Production of a Polyhydroxyalkanoate Biopolymer in Insect Cells with a Modified Eucaryotic Fatty Acid Synthase

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A novel pathway for the synthesis of poly-3-hydroxybutyrate has been engineered by simultaneous delivery of two genes into insect cells (*Spodoptera frugiperda*) by use of individual baculovirus vectors. This system includes expression of a dehydrase-domain mutant rat fatty acid synthase cDNA and the *phbC* gene encoding polyhydroxyalkanoate synthase from *Alcaligenes eutrophus*. The dehydrase-deficient fatty acid synthase provides de novo synthesis of *R*-(–)-3-hydroxybutyryl-coenzyme A as a premature termination product rather than palmityl-coenzyme A, the normal product of wild-type rat fatty acid synthase. High levels of this mutant multifunctional protein provide a suitable precursor pool of *R*-(–)-3-hydroxybutyryl-coenzyme A for conversion to poly-3-hydroxybutyrate in insect cells coexpressing the *phbC* gene product. This strategy for redesigning a poly-3-hydroxybutyrate biosynthetic pathway suggests a new method for generating structurally diverse polyhydroxyalkanoates by metabolic engineering.

Polyhydroxybutyrate (PHB) is a linear polyester of *R*-(–)-3-hydroxybutyric acid that was first discovered almost 70 years ago (24). It serves as a carbon or energy reserve under limiting nutrient conditions in a variety of gram-positive and gram-negative bacteria (5). PHB is often visualized as electron-dense granules in bacterial cells that can produce the material at levels up to 90% dry cell weight. PHB was the first identified member of a class of biodegradable thermoplastic storage molecules collectively known as polyhydroxyalkanoates (PHAs). Wallen and Rohwedder (47) identified two 3-hydroxy fatty acids in activated sewage sludge, and later, the presence of at least six 3-hydroxy fatty acids was shown to exist in polymer hydrolysates of batch-grown *Bacillus megaterium* (3, 8, 13). The structural characteristics imparted by various alkyl groups result in unique physical properties for different PHA polymers (2, 32, 41).

Although PHB represents the archetypical form of a biodegradable PHA, its unfavorable physical properties (i.e., brittle, low thermal decomposition temperature, low impact strength) preclude significant use of the homopolymeric form. However, copolymers derived from various proportions of 3-hydroxybutyrate and 3-hydroxyvalerate monomers have enhanced physical properties (i.e., greater flexibility, higher thermal decomposition temperature, and higher impact strength) (14). Such copolymers are synthesized when cells are grown in the presence of propionic acid, with the crystallinity of the polymer decreasing as the proportion of hydroxyvalerate increases (14).

Significant progress has been made recently in the cloning, analysis, and expression of genes involved in production of PHB (11, 29, 30, 32, 40, 43). This work has provided an important foundation from which to consider new approaches for production of useful PHAs. In addition, high-level production of PHB has recently been demonstrated in plants by introducing the genes encoding acetoacetyl-coenzyme A (acetoacetyl-CoA) reductase (*phbB*), PHA synthase (*phbC*), and β -ketothiolase (*phbA*) into *Arabidopsis thaliana* (27, 31, 32).

Although strategies exist for generating PHAs with useful physical properties, they are limited by the expense of specialized feedstocks for bacterial fermentation. There would be considerable advantages to a metabolic engineering strategy which provides synthesis of PHA monomers intracellularly, compared with the traditional approach of feeding specialized precursor feedstocks to bacterial fermentation systems. Toward this end, we considered the possibility that acyl chain biosynthesis by fatty acid and polyketide synthases (PKSs) (15) may provide a versatile overall strategy. The model for this type of approach is represented by the multifunctional rat fatty acid synthase (FAS) (36). It is a homodimer with a subunit structure consisting of 2,505 amino acid residues and a molecular mass of 272,340 Da. Each subunit consists of seven catalytic activities in separate domains (1). Studies of rat FAS led to the recognition of multifunctional proteins as a new class of enzymes with two or more activities associated with a single polypeptide (21, 22, 42). Further studies involving limited proteolysis of these large multifunctional proteins revealed that some isolated domains retained activities associated with the FAS (33, 37, 44).

An important issue for producing novel PHAs that have utility and retain their quality as biodegradable and renewable materials is the ability to generate in vivo a diverse range of hydroxyacyl-CoA monomers. Traditional strategies involve feeding short- and long-chain fatty acid monomers to bacterial cultures that are incorporated into the polymer chain to give random copolymers with enhanced physical properties (2). Current technology is limited to the monomeric diversity generated by cells that produce PHAs. *R*-(–)-3-hydroxybutyryl-CoA is a common substrate in many bacteria; however, precursor feeding studies have revealed that some PHA synthases are relatively nonspecific and may polymerize 3-hydroxy-, 4-hydroxy-, and 5-hydroxyalkyl-CoA esters (6, 12, 39, 45). Furthermore, methyl-substituted and unsaturated 3-hydroxyalkyl-CoA esters have also been incorporated into PHA polymers in *Pseudomonas oleovorans* (9, 10, 17). A primary challenge for future development of technologies to produce structurally diverse hydroxyacyl-CoA units for homopolymer and copolymer PHAs is the rational construction of monomer pathways that provide an intracellular precursor pool for the PHA syn-

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FIG. 1. Comparison of the natural and engineered pathways for PHB synthesis. The three enzymatic steps of PHB synthesis in bacteria involving β -ketothiolase, acetoacetyl-CoA reductase, and PHB polymerase are shown on the left. The engineered pathway for PHB synthesis in *Sf21* cells is shown on the right. This involves condensation of acetyl-CoA and malonyl-CoA followed by ketoreduction of acetoacetyl-ACP to give β -hydroxybutyryl-ACP (16). These reactions are both performed by the rat FAS DH⁰ multifunctional protein (represented by the open arrow) with catalytic sites abbreviated as follows: KS, β -ketoacyl synthase domain; AT, acyltransferase domain; DH⁰, nonfunctional DH domain; KR, ketoreductase domain; ER, enoylreductase domain; TE, thioesterase domain. SCoA, S-coenzyme A.

thase. Although this already occurs in some bacteria through the β -oxidation pathway of fatty acids, it provides only a limited array of PHA monomers that vary in alkyl chain length but not in the degree of alkyl group branching, unsaturation, or functionalization along the acyl chain. By redirecting a FAS (or polyketide synthase) (4, 16, 20) multifunctional protein toward premature termination products, it may be possible to generate a predetermined and structurally diverse array of acyl chains for the synthesis of high-utility biodegradable PHAs.

Recently, expression of functionally active rat FAS in a baculovirus-insect cell expression system allowed identification of the dehydrase (DH)-domain active-site His-878 (19). Following site-directed mutagenesis, *in vitro* studies showed that the DH mutant with His replaced by Ala at position 878 (His-878 $\rightarrow$ Ala) produced 3-hydroxybutyryl-CoA as a premature termination product from acetyl-CoA, malonyl-CoA, and NADPH. We therefore considered the possibility that the rat FAS DH mutant could be a model for a redirected multifunctional protein to provide monomer substrates for PHA biosynthesis.

This report describes the construction of a novel metabolic pathway for the production of PHAs in insect cells. The approach utilizes a DH-deficient (DH⁰) form of rat FAS to pro-

duce the PHB monomer, *R*-(−)-3-hydroxybutyrate. This multifunctional protein effectively replaces the β -ketothiolase and acetoacetyl-CoA reductase activities of the natural procaryotic PHB pathway. Coexpression of the mutant rat FAS cDNA with the wild-type *Alcaligenes eutrophus phbC* gene results in PHB production in insect cells.

MATERIALS AND METHODS

Plasmids, strains, and culture conditions. Plasmid pFAS206 and a recombinant baculoviral clone encoding FAS206 (19) were generous gifts of A. Joshi and S. Smith. Plasmid pAet41 (30), the source of the *Alcaligenes eutrophus* PHA synthase, was kindly provided by A. Sinskey (Massachusetts Institute of Technology, Cambridge). Baculovirus transfer vector pBacPAK9 and linearized baculoviral DNA were obtained from Clontech Inc. (Palo Alto, Calif.). Restriction enzymes, T4 DNA ligase, *Escherichia coli* DH5 α competent cells, lipofectin reagent, Grace's insect cell medium, fetal bovine serum, and antibiotic-antimycotic reagent were obtained from GIBCO-BRL (Grand Island, N.Y.). *Spodoptera frugiperda* (strain Sf21) and BTI-TN-5B1 *Trichoplusia ni* insect cells were generous gifts from G. Franzen, R&D Systems (Minneapolis, Minn.) and Stefan Harsch (Department of Veterinary Pathobiology, University of Minnesota), respectively. Tissue culture dishes were obtained from Corning Inc. (Corning, N.Y.). Spinner flasks were obtained from Bellco Glass Inc. (Vineland, N.J.). SeaPlaque GTG agarose was obtained from FMC Bioproducts Inc. (Rockland, Maine).

Construction of pBPphbC. The *phbC* gene was excised from pAet41 on an approximately 1.8-kb fragment (30) by digestion with *Bst*BI and *Stu*I, purified as

FIG. 2. SDS-PAGE and Coomassie brilliant blue R staining of proteins synthesized at various time points during infection of Sf21 cells. Approximately 0.5 mg of total cellular protein from various samples was fractionated on a 10% polyacrylamide gel. Samples include uninfected cells on days 0, 1, 2, and 3 (lanes 1 to 4, respectively), infection with BacPAK6::phbC alone on days 0, 1, 2, and 3 (lanes 5 to 8, respectively), infection with baculoviral clone containing rat FAS206 alone (19) on days 0, 1, 2, and 3 (lanes 9 to 12, respectively), and doubly infected cells (rat FAS206, BacPAK6::phbC) on days 0, 1, 2, and 3 (lanes 13 to 16, respectively). (A) Rat FAS DH⁰ protein; (B) PHA synthase. Migration of molecular mass markers (in kilodaltons) is indicated on the left.

described earlier (48), and ligated to pBacPAK9 digested with *Bst*BI and *Stu*I. This resulted in pBPphbC, the baculovirus transfer vector used in formation of recombinant baculovirus particles carrying *phbC*.

Insect cell maintenance and recombinant baculovirus formation. Sf21 cells were maintained (28) at 26 to 28°C in Grace's insect cell medium supplemented with 10% fetal bovine serum, 1.0% pluronic F68, and 1.0% antibiotic-antimycotic reagent. Cells were maintained in suspension at 0.2×10^6 to 2.0×10^6 /ml in a 60-ml total culture volume in 100-ml spinner flasks at 55 to 65 rpm. Cell viability during the culture period was typically 95 to 100%, as determined by trypan blue dye exclusion. The procedures for use of the transfer vector and baculovirus were essentially those described by the manufacturer (Clontech). Purified pBPphbC and linearized baculovirus DNA were used to cotransfect Sf21 cells by the liposome-mediated method (7). Four days later, cotransfection supernatants were harvested for subsequent plaque purification. Recombinant viral clones were purified from plaque assay plates containing 1.5% SeaPlaque GTG after 5 to 7 days at 28°C. Recombinant viral clone stocks were then amplified in T25 flask cultures (4 ml, 3×10^6 /ml on day 0) for 4 days; infected cells were identified morphologically and then screened for production of PHA synthase by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 10% polyacrylamide gels (23) stained with Coomassie brilliant blue R.

Insect cell double-infection protocol. Four 100-ml spinner flasks were each inoculated with 8×10^7 cells in 50 ml of fresh insect cell medium. To flask 1, an additional 20 ml of fresh insect cell medium was added (uninfected control); to flask 2, 10 ml of BacPAK6::phbC viral stock ($\approx 10^8$ PFU/ml) and 10 ml of fresh insect cell medium were added; to flask 3, 10 ml of BacPAK6::FAS206 viral stock ($\approx 10^8$ PFU/ml) and 10 ml of fresh insect cell medium were added; and to flask 4, 10 ml of BacPAK6::phbC viral stock ($\approx 10^8$ PFU/ml) and 10 ml of BacPAK6::FAS206 viral stock ($\approx 10^8$ PFU/ml) were added. These viral infections were carried out at multiplicities of infection of approximately 10. Cultures were maintained under normal growth conditions, and 15-ml samples were removed at 24-, 48-, and 72-h time points. Expression of both rat FAS and PHA synthase is under the control of the very late polyhedrin promoter. Very late baculovirus gene expression commences at approximately 24 h postinfection and continues through 48 to 60 h postinfection, after which most cells are no longer viable (28). We maintained the cultures to 72 h in an effort to maximize PHB production. Cells were collected by gentle centrifugation at $1,000 \times g$ for 5 min, the medium was discarded, and the cells were immediately stored at -70°C .

PHB assay. PHB was assayed from Sf21 cell samples by the propanolysis method of Riis and Mai (34). Cell pellets were thawed on ice, resuspended in 1 ml of cold distilled deionized H₂O, and transferred to 5-ml screw-top test tubes with Teflon seals. Two milliliters of distilled deionized H₂O was added, the cells were washed and centrifuged, and then 3 ml of acetone was added and the cells were washed and centrifuged. The samples were then desiccated by placing them in a 94°C oven for 12 h. The following day, 0.5 ml of 1,2-dichloroethane, 0.5 ml

of acidified propanol (20 ml of HCl, 80 ml of 1-propanol), and 50 μ l of benzoic acid standard were added and the sealed tubes were heated to 100°C in a boiling water bath for 2 h with periodic vortexing. The tubes were cooled to room temperature, and the organic phase was submitted for gas chromatographic (GC) analysis with a Hewlett-Packard 5890A GC equipped with a Hewlett-Packard 7673A automatic injector and a fused silica capillary column (DB-WAX 30W; 30 m long). Positive samples were further subjected to GC-mass spectrometry (GC-MS) analysis for the presence of propylhydroxybutyrate with a Kratos MS25 GC-MS. The following parameters were used: source temperature, 210°C; voltage, 70 eV; accelerating voltage, 4 keV.

Confocal microscopy and immunofluorescence. BTI-TN-5B1-4 *T. ni* cells were cultured for 44 h on 12-mm-diameter glass coverslips that had been treated with 5 mM EDTA. The cells were washed in phosphate-buffered saline (PBS; 137 mM NaCl, 1.47 mM KH₂PO₄, 8.17 mM Na₂HPO₄, 2.7 mM KCl [pH 7.0]) for 5 min and then fixed with paraformaldehyde (4% paraformaldehyde, 100 mM PBS [pH 7.0]) on ice for 60 min. After fixation, the cells were washed three times for 5 min each in PBS, permeabilized in methanol for 10 min at -20°C , and allowed to air dry. The cells were then treated with blocking buffer (5% glycerol, 5% normal goat serum [Gibco-BRL], 1% bovine serum albumin, 1% cold-water fish gelatin [Sigma Chemical Co., St. Louis, Mo.], 5% dimethyl sulfoxide in PBS [pH 7.2]) for 30 min at 37°C. They were then incubated in the primary antibody (rabbit anti-PHA synthase [48] diluted 1:200 in blocking buffer) overnight at 4°C. After primary antibody incubation, the cells were washed three times for 5 min each in PBS and incubated in the secondary antibody (Cappel goat anti-rabbit fluorescein isothiocyanate 55646 diluted 1:400 in blocking buffer) for 2 h at 37°C. After secondary antibody incubation, the cells were again washed three times for 5 min each time in PBS, allowed to air dry, and mounted on glass slides with VectaShield mountant (Vector Labs, Burlingame, Calif.). Immunofluorescent microscopic images were obtained with a Bio-Rad MRC-600 confocal microscope which was equipped with a krypton-argon laser, a Nikon Diaphot inverted microscope, and a Nikon 60 $\times$ Plan Apo objective (NA 1.4).

RESULTS

Coexpression of rat FAS DH⁰ cDNA and *phbC* in insect cells. To engineer a monomer pathway for PHA biosynthesis, a strategy was devised to generate PHB by combining the metabolic potential of a multifunctional FAS with PHA synthase from *Alcaligenes eutrophus* (30). Previous studies di-

FIG. 3. GC analysis of PHB accumulation in Sf21 cells. GCs from various samples are superimposed and labeled 1 to 7. The PHB standard (Sigma) is chromatogram 7, showing a propylhydroxybutyrate elution time of 10.0 min (arrow labeled S). The GC of extracts of the uninfected (chromatogram 1) and single-infected (chromatogram 2, rat FAS DH⁰, day 3; chromatogram 3, PHA synthase, day 3) samples are shown at the bottom. GCs of extracts of double-infected cells at days 1 (chromatogram 4), 2 (chromatogram 5), and 3 (chromatogram 6) are also shown exhibiting a peak eluting at 10.0 min (arrow labeled X). Peak, double-infected, day 3 extract (chromatogram 6) was used for MS analysis.

FIG. 4. GC-MS (electron impact) analysis of PHB. The characteristic fragmentation of propylhydroxybutyrate at m/z of 43, 60, 87, and 131 is shown. (A) PHB standard (Sigma) from bacteria; (B) peak X from double-infected, day 3 (Fig. 3, chromatogram 6) Sf21 cells expressing rat FAS DH⁰ protein and PHA synthase.

rected toward the identification of a DH-domain active site in the rat FAS resulted in generation of a mutant form of the multifunctional protein that gave 3-hydroxybutyryl-CoA production in vitro (19). However, it was not clear whether a stable pool of 3-hydroxybutyryl-CoA would be formed in vivo to provide suitable substrate levels for polymerization by PHA synthase.

The strategy for engineering a PHB pathway in insect cells compared with the normal pathway for PHB synthesis in *Alcaligenes eutrophus* is shown in Fig. 1. We decided to investigate whether the His-878→Ala rat FAS DH⁰ multifunctional protein (19) could replace the procaryotic β -ketothiolase and acetoacetyl-CoA reductase functions for 3-hydroxybutyryl-CoA synthesis. To combine the metabolic functions of the multifunctional rat FAS DH⁰ protein and the *Alcaligenes eutrophus* PHA synthase, it was essential to test individual expression of the corresponding genes in Sf21 cells. Production of the active rat FAS DH⁰ polypeptide in Sf9 cells has been reported previously (19). However, synthesis of active PHA synthase in Sf21 cells remained to be established, and a baculovirus transfer vector (pBPphbC) containing the corresponding gene was generated (see Materials and Methods) (48). Following recombination with linearized baculovirus DNA, a recombinant baculovirus clone which produced high levels of soluble PHA synthase as determined by SDS-PAGE was identified (Fig. 2). Recent studies have shown that *Alcaligenes eutrophus* PHA synthase must be modified with phosphopantetheine to be catalytically active (11). Since it was unclear whether this procaryotic gene product would be properly modified in insect cells, a series of experiments was performed that confirmed high-level activity of the PHA synthase obtained from Sf21 cell extracts (48).

Once activity of the *phbC* gene product had been established, the baculovirus clones containing the rat FAS DH⁰

cDNA and BacPAK6:*phbC* were employed in a double infection strategy to determine if PHB would be produced in insect cells. For this to occur, the *R*(-)-3-hydroxybutyryl-CoA released from rat FAS DH⁰ protein must be trapped by PHA synthase and converted to polymer at a rate higher than that of β -oxidation, which would regenerate acetyl-CoA. Another key issue was the stereochemical configuration of the 3-hydroxyl group which must be in the *R* form to be recognized as a substrate by PHA synthase. Fortunately, previous biochemical studies on eucaryotic FASs indicated that the *R* form of 3-hydroxybutyryl-CoA would be generated (35, 46). SDS-PAGE of protein samples from a time course of the uninfected (control), single-infected, and double-infected Sf21 cells was performed (Fig. 2). From these data, it is clear that the rat FAS DH⁰ and PHA synthase polypeptides are efficiently coexpressed in the double-infected Sf21 cells. It is apparent that coexpression results in ~50% reduced levels of both polypeptides compared with that of Sf21 cells that are producing the individual proteins. Western blot (immunoblot) analysis using anti-rat FAS (18) and anti-PHA synthase antibodies (48) confirmed simultaneous production of the corresponding proteins (data not shown).

Characterization of PHB production in insect cells. To determine if de novo synthesis of PHB was occurring in Sf21 cells that coexpress the rat FAS DH⁰ and PHA synthase, fractions of these samples were extracted and the extract was subjected to propanolysis and analyzed for the presence of propylhydroxybutyrate by GC (Fig. 3). A unique peak with a retention time that coincided with that of a propylhydroxybutyrate standard was detected only in the double-infection samples at 48 and 72 h, in contrast to the individually expressed gene products and uninfected controls, which were negative. These samples were analyzed further by GC-MS to confirm the identity of the product. Figure 4 shows MS data corresponding to the

material obtained from peak 10.1 in the GC compared with that of a propylhydroxybutyrate standard. The results show clearly that PHB synthesis is occurring only in Sf21 cells coexpressing the rat FAS DH⁰ cDNA and the *phbC* gene from *Alcaligenes eutrophus*. Integration of the peak in the GC corresponding to propylhydroxybutyrate revealed that approximately 1 mg of PHB was isolated from a 1-liter culture of Sf21 cells (approximately 600 mg [dry cell weight] of Sf21 cells).

PHB granule formation in insect cells. To provide further evidence that PHB was being produced in insect cells, *T. ni* insect cells coexpressing rat FAS DH⁰ cDNA and *phbC* were analyzed for the presence of granules. This was accomplished by cell fixation and treatment with anti-PHA synthase antibodies. Granules were clearly observed (Fig. 5A) under these conditions, in contrast to uninfected wild-type *T. ni* cells (Fig. 5B) or in cells individually producing rat FAS DH⁰ or PHA polymerase polypeptides alone (data not shown). PHB granules of similar sizes and shapes have been observed in bacterial cells (26), transgenic plants (31), and chloroplasts of plant mesophyll cells (27). Taken together, the isolation of PHB and visualization of granules provide compelling evidence of biopolymer formation in insect cells by use of a novel redesigned PHA metabolic pathway.

DISCUSSION

Previous work by Joshi and Smith (19) established the ability of the rat FAS protein with a His-878→Ala mutation to produce 3-hydroxybutyryl-CoA. This analysis identified the active-site residue of the DH domain in the rat FAS multifunctional protein, thereby confirming the physical location of this elusive catalytic step in fatty acid keto group processing. The results suggested that the rat FAS DH⁰ protein, although compromised in its absolute function, was still capable of mediating acyl transfer, catalyzing a single-cycle condensation reaction involving acetyl-CoA and malonyl-CoA, and performing ketoreduction on the nascent acetoacetyl-acyl carrier protein (acetoacyl-ACP) intermediate to give 3-hydroxybutyryl-ACP. Although not explicitly addressed, the identification of 3-hydroxybutyryl-CoA in vitro suggested strongly that the premature termination product was released from the mutant protein by transfer to a CoA acceptor (19). The work described in this report was driven by the hypothesis that premature hydroxyacyl-ACP chain elongation intermediates synthesized by FAS (or PKS) multifunctional proteins may provide (after transfer to a CoA acceptor) a novel source of substrates for PHA synthesis.

An important aspect of the current study was expressing in active form the rat FAS DH⁰ multifunctional protein in concert with the procaryotic PHA synthase from *Alcaligenes eutrophus*. Although expression of the eucaryotic multifunctional protein in active form had been described previously (18, 19), the bacterial enzyme had been expressed only in *E. coli*, albeit relatively poorly. Moreover, recent studies have revealed that catalytically active PHA synthase (11), as with FAS (15), PKS (15), and peptide synthetases (38), requires phosphopantetheine modification of one or more Ser residues. Therefore, the proposed redesigned PHA metabolic pathway required cosynthesis of posttranslationally modified eucaryotic and procaryotic gene products in insect cells. It was therefore significant that PHA synthase can be produced at high levels (~500-fold improvement over that in *E. coli*) in active form by baculoviral expression of the *phbC* gene in insect cells (48). In addition, coexpression of multiple gene products in insect cells has been described previously (25).

Although the expression of both rat FAS DH⁰ and PHA

FIG. 5. Immunofluorescent localization of PHB granules in insect cells producing the rat FAS DH⁰ and PHA synthase polypeptides. (A) *T. ni* cells coinfected with recombinant baculovirus containing the rat FAS DH⁰ cDNA and the PHA synthase gene. Immunofluorescent PHB granules are indicated by the arrows. (B) Uninfected *T. ni* cells. Bars, 10 μ m.

synthase proteins had been achieved previously in a highly active form, several additional requirements had to be met for successful functioning of the redesigned PHA pathway. First, the stereochemistry of the hydroxyl group required the *R* configuration for the 3-hydroxybutyryl-CoA to be a substrate for

PHA synthase. The *R* configuration for the hydroxyl group during keto group processing in the multifunctional FAS had been established previously. However, it was not clear, a priori, whether synthesis of the 3-hydroxybutyryl-ACP chain elongation intermediate and transfer to a CoA acceptor followed by in vivo polymerization would be more rapid than β -oxidation of 3-hydroxybutyryl-CoA to two molecules of acetyl-CoA. The results of this study show that PHA synthase effectively competes for its substrate and suggests that the kinetics of polymerization is at least similar to or more rapid than catabolism of 3-hydroxybutyryl-CoA in insect cells.

This work provides an important starting point for the design of more complex acyl-CoA monomer synthase from FAS or PKS multifunctional proteins in which premature termination of a biosynthetic pathway is specifically engineered to provide suitably modified acyl-CoA monomers for PHA synthesis. This strategy may be applicable to both procaryotic and eucaryotic (including plant) expression systems, since the formation of biopolymer will not be dependent on specialized feedstocks. Thus, once an engineered PHA monomer pathway gene cassette is introduced into a bacterial or plant system, and coexpression with the appropriate PHA polymerase is achieved, novel biopolymer formation may be explored. Thus, the strategy described herein suggests a new way to harvest metabolic diversity provided by FAS or PKS multifunctional proteins involved in the construction of hydroxyacyl-CoA chains and to tap a significant new source of diversity for novel PHA bioplastics.

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