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Title: Role of PhaC type I and type II enzymes during PHA biosynthesis.

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Abstract: PHA synthases (PhaC) are grouped into four classes based on the kinetics and mechanisms of reaction. The grouping of PhaC enzymes into four classes is dependent on substrate specificity, according to the preference in forming short chain length (scl) or medium chain length (mcl) polymers: class I, class III, and class IV produce scl-PHAs depending on propionate, butyrate, valerate and hexanoate precursors, while class II phaC synthesize mcl-PHAs based on the alkane (C6 to C14) precursors.

PHA synthases of class I, in particular $PhaC_{Cs}$ from *Chromobacterium* USM2 and $PhaC_{Cn}$/*Re*PhaC1 from *Cupriavidus necator*/R. *eutropha,* have been analysed and the crystal structures of the C-domains have been determined. $PhaC_{Cn}$/*Re*PhaC1 was also studied by small angle X-ray scattering (SAXS) analysis. Models have been proposed for dimerization, catalysis mechanism, substrate recognition and affinity, product formation and product egress route. The assays based on amino acid substitution by mutagenesis have been useful to validate the hypothesis on the role of amino acids in catalysis and in accommodation of bulky substrates, for the synthesis of PHB co-polymers and medium chain length-PHA polymers with optimized chemical properties.

Keywords: PhaC synthase, classification, dimerization, substrate binding, exit cavity, C3-C14 alkanes, polymer composition.

1. Introduction

Polyhydroxyalkanoates (PHAs) are biodegradable polyesters produced in several Gram-negative and Gram-positive bacteria, in archea and cyanobacteria [1-8]. PHAs are polymers containing various alkanes, such as3-hydroxy propionate (3HP), butyrate (3HB), valerate (3HV), hexanoate (3HHx), heptanoate (3HHp), octanoate (3HO), nonanoate (3HN), decanoate (3HD), dodecanoate (3HDD), in addition to 4-hydroxybutyrate (4HB) or 5-hydroxyvalerate (5HV), and produced through the availability of the corresponding CoA thioester substrates [9-14].

PhaC synthases, the polymerizing enzymes, are grouped into four classes based on substrate specificity, and the preference in forming short chain length (scl-) or medium chain length (mcl-) polymers: class I, class III, and class IV produce principally scl-PHAs, while class II phaC synthesize mcl-PHAs [7, 15].

The PHA biosynthesis genes include: *PhaPs*, genes coding for Phasins, the granule assembling proteins, *PhaM*, encoding the activator and accelerator of the catalytic activity of PHA synthase, encoded by *PhaC*; *phaA*, encoding the acetoacetyl-CoA β-thiolase, *phbB* coding for the

acetoacetyl-CoA reductase, *phaG*, coding for 3-hydroxyacyl-carrier protein-CoA transferase, *phaJ*, encoding the enoyl-CoA hydratase, and *PhaZ*, encoding the PHA depolymerising enzyme.

PhaC proteins possess the so called lipase box, G-X-S-X-G, and a secondary structure containing the α/β hydrolase fold, a characteristic succession of alpha helices and beta strands, typical of lipases. In PhaC enzymes, the lipase box has the conserved sequence G-G/S-X-C-X-G/A-G, renamed the PhaC box consensus sequence. The Cys in the lipase box-like sequence is the catalytic amino acid, forming the covalently bond intermediate, Cys-S-H3B [7, 16].

PHA synthase activity is based on the catalytic triad C-H-D, cysteine, histidine, aspartate, also responsible for catalysis in lipases (S-H-D). In the catalytic triad, the negatively charged Asp_{447} in $PhaC_{Cs}$ from *Chromobacterium* spp. [17, 18] and Asp_{480} in $PhaC_{Cn}$ from *Cupriavidus necator* [19, 20] assist to enhance the basicity of His_{477} and His_{508}, respectively, by formation of a direct hydrogen bond. Previous reports suggest that the Asp residue of the triad acts as a general base catalyst to accelerate deprotonation of the 3-hydroxyl group of HB in the step involving elongation of the PHA product.

PHA synthases are grouped into four classes based on the kinetics and mechanism of reaction. The grouping of PhaC enzymes into each class is dependent on the structure of the PhaC, alone or in association to other subunits, and the substrate specificity: class I, class III, and class IV produce scl-polymers depending on propionate (3HP), butyrate (3HB, 4HB), valerate (HV) and hexanoate (HH) precursors (C3 to C6 carbons), while class II phaC enzymes synthesize mcl-polymers depending on hexanoate (3HH), heptanoate (3HHp), octanoate (3HO), decanoate (3HD), undecanoate (3HUD), dodecanoate (3HDD) (C6 to C12), and availability of the corresponding CoA thioester substrates, originating from three different metabolic pathways [9, 10, 20].

While some bacterial species produce mainly 3HB polymers, other species can synthesize various PHAs, depending on availability of intermediate precursors [1, 3, 9]. The structure and properties of the polymers are affected by the monomers that are incorporated. PHAs can be molded into films and hollow bodies. Polyhydroxybutyrate (PHB) is brittle, fragile, and stiff, with low elongation ability, and a break point below 15%. The incorporation of 3-hydroxyvalerate or other co-monomers can decrease the brittleness of PHB. The thermal, rheological and barrier properties of PHAs show good application potential in thermoplastic materials. The synthesis of copolymers is a frequent strategy to improve the properties of PHAs, to improve the plastics flexibility and lower the glass transition temperature (Tg) and the melting temperature (Tm) [21]. P3HB-4HB polymers and conventional thermoplastic used for packaging show high tensile strength and higher elongation at break. HBV-containing mcl-PHAs are elastic, have low melting point, a relatively low degree of crystallinity, and with various tensile strength. PHB copolymer containing 3-hydroxyvalerate unit P(3HB-*co*-3HV) has been developed with improved mechanical properties. To this aim, either optimization of substrate availability (feedstock, successive addition of precursors), and efficiency of enzymes, through genetic engineering and selection of PHA synthases, have been applied.

Class I and class II PHA synthases

Classes I and II PHA synthases are formed by a single protein (PhaC), of about 60 kDa.

In class I, the active PhaC enzyme is a dimer, with catalytic (CAT) domains facing each other, with N-domains making direct contacts, sustaining protein interaction and dimerization. It was hypothesized that a partially folded catalytic domain is partially occupied by the lid and cat domain secondary structure, that change their conformation in presence of activation factors, to open the catalytic domain and to allocate the 3HB-CoA in its binding site (Chek et al., 2017).

PhaC sequences differ in their length. In *Cupriavidus necator* (formerly *Ralstonia eutropha*) the class I enzyme PhaC$_{Cn}$/*Re*PhaC, the sequence is composed of a 191 amino acids N-terminal domain proteolytically cleaved after arginine, and a C-domain, containing the catalytic site, composed of 398 amino acids, for a total of 589 residues (Figure 1). In *C. necator*, PhaC$_{Cn}$ contains Cys$_{319}$, Asp$_{480}$ and His$_{508}$, located between the beta 6 and alpha 3 turn, the beta 10 and alpha 7 turn, and at the end of the beta 11 strand, respectively. In the between of Cys$_{319}$ and Asp$_{480}$ is located the D-loop and the helix-turn-helix motif (HTH), formed by α4, α5, α6, and β7-β8 stretches [18, 19]. (Wittenborn et al. ,2016, Kim et al., 2017a). PhaC$_{Cn}$/*Re*PhaC1 was also studied by small angle X-ray scattering (SAXS) analysis that confirmed the previous findings on the protein assembled as a dimer [22].

The PhaC synthase from *Chromobacterium* spp., phaC$_{Cs}$, was extensively studied [16, 17]. PhaC$_{Cs}$ has a peculiarity of utilization of 3HB, 3HV, and 3HH, producing scl-PHA polymers with mixed composition, with ability to incorporate C5 and C6 alkanes into the PHA polymer. PhaC$_{Cs}$ was found highly active, with fast polymerization rate [17]. PhaC$_{Cs}$ is shorter in length (for about 29 amino acids) in respect to PhaC$_{Cn}$ and this produces differences in numbering of amino acids. PhaC$_{Cs}$ structure has a substrate-binding site hidden by a partially disordered protein domain, the CAP domain [16, 17]. Cysteine$_{291}$, at the end of the ß6 sheet, is followed by the CAP domain, containing the LID structure, that close the accessibility of the substrate access pocket. The β sheets in the CAP domain have been renumbered with Greek letters in the PhaC$_{Cs}$ sequence. Thus, from the N-terminal sequence, up to the β6-α3 turn, the two PHA synthases conserve the same numbering in their secondary structures, but the successive α-β turns are differently numbered. In PhaC$_{Cs}$, the core subdomain contains six ß-strands (ß8 to ß13) and four a-helices (α4 to α7), whose number does not correspond to those in the PhaC$_{Cn}$-CAT structure.

In PhaC$_{Cs}$ the catalytic triad is composed of Cys$_{291}$, His$_{477}$ (located after the β9 sheet, in respect to the β11 sheet in PhaC$_{Cn}$) and Asp$_{447}$ (located at the turn formed by ß8 strand and α4 helix, the ß8-α4 fold, in respect to the β10-α7 fold in PhaC$_{Cn}$).

In other species, such as *Delftia acidovorans* (previously *Comamonas acidovorans*) the PHA synthase contains a large insert of 40 amino acid residues shown to improve the specific activity of the enzyme, located in the α/β hydrolase fold, following the catalytic cysteine after the β6 turn [25].

Aeromonas spp., such as *A. caviae, A. hydrophila*, and *A. punctata*, possess PhaC enzymes belonging to class I. The enzyme PhaC$_{Cc}$ from *Caulobacter crescentus* (*C. vibrioides*) [26], displayed to accommodate alkanes with various alkyl side-chain length.

Class II phaC synthases have been extensively studied, and are widely distributed in bacteria: in *Pseudomonas* spp. (*P. putida, P. mendocina, P. oleovorans, P. campisalis*; *P. stutzeri)* there are two phaC genes, of which PhaC1 is the active enzyme under physiological conditions. PhaC synthases have been reported in *Halomonas* spp., such as *H. campisalis*, *Halomonas* sp. O-1 and *Halomonas elongata* DSM2581 [27], and in *P. stutzeri*, that can be exploited in polymerization of mcl-PHAs [28]. There are two PHA synthases, PhaC1 and PhaC2, in *P. oleovorans*, of which PhaC2 has a higher affinity for 3-hydroxyhexanoate (3HH) monomers.

Class II phaC enzymes differ from PhaC$_{Cn}$, as prototype of class I PHA synthases, for about 28 amino acids, reaching the C-terminal (1-559) with about 30 amino acids shorter sequence. The catalytic triad has been renumbered as Cys$_{296}$, Asp$_{452}$, His$_{453}$ and His$_{480}$ in *Pseudomonas* spp., prototype for Class II PHA synthases.

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Class III and Class IV PHA synthases

Class III PHA synthases are made of two subunits, namely, a catalytic subunit PhaC (40–53 kDa) and a second subunit PhaE (ranging from 20 to 40 kDa), which form the PhaEC complex, in which the PhaE subunit is necessary for PHA polymerization. Class III phaC are structured as tetramers, such as phaEC from *Allochromatium vinosum*, (with catalytic triad Cys_{149}, Asp_{302}, His_{331}), whose enzyme activity has been studied using substrate analogs [15]. The authors performed molecular docking and *in silico* studies, that are in agreement with the crystal structure of synthases available [15], based on homology models built using CPHmodels3.0, SWISS-MODEL and I-TASSER, performing structure-guided sequence profiles. The results of their analysis, referring to $PhaC_{Cn}$ and PhaC class III from *Allochromatium vinosum*, describe the presence of an active site of cysteine, that is buried in a pocket: the authors, by comparison with other enzymes with known crystal structure (lipases), postulated the presence of the substrate entrance and product exit channels [15].

There is also an Archaeal type, PhaC class IIIA: this group is represented by *Haloarcula marismortui*: Archea present good perspectives of exploitation for polymer production, given by the easiness of PHB extraction.

Class IV PHA synthases from *Bacillus* spp. are composed of a catalytic subunit PhaC (41.5 kDa) and a PhaR (22 kDa) subunit, similarly to class III synthases composed of phaE and phaC units. Class IV PHA synthases are classified as *B. megaterium* type (IV_m) [23], *Bacillus cereus* type (IV_c) [22], and *B. bataviensis* type (IV_b), with 33% homology to the other phaC sequences [5]. In *E. coli* expressing PhaRC from *B. cereus* YB-4, the biosynthesized PHA undergoes synthase-catalyzed alcoholytic cleavage using endogenous and exogenous alcohols. This alcoholysis is thought to be shared among class IV synthases, and this reaction is useful for regulation of PHA molecular weight and for modification of the PHA carboxy terminus.

The catalytic cysteine in the active site is C_{151} in *B. cereus*, and C_{147} in *B. megaterium* type IV enzymes. As shown for PhaCYB4 from *B. cereus* YB-4, the involvement of Cys_{151}, Asp_{306}, and His_{335} in polymerization activity was shown by site-directed mutagenesis [5].

Diversity and spread of phaC in bacteria

PHA synthase genes can be identified in environmental bacterial strains for a preliminary screening, before knowledge on PHA synthesis ability due to the presence of the gene, using PCR amplification with conserved primers [7, 29]. Through PCR analyses, *phaC* genes were detected in a collection of bacterial strains isolated from soils and from marine environments. In samples of environmental strains, we amplified phaC gene sequences in colonies from environmental isolates, and performed DNA sequencing of ribosomal DNA to identify the strains at species level (unpublished results): with this method several species were classified for ability to produce PHA, namely *P. oleovorans, P. fluorescens, P. sihuiensis, P. putida, Comamonas testosteroni, Aeromonas hydrophila*, as well as *Cupriavidus necator*. It is envisaged that PCR screening using various different primer sets can be optimized to find new phaC polymorphisms and potential novel PHA synthase sequences. Quelas reported the presence in *Bradyrhizobium japonicum* USDA110 of five polyhydroxyalkanoate (PHA) synthases (PhaC), distributed into four different PhaC classes [30], and characterized the requirements for two of the genes in legume nodules under various physiological conditions.

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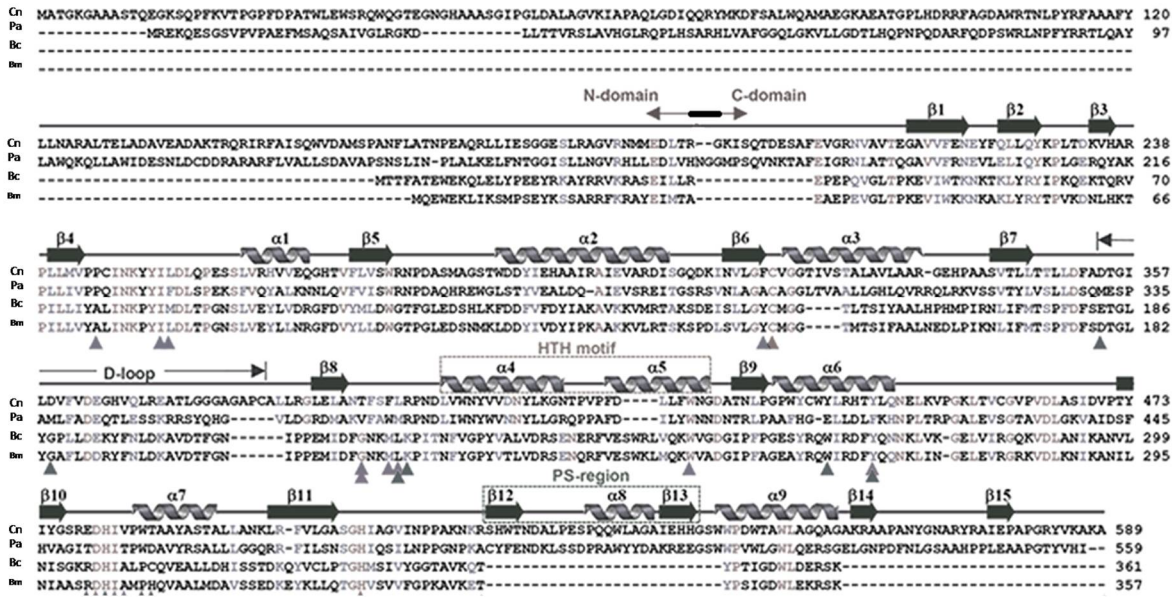

Figure 1. Amino acid sequence and secondary structure of *Cupriavidus necator* PhaC$_{Cn}$, aligned with the PHA synthase of class II (*P. aeruginosa*), class IVm and class IVb, from *Bacillus megaterium* and *B. cereus*, respectively.

Crystal structure

In two publications appeared almost contemporarily, two teams reported on the crystal structure of PhaC$_{Cn}$-CAT, the catalytic domain of PhaC from *Cupriavidus necator* [18, 19, 24]. PhaC$_{Cn}$-CAT was shown to dimerize, and to adopt a partially open form maintaining a narrow substrate access to the active site. PhaC$_{Cn}$ needs PhaM, the primer of PHA synthesis, to start and accelerate polymer synthesis, and this may be due to increased accessibility of 3HB-CoA substrate to the active site. Wittenborn obtained the crystal structure of PhaC$_{Cn}$(C$_{319}$A), a construct in which the active site cysteine (Cys$_{319}$) was mutated to alanine to improve protein stability in the absence of detergent. During the crystallization, proteolysis of PhaC$_{Cn}$ occurred after the N-domain (R$_{192}$), leading to the crystal structure of the C-domain: PhaC$_{Cn}$-CAT is formed by two Core subdomains (G$_{143}$-F$_{352}$, L$_{450}$-A$_{589}$), flanking on both sides a Dimerization domain (A$_{353}$-L$_{549}$) containing the dimerization loop (D-loop) and the Helix-loop-helix (HTH) domain; in addition, in the terminal Core sub-domain there is an Extended C-terminal region (EC: R$_{521}$-A$_{589}$), that is missing in class IV PhaC (figure 1), and a Protruding Structure, PS, that elongates from the Extended C-region.

The catalytic domain of PhaC$_{Cn}$ contains the residues 201–368 and 378–589 (with residues 369–377 devoid of any structure), showing an α/β-hydrolase fold, featuring a central mixed β-sheet flanked by α-helices on both sides. This architecture is similar to that seen in lipases. The CAT domain in the PhaC$_{Cn}$ sequence is structured by the presence of the β1-4 sheets, the α1 helix, the β5 sheet, α2 helix, β6 sheet facing the lipase box, followed by the α3-β7 fold: after this structure there is the D-loop; after the β8 sheet, there is the helix-loop-helix (HTH), composed of the α4 and α5 helices facing each other, and the β9-α6 fold, where the Dimerization subdomain ends (L$_{449}$); as for the other amino acids of the catalytic triad, the aspartate is located between the β10-α7 fold, and the histidine is located after the β11 sheet. The active site of PhaC$_{Cn}$ is accessible via a water-filled channel, with a size of 12.5 Angstrom, that can accommodate the 3HB-CoA substrate and/or short PHA oligomers.

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Two PhaC monomers interact through the dimerization surfaces (A_{353}-E_{445}), containing hydrophobic amino acids, by means of interaction between one monomer helix-loop-helix motif (HTH) and the D-loop of the second monomer [18, 19].

In the report on the crystal structure obtained from the catalytic domain of PhaC from *Chromobacterium* sp. USM2, $PhaC_{Cs}$-CAT was compared to the $PhaC_{Cn}$-CAT crystal structure [17]. Considering the two structures described, in $PhaC_{Cs}$-CAT a difference in the accessibility of the active site has been evidenced. Chek showed that in the $PhaC_{Cs}$-CAT dimer, the CAP and LID domains close the access to the substrate binding site [17]. The structure proposed by Chek and colleagues describing a $PhaC_{Cs}$ active site covered by the CAP subdomain, differs from the partially open form of the $PhaC_{Cn}$ catalytic domain reported by Wittenborn. The CAP domain occupies partially the access to the substrate binding pocket, and the LID domain needs to slide away in order to free the access for 3OH-alkanoyl-S-CoA units. Both catalytic domains of $PhaC_{Cs}$ and $PhaC_{Cn}$ form a dimer mediated by the CAP subdomain. The difference between the closed and partially open form is provided by the conformation of the CAP subdomain. The CAP subdomain undergoes a conformational change during catalytic activity with rearrangement of the dimeric form.

The main difference between the two crystal structures was found in the folding of αB′ and ηB′ helices and their linker loop of $PhaC_{Cs}$-CAT, while the corresponding positions in $PhaC_{Cn}$-CAT, show a long α4 helix that presents a partial access to the active site. The region Leu_{402}–Asn_{415} forming the α4 helix in $PhaC_{Cn}$-CAT is conserved among Class I and II PHA synthases, whereas the corresponding segment, Leu_{369}–Lys_{382} of $PhaC_{Cs}$-CAT, displays a disordered structure.

Catalytic mechanism

The models proposed for the available PhaC structures, hypothesize the presence of a substrate entrance tunnel, that accommodates HB-CoA, with a size of about 12.5-13 Å, and a product egress tunnel, positioned perpendicularly to the entrance tunnel. Various catalytic mechanisms for PHA synthases have been proposed, in the context of dimerization of PHA synthases of class I and II [17].

One mechanism is referred to as the non-processive ping-pong model: this mechanism requires two cysteines in the active sites in the dimer, for PHA chain elongation, with chain transfer from one cysteine to the second active site. The ping-pong mechanism requires two thiol groups located at a distance short enough to shuttle back and forth the growing (3HB)*n* chain between the two thiols. The dimeric structures described by Wittenborn and by Kim for $PhaC_{Cn}$-CAT, and by Chek for $PhaC_{Cs}$, show that the two active sites are too distant (33 and 28.1 Angstrom, respectively) for successive chemical reactions.

The distance between the active sites in the dimer seems to favor the mechanism based on a single active site for each elongation reaction. In the model described by Chek, the dimer, composed by two units of $phaC_{Cs}$ through the contacts between the two CAP domains and the two N-domains, presents two channels leading to the two active sites. The dimeric structure proposed by Chek [17], favors the involvement of one active site for each processing step. In the model, the substrate enters the substrate-binding tunnel, while chain product is elongated along a path near the protein surface, with a sliding mechanism of the PHA polymer under synthesis along a V shaped cavity within the enzyme. In the proposed structure, the enzyme moves along the extremity of the forming polymer to add new 3HB units, rather than hosting the polymer into a product egress channel. The mechanism involves a processive model that requires a single active site for PHA chain elongation and a non-covalent intermediate, in addition to a covalent intermediate bound to the Cys residue at the active center during the catalytic cycle. The enzyme dimer, through interactions with other partners, with substrate, phasins and phaM, move the CAP domains to flip away, opening the active site entrance, and freeing the product channel, and the two Core units contemporarily accept the substrate and produce the 3HBn polymers. The process occurs with a two step catalysis mechanism

that allows the intermediates to be located in the enlarged cavities partially freed from the CAP occupancy. The arrangement of the dimer, different from that of the PhaC*Cn*-CAT dimer, may allow to the CAP subdomains to undergo a conformational change during catalytic activity with rearrangements in the dimer, that facilitate substrate entry, intermediate product formation, and product exit from the active site. According to the crystal structure of the PhaC$_{Cn}$-CAT dimer [18, 19] the substrates enter through the substrate-binding tunnel: the first 3HB-CoA is attacked by the nucleophilic Cys-SH to produce a 3HB-Cys covalent bond, as in the aforementioned model, and frees CoA-SH, that is released from the product egress tunnel. A second 3HB-CoA attacks 3HB-Cys thioester bond with the hydroxyl group in 3HB to produce a (3HB)2-CoA intermediate, reaction that frees the Cys residue in the active center. The Cys residue again attacks the thioester bond of the (3HB)2-CoA intermediate to produce (3HB)$_{n+1}$, covalently bound to the Cys residue and release of free CoA. In this model, the growing 3HB polymer is bound to the enzyme at the end of each cycle. This model cannot allow to position large molecules such as (3HB)n-CoA intermediate within the substrate binding site, that has a cavity of 12.5 Angstrom.

An alternative model has been proposed with a succession of reactions slightly different. The model proposed for PhaC$_{Cn}$, by Wittenborn, implies that newly entered 3HB-CoA produces 3HB-Cys; then (3HB)2-CoA enters the active site to produce (3HB)3-CoA, which is again released from the active site. When a new (3HB)2-CoA substrate binds, the HB hydroxyl group is deprotonated by His$_{508}$, facilitated through modulation of the histidine basicity by Asp$_{480}$. The newly formed HB alkoxide attacks the Cys-HB thioester, generating a noncovalent, CoA-bound intermediate. However, if the (3HB)3-CoA produced is held in the active site and attacked by the active Cys residue again to produce (3HB)3-Cys, chain elongation would then require an inter-subunit reaction. Again, (3HB)n-Cys adducts would require a larger active site cavity.

Mutation and amino acid substitution studies

Several studies focused on PHB synthases with mutations enabling the enzymes to accelerate the reaction kinetics [31-33] and ability to accept bulk substrates as precursors for the production of mcl-PHAs and grafted copolymers.

Nomura and Taguchi [34] reviewed the attempts to engineer various classes of PHA synthases, either by mutagenesis or by evolution, in class I and Class II enzymes. The methods utilized either random mutagenesis, intragenic suppression mutagenesis, gene shuffling, random mutagenesis combined with Site-specific saturation mutagenesis and recombination, localized semi-random mutagenesis, PCR-mediated random chimeragenesis, intragenic suppression mutagenesis, site-specific saturation mutagenesis.

Many authors described mutations in amino acids positioned in various domains of different PHA synthases, most often finding a decrease in production of mcl-PHA and higher synthesis of scl-PHA. Beneficial effects of mutagenesis studies of Glu$_{130}$ and Ser$_{477}$ have been described [35-37]. For instance, the E$_{130}$D substitution and S$_{477}$X mutation in type II PHA synthase showed an enhancement of PHA production and alteration of polymer molecular weight.

A mutational study of PhaC$_{Cs}$ reported by Chuah [40], showed that in PhaC$_{Cs}$ Ala$_{479}$ is a critical residue required for substrate specificity, as determined by various site-specific mutational assays both *in vivo* and *in vitro*, and production tests of copolymers such as P(3HB-*co*-3HHx). In PhaC$_{Cn}$ and in other Class I enzymes this position corresponds to the conserved residue Ala$_{517}$. In the structure proposed by Chek, Ala$_{479}$ is located within α5 helix and the side chain protrudes into a depression of the molecular surface formed by loops (β4-α1, β9-α5 and α5-β10 loops) from the core subdomain, and is partially covered by the helix ηB' and the following loop of the LID region from the CAP subdomain: the A$_{479}$ mutation results in weakening of the interactions between the LID region and the core subdomain, and stabilizes the active form of this enzyme by releasing the LID region from

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the active site. Since Ala$_{479}$ is surrounded by polar residues (Ser$_{475}$ and Arg$_{490}$), it is supposed that replacement of Ala$_{479}$ with Ser or Thr facilitates hydrogen-bonding interactions with the polar residues and stabilization of α5 helix harboring the active residue His$_{477}$, important for enzyme activity.

A mutagenesis study of class I PHA synthases showed that the F$_{420}$S mutation in PhaC$_{Cn}$ increased the specific activity with a shortened lag phase [41]. This residue corresponds to Phe$_{387}$ of PhaC$_{Cs}$, which is conserved among Class I and II PHA synthases, and is located in αC helix of the CAP domain. Phe$_{387}$ is involved in dimerization by participating in an intermolecular nonpolar interaction linking the αC helix to the LID region, suggesting that the mutation may affect the conformational stability and/or conformation transition of the LID region.

The CAP subdomain provides αC and αD helices as building blocks of the active site cavity filled with a cluster of water molecules. In the structure obtained by Chek [17], the C-terminal portion of the LID region of the CAP subdomain is disordered and is followed by αC helix docked to the core subdomain. Two highly conserved residues, Trp$_{392}$ and Asp$_{395}$, are present in αC helix. Trp$_{392}$ of PhaC*Cs* is located in the αC helix of the CAP subdomain and faces Site B of the channel.

Amara and Rhem attempted to modify the activity of PhaC from *Pseudomonas* species [37]. The conserved residue Trp$_{398}$ was replaced, such as Trp$_{398}$Phe and Trp$_{398}$Ala, and the mutation resulted in inactivation of the enzyme. Using the threading model of enzyme structure, the authors located the Trp residue as exposed on the surface, in agreement with the results shown by Chek for class I enzymes [42-46].

Tyr$_{412}$ in PhaC$_{Cs}$, and Tyr$_{446}$ in the α6 helix in PhaC$_{Cn}$, are residues conserved in Class I, III and IV PHA synthases, while Phe occupies this position in Class II synthases: in addition to this amino acid position, there is a second substitution that seems to have a role in accommodating larger substrates. Tyr$_{438}$ is conserved in Class I, III and IV enzymes, while in Class II PhaC this position is occupied by His: this may contribute to a reduction of size, eliminating the bulky side chain (phenol ring), and determining changes in polar interactions with other amino acids facing the substrate entrance tunnel; these amino acids interactions may account for the property to accommodate large substrates in class II enzymes.

PhaC1 and PhaC2 from *Pseudomonas stutzeri* [39], have been applied to produce mcl-PHAs in engineered bacteria. *Ps*PhaC2 with four point mutations, at E$_{130}$D, S$_{325}$T, S$_{477}$G, and Q$_{481}$K was used to accommodate substrates with various shapes and structures, to produce mcl-PHAs and block copolymers. The putative catalytic residues Cys$_{296}$, Asp$_{452}$, His$_{453}$ and His$_{480}$ were replaced by site-specific mutagenesis [37]. Considering the *Pseudomonas* mcl-PHA synthases, the His$_{480}$Gln substitution did not affect enzyme activity, posing the doubt that His is not a component of the catalytic triad. As for a second conserved histidine, when His$_{453}$ was replaced by Gln, the modified enzyme showed only 24% of wild-type in vivo activity, which make suppose that His$_{453}$ might be part of the catalytic triad in class II PHA synthases [37]. However, no other study confirmed the involvement of His$_{453}$ in class II PhaC2 catalysis.

Sheu studied the increase of PHA synthase thermostability and activity, using chimeric constructs, indicating that some amino acid substitutions may stabilize the enzyme at higher temperature [31].

Production of PHA in fermentors

Various companies are involved in production of bioplastics for industrial applications. The methods are various, either using patented strains, engineered PHA synthases, and growth conditions favoring the high yield and high PHA content/dry cell weight. In the field of monitoring

the endpoint step of PHA synthesis, and bacteria collection, various methods have been established, from lipid staining [47] and analysis of fluorescence intensity, to physic-chemical analyses (Raman, FTIR spectra). Since bacterial cultures require sterilization that is costly at industrial scale, methods based on halophilic strains have been proposed as to circumvent the sterilization process. Extraction of PHAs from bacteria requires costly procedures, therefore researchers used Archea or cyanobacteria that have PHA granules easily extracted, decreasing the costs of production.

Progress and advancements in PHA field

Recent advancements on PHA granule structure and composition have been achieved [48]. The high molecular weight storage PHB consists of > 10^3 3HB residues (storage PHB). PHB granules *in vivo* are covered by a surface layer that is distinct from the polymer core. PHA granules are structured through the action of various proteins on the surface. The granules, named also carbonosomes, represent supramolecular complexes with specific functions. In addition to Phasins (such as PhaP2, PhaP3, PhaP4), among the proteins identified during PHA granule isolation, there are the PHB synthase (PhaC1), PhaM, the activator of PhaC, Acetyl-CoA acetyltransferase, and acylCoA synthetase: their presence may be explained by the need to avoid accumulation of CoA-SH, produced by the PHA synthase during polymer synthesis, since an excess of CoA would inhibit the enzyme. The most accurate model for PHA synthesis within bacterial cell is the Scaffold Model: it assumes that PHB synthase of nascent PHB granules is attached to a scaffold within the cell. PHB granules have been localized in the cell centre, along with the length axis of the bacteria. PhaM, that specifically interacts with PhaC1 and with phasin PhaP5, interacts also with DNA and with the nucleoid *in vitro* and *in vivo*, and this may explain why PHB granules have been found attached to the bacterial nucleoid.

Conclusions

In this review, we reported on the classification of PHA synthases, the proposed structures and role of individual amino acids in the catalysis and mechanism of activity of class I and class II PHA synthases, presenting the information available on the other types of enzymes. We have reviewed the engineering attempts and the effect of modification of key amino acids on the enzymatic activity and product formation. It is expected that PHA synthases may be further improved to produce effectively and at convenient costs tailor-made polymers.

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