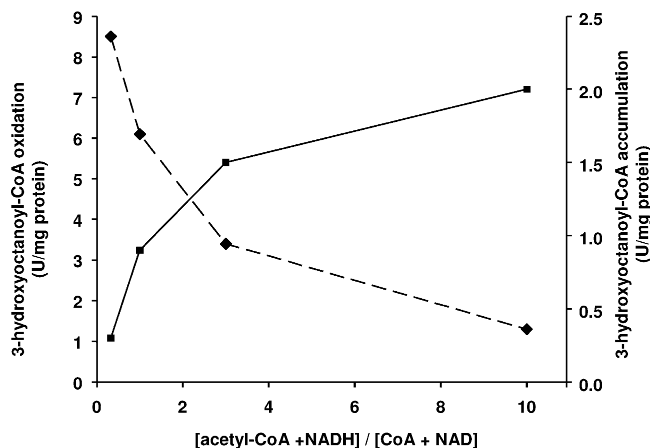

Figure 5. Influence of [acetyl-CoA]/[CoA] and [NADH]/[NAD] ratios on the oxidation and formation of 3-hydroxyoctanoyl-CoA. *R*-3-hydroxyoctanoyl-CoA oxidation (-◆-, left scale) and 3-hydroxyoctanoyl-CoA accumulation (-■-, right scale) were followed using different ratios of [acetyl-CoA]/[CoA] and [NADH]/[NAD]. Assay conditions: 100 mM Tris-HCl pH 9, 0.5 mg/mL BSA, 250 mM NaCl, and 0.125% Triton X-100 with either 0.5 mM *R*-3-hydroxyoctanoyl-CoA (to measure *R*-3-hydroxyoctanoyl-CoA depletion) or 0.5 mM 2-octenoyl-CoA (to measure 3-hydroxyoctanoyl-CoA formation). Acetyl-CoA, CoA, NAD, and NADH were added such that [acetyl-CoA] + [CoA] = 1 mM, [NAD] + [NADH] = 1 mM, [CoA] = [NAD], [acetyl-CoA] = [NADH].

PHA Metabolism and Fatty Acid Oxidation. In this study, we observed that PHA depolymerase (PhaZ) attached to PHA granules released 3-hydroxycarboxylic acids and also seemed to function under conditions when PhaZ activity would appear not to be required (Figure 3). Indeed, it was suggested that PHA polymerase (PhaC) and PHA depolymerase are organized in a complex to function efficiently and PhaZ is needed for the optimal production of PHA.[44] The released 3-hydroxycarboxylic acids are (or can be) activated to 3-hydroxyacyl-CoAs by an acyl-CoA synthetase via an ATP-dependent reaction.[16] This metabolite is a substrate for PhaC as well as the β-oxidation cycle. Depending on the metabolic state of the cell, 3-hydroxyacyl-CoA is either incorporated into nascent PHA polymer chains or oxidized via the β-oxidation pathway to make the stored carbon available to the central metabolism.

When cells contain high levels of acetyl-CoA, ATP, and NADH (high energy metabolites), cells cannot utilize these compounds for growth, resulting in high [acetyl-CoA]/[CoA] and [NADH]/[NAD] ratios. The β-oxidation cycle is inhibited and 3-hydroxyacyl-CoA accumulates. Since the activity of PhaC is stimulated by the high [3-hydroxyacyl-CoA]/[CoA] ratio, the 3-hydroxycarboxylic acids are channeled into PHA. The activity of PhaZ is not influenced by the changing metabolite concentrations and continues to degrade the polymer to some extent. Due to the limited availability of free CoA in the cell, part of the released monomers are excreted rather than activated again by the acyl-CoA synthetase (Figure 3). When the concentration of high energy metabolites is low, [acetyl-CoA]/[CoA] and [NADH]/[NAD] ratios in cells are low, which stimulate fatty acid oxidation. As a consequence, the 3-hydroxyacyl-CoA formed by the sequential action of PhaZ and acyl-CoA synthetase is mainly degraded via the fatty acid oxidation pathway rather than being polymerized again.

Physiological Role of Simultaneous PHA Synthesis and Degradation. Our results, as well as earlier data from Doi et al.,[19] Zinn[48] and Uchino et al.,[20] show that there is simultaneous polymerization and degradation of PHA. 3-Hydroxycarboxylic acids are released by PhaZ and excreted in the medium, while

simultaneously 3-hydroxyacyl-CoA is incorporated in the polymer. This results in a cyclic metabolic pathway of which the net balance is the hydrolysis of ATP to AMP and pyrophosphate. The metabolic advantage of such a mechanism is not clear, as a significant amount of energy is wasted in what appears to be a futile cycle. It could, however, be that this cycle provides the cell with a valuable regulatory mechanism to control fatty acid flux through the fatty acid oxidation pathway. Under fluctuating environmental concentrations of carbon and other vital nutrients, the cell can adapt very rapidly without the need for the induction of a cascade of genes. Instead, PHA acts as a metabolic buffer that can easily absorb or release carbon as required.

Conclusion

An interesting finding of this study is that the activities of three PHA granule-attached catalytic proteins, namely, PHA polymerase, depolymerase, and acyl-CoA synthetase, are needed for optimal PHA production. Even during the PHA accumulation phase, the PHA depolymerase is active, further confirming that PHA synthesis and degradation is a simultaneous process. Our experiments performed with different physiological compounds demonstrate that PHA polymerase activity of *P. putida* Gpo1 is sensitive to the ratio of [*R*-3-hydroxyacyl-CoA]/[CoA] in which free CoA was found to be a mild competitive inhibitor, while fatty acid oxidation is regulated by the [acetyl-CoA]/[CoA] and [NADH]/[NAD] ratios, with high ratios resulting in accumulation and low ratios leading to rapid oxidation of 3-hydroxyacyl-CoA. These results suggest that the balance between PHA synthesis and fatty acid oxidation is controlled mainly by the [acetyl-CoA]/[CoA] and [NADH]/[NAD] ratios in cells. Because PHA metabolism involves not only catalytic proteins, but also structural proteins such as phasins and regulatory proteins, it will be necessary to study the influence of the latter proteins on PHA synthesis and degradation in the next steps.

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