Research Article

Structure and function of the N-terminal domain of *Ralstonia eutropha* polyhydroxyalkanoate synthase, and the proposed structure and mechanisms of the whole enzyme

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Polyhydroxyalkanoates (PHAs) are natural polyesters synthesized by numerous microorganisms as energy and reducing power storage materials, and have attracted much attention as substitutes for petroleum-based plastics. In an accompanying paper, the authors reported the crystal structure of the C-terminal domain of *Ralstonia eutropha* PHA synthase (PhaC1). Here, the authors report the 3D reconstructed model of full-length of *R. eutropha* PhaC1 (*Re*PhaC1) by small angle X-ray scattering (SAXS) analysis. The catalytic C-terminal domain of *Re*PhaC1 (Re*PhaC1*CD) dimer is located at the center of Re*PhaC1*, and the N-terminal domain of *Re*PhaC1 (Re*PhaC1*ND) is located opposite the dimerization subdomain of Re*PhaC1*CD, indicating that Re*PhaC1*ND is not directly involved in the enzyme catalysis. The localization studies using Re*PhaC1*, Re*PhaC1*ND and Re*PhaC1*CD revealed that Re*PhaC1*ND plays important roles in PHA polymerization by localizing the enzyme to the PHA granules and stabilizing the growing PHA polymer near the active site of Re*PhaC1*CD. The serial truncation study on Re*PhaC1*ND suggested that the predicted five α-helices (N-α3 to N-α7) are required for proper folding and granule binding function of Re*PhaC1*ND. In addition, the authors also report the SAXS 3D reconstructed model of the Re*PhaC1*/Re*PhaM*MC complex (Re*PhaM*MC, PAKKA motif-truncated version of Re*PhaM*). Re*PhaM* forms a complex with Re*PhaC1* by interacting with Re*PhaC1*ND and activates Re*PhaC1* by providing a more extensive surface area for interaction with the growing PHA polymer.

Keywords: 3D reconstructed model · Enzyme mechanism · PHA synthase · PhaM · Polyhydroxyalkanoates

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1 Introduction

Biopolymers derived from organisms have attracted increasing attention with their diverse material properties for industrial and medical applications [1]. Among them, polyhydroxyalkanoates (PHAs), the representative bacterial polyesters have been heavily studied for several decades because of their characteristics, biocompatibility, biodegradability and similar properties with performance plastics [1–3]. These polymers are found in many

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bacteria including *Ralstonia* and *Pseudomonas* [1–3] in the form of intracellular granules. These bacteria accumulate PHAs as energy and redox storage materials under nutrient-limited conditions with excess carbon sources [1–3].

Several enzymes are related in PHA biosynthesis, PhaA (β-ketothiolase, a PHA biosynthetic enzyme), PhaZ (depolymerase), PhaR (regulatory protein), PhaP (granule-associated protein), and so on. The key enzyme, PHA synthase (PhaC) polymerizes various coenzymes A thioesters of hydroxycarboxylate into PHAs [3, 4]. Many studies have been reported the roles of PhaC in PHAs molecular weight, polydispersity, monomer composition, and productivity [4–6]. Therefore, many attempts were made to elucidate the polymerization mechanism based on the structural information of PhaC. However, due to the difficulties of obtaining crystal structure, most studies were achieved by prediction based on sequence alignments and modeling the 3D protein structure using homologous proteins such as lipase [7, 8].

Very recently, the crystal structure of C-terminal domain of PhaC (200–589 amino acid residues) from *Ralstonia eutropha* has been independently resolved by our group (see the accompanying paper; [9]) and also by Wittenborn et al. [10]. The *R. eutropha* is a representative poly(3-hydroxybutyrate) (PHB) producer. The *R. eutropha* PHA synthase belongs to the class I PHA synthase which utilizes coenzyme A thioesters of short-chain-length hydroxy-carboxylic acids (C3–C5) and comprises one type of subunit PhaC1 [4]. From the crystal structure, C-terminal domain functions were concluded as a catalytic domain representing the active sites consisting of catalytic triad, His508, Asp480 and Cys319 [9, 10]. However, the C-terminal domain without the N-terminal domain (1–199 amino acid residues) showed only negligible amount of activity by in vivo experiments and modeling the 3D protein structure using homologous proteins such as lipase [7, 8].

To determine the molecular weights and overall surface structures of RePhaC1<sub>CD</sub>, RePhaC1<sub>F</sub> and the RePhaC1<sub>F</sub>/RePhaM<sub>AC</sub> complex in solution, we performed SAXS experiments. SAXS measurements were carried out at the 4C SAXS II BL of the PAL (Pohang, Republic of Korea). A light source from an In-vacuum Undulator 20 (VU20: 1.4-m length, 20-mm period) of the Pohang Light Source II storage ring was focused with a vertical focusing toroidal mirror coated with rhodium and monochromatized with a Si (111) double-crystal monochromator (DCM), yielding an X-ray beam wavelength of 0.734 Å. The X-ray beam size at the sample stage was 0.2 (V) × 0.6 (H) mm<sup>2</sup>. A two-dimensional (2D) charge-coupled detector (Mar USA, Inc.) was employed. Sample-to-detector distances (SDDs) of 4 m and 1 m were used for SAXS. The magni-
tude of the scattering vector, \( q = (4\pi/\lambda) \sin \theta \), was 0.09 / nm < \( q < 5.00 / \text{nm} \), where \( 2\theta \) is the scattering angle, and \( \lambda \) is the wavelength of the X-ray beam source. The scattering angle was calibrated with polyethylene-b-polybutadiene-b-polystyrene (SEBS) block copolymer standard. We used quartz capillaries with an outer diameter of 1.5 mm and wall thickness of 0.01 mm as solution sample cells. All scattering measurements were conducted at 4°C. The SAXS data were collected in six successive frames of 0.1 min each to monitor radiation damage. For accurate SAXS measurements, a small concentration range of 0.5–1.5 mg/mL was used and finally 5 mg/mL of sample was used. Each 2-D SAXS pattern was radially averaged from the beam center and normalized to the transmitted X-ray beam intensity, which was monitored with a scintillation counter placed behind the sample. The scattering of specific buffer solutions was used as the experimental background.

Radius of gyration (\( R_g \)) values were estimated from the scattering data using Guinier analysis [13]. The molecular mass (MM) was calculated from the scattering curve based on the QR method [14]. The pair distance distribution \( p(r) \) function was obtained through the indirect Fourier transform method using the program GNOM [15].

### 2.3 Reconstruction of 3D structural models

To reconstruct the molecular shapes, the ab initio shape determination program GASBOR [16] was used. For each model reconstruction, ten independent models were selected, and the averaged aligned model was filtered at a given cut-off volume using the program DAMAVER [17]. The final models were obtained by imposing P2 symmetry restriction. The SAXS curves were calculated from the atomic models using the program CRYSOL [18]. For comparison of the overall shapes and dimensions, the ribbon diagrams of the atomic crystal models were superimposed onto the reconstructed dummy atom models using the program SUPCOMB [19].

### 2.4 PHB polymerization activity assay

The in vitro PHB polymerization activities of PHA synthases and their variants were measured by monitoring the decrease in absorbance at 236 nm due to the cleavage of the thioester bond in 3-HB-CoA [20]. The reaction mixture comprised 800 nM \( \text{RePhaC1} \) enzyme, 50 mM sodium phosphate buffer (pH 7.0), and 2 mM DTT, with or without 0.02% w/v Triton X-100. The reaction was initiated by adding 30 \( \mu \text{M} \) DL-3-hydroxybutyryl-CoA (Sigma) to the reaction mixture. The molar extinction coefficient \( (\varepsilon = 23604500 / \text{M/cm} \text{ at 236 nm}) \) was used to calculate the activity. To measure \( \text{RePhaC1}_{F} \) activation by \( \text{RePhaM} \), \( \text{RePhaC1}_{F} \) was pre-incubated with 80 nM \( \text{RePhaM}_{P} \) or \( \text{RePhaM}_{MC} \) for 30 min at 4°C before the activity measurement.

### 2.5 In vivo PHB biosynthesis in \( E. \text{coli} \)

All bacterial strains and plasmids used in this study are listed in Table 1. For in vivo PHB biosynthesis, \( E. \text{coli} \) K-12 W3110 strain was used. To construct the PHB biosynthetic pathway in \( E. \text{coli} \), the \( \text{RephaC1} \) and \( \text{RephaAB} \) genes were expressed by the pCnCAB plasmid [21]. For the construction of plasmids expressing variants of PHA synthase, the \( \text{RephaC1} \) gene was replaced with \( \text{RephaC1}_{CD} \) (pCnCAB plasmid) or \( \text{RephaC1} \) variants harboring point mutations for site-directed mutagenesis.

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**Table 1. Bacterial strains and plasmids used in this study**

<table>
<thead>
<tr>
<th>Strain/plasmid</th>
<th>Relevant characteristics*</th>
<th>Source/reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>W3110 Coli Genetic Stock Center, strain No.4474</td>
<td></td>
<td>CGSCb)</td>
</tr>
<tr>
<td>BL21(DE3) F- ompT hsdSb (rB mB -) gal dcm (DE3)</td>
<td></td>
<td>Novagenc)</td>
</tr>
<tr>
<td>pCnCAB</td>
<td>Ap(^{R}), ( \text{RephaCB} ) promoter, ( \text{RephaCB} ) transcriptional terminator, derivative of pBluescript II KS(+)</td>
<td>[21]</td>
</tr>
<tr>
<td>pCnCAB(_{CD})</td>
<td>Ap(^{R}), ( \text{RephaC1} ) was replaced by ( \text{RephaC1}_{CD} ), derivative of pCnCAB</td>
<td>This study</td>
</tr>
<tr>
<td>pTac15k</td>
<td>Km(^{R}), p15A origin, lac promoter, pACYC177 derivative,</td>
<td>Lab stock</td>
</tr>
<tr>
<td>pCnCAB</td>
<td>Ap(^{R}), ( \text{RephaCAB} ) promoter, ( \text{RephaAB} ) transcriptional terminator, derivative of pCn-CAB</td>
<td>[24]</td>
</tr>
<tr>
<td>pCnCAB-K</td>
<td>Km(^{R}), ( \text{RephaCAB} ) promoter, ( \text{RephaAB} ) transcriptional terminator, derivative of pTac15k</td>
<td>This study</td>
</tr>
<tr>
<td>pET-22b( (+) )</td>
<td>Ap(^{R}), T7 promoter, CoEl1 origin, expression vector</td>
<td>Novagen(^{c)}</td>
</tr>
<tr>
<td>pET22/hC(_{F})</td>
<td>Ap(^{R}), T7 promoter, ( \text{RephaC1}_{F} ) with 6-histag, derivative of pET-22b( (+) )</td>
<td>This study</td>
</tr>
<tr>
<td>pET22/hC(<em>{F})(</em>{CD})</td>
<td>Ap(^{R}), T7 promoter, ( \text{RephaC1}<em>{F} ) with 6-histag and ( \text{RephaC1}</em>{CD} ) with 6-histag, derivative of pET-22b( (+) )</td>
<td>This study</td>
</tr>
<tr>
<td>pET22/hC(<em>{F})(</em>{CD})</td>
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<td>This study</td>
</tr>
</tbody>
</table>

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*Abbreviations: Ap, ampicillin; Km, kanamycin; Cm, chloramphenicol; \(^{R}\), resistance

b) Coli Genetic Stock Center, New Haven, CT.

c) Novagen, Inc., Madison, WI.
studies. For in vivo polymer production, seed cultures were prepared in 25 mL test tubes containing 10 mL of LB medium at 30°C overnight in a rotary shaker at 250 rpm. A total of 1 mL of the seed culture was inoculated into a 250 mL flask containing 100 mL of LB medium supplemented with 20 g/L of glucose. Ampicillin (each at 50 µg/mL) was added to the medium depending on the resistance markers of the employed plasmids. The content and monomer compositions of the synthesized polymers in E. coli cells were analyzed by gas chromatography (GC) [22, 23]. The cultured cells were washed twice with distilled deionized water, and the cells were lyophilized. The lyophilized cell pellet was subjected to methanolation with benzoic acid as an internal standard in acidified methanol with 15% v/v sulfuric acid. The resulting methyl ester of 3-hydroxybutyrate was measured by GC. The GC analysis was performed by injecting 1 µL of sample into an Agilent 6890N GC system (Agilent Technologies, Palo Alto, CA, USA) equipped with an Agilent 7683 automatic injector, a flame ionization detector, and a fused silica capillary column (ATTTM-Wax, 30 m, ID 0.53 mm, film thickness 1.20 µm, Alltech, Deerfield, IL) using nitrogen as a carrier gas. The GC oven temperature was initially maintained at 80°C for 5 min and ramped to 230°C at 7.5°C/min. The temperature was increased with a gradient of 10°C/min until reaching 260°C and held for 5 min. The injector and detector were maintained at 250°C and 300°C, respectively.

2.6 Localization of RePhaC1

The bacterial strains and plasmids used in this study are listed in Table 1. To investigate the localization of RePhaC1, E. coli BL21(DE3) (Novagen, Inc., Madison, WI) was used. To construct plasmids pET22:hcF and pET22:hCCD, the RePhaC1F and RePhaC1CD genes, respectively, were cloned into pET-22b (+). For pET22:hcF′-hCCD, both RePhaC1F and RePhaC1CD genes were cloned into pET-22b (+). To construct pChnAB-K, phaAB genes were amplified from pChnAB [24] and were cloned into pTac15k. Cell cultivation and protein purification were performed as described above. To solubilize the pellet fraction of the cell lysate, the cell pellet was resuspended with buffer B (40 mM Tris-HCl, pH 8.0, 5 mM BME and 2% w/v Triton X-100) by vigorous vortexing and incubated for 1 h. Cell debris was removed by centrifugation at 13,500 × g for 1 h, and the buffer B-solubilized fraction was applied to a Ni-NTA agarose column (QIAGEN). After washing with buffer B containing 20 mM imidazole, proteins were eluted with buffer B containing 300 mM imidazole.

2.7 Transmission electron microscopy (TEM)

To confirm the synthesis of PHB granules in the cell cytoplasm, TEM analysis was performed. The samples were prepared as previously described [25, 26]. Briefly, approximately 5 mL of cell culture was harvested and washed twice with 50 mM potassium phosphate buffer (pH 7.5). Cells were then resuspended in 2.5% glutaraldehyde-supplemented phosphate buffer for fixation. The fixed cells were stained with uranyl acetate, dehydrated, and the sample was dissected [26]. TEM analysis was performed using an FEI Tecnai G2 Spirit optical system at the Korea Basic Science Institute (KBSI).

2.8 Electrophoretic gel mobility shift assay (EMSA)

The RePhaM F and RePhaM AC proteins were purified as described above for RePhaC1. EMSAs were carried out using the recombinant RePhaM F and RePhaM AC proteins. To prevent the artifact of natural binding of PhaM to Ralstonia DNA, a totally different DNA (PCR-amplified Clostridium acetobutylicum crotonase gene) was incubated with increasing amounts of purified 6-His-tagged RePhaM F and RePhaM AC for 20 min at room temperature and applied on a 1% w/v agarose gel using 0.5x Tris-acetate-ethylenediaminetetraacetic acid (EDTA) (TAE) running buffer. Bound and unbound fractions were detected using a changeable ultraviolet (UV) trans-illuminator.

2.9 Protein pull-down assay

To observe the complex formation between RePhaC1 and RePhaM, protein pull-down assays were performed. The coding genes for RePhaC1 proteins (RePhaC1F, RePhaC11ND, and RePhaC1CD) were cloned into pET30a to apply a 6-His-tag at the C-terminus, and those for RePhaM proteins (RePhaM F and RePhaM AC) were cloned into pET21a to express proteins without a fusion tag. The resulting plasmids were combinatorially co-transformed into E. coli B834. Cells were cultured, and cell extracts were prepared as described above for RePhaC1. Cell extracts were applied to Ni-NTA agarose column and then thoroughly washed to eliminate nonspecific binding among proteins. Co-elution of RePhaM with the 6-His-tagged RePhaC1 was monitored by SDS-PAGE.

3 Results and discussion

3.1 SAXS modeled structure of RePhaC1 F

In order to determine the structure of full-length RePhaC1 (RePhaC1_F), we first attempted to obtain the crystal structure of the protein. Since RePhaC1_F is cleaved into two domains at Arg192 by proteolysis, we introduced a R192A mutation in RePhaC1_F (RePhaC1_F(R192A)) and obtained its crystals. However, the RePhaC1_F(R192A) crystals diffracted X-rays very poorly, and this could not be improved after repeated trials (data not shown). As an alternative, small-angle X-ray scattering (SAXS) analysis was performed. We first performed SAXS analysis using the C-terminal
domain of *RePhaC1 (RePhaC1\textsubscript{CD}).* The result showed that *RePhaC1\textsubscript{CD} exists as a dimeric form with a similar shape to the dimeric form in the crystal structure (Figs. 1A–E). These results indicate that the dimeric form of *RePhaC1\textsubscript{CD} observed in the crystal structure reported previously is the active form of the enzyme. We then performed SAXS experiment using *RePhaC1\textsubscript{F}.* As observed in SAXS analysis of *RePhaC1\textsubscript{CD},* that of *RePhaC1\textsubscript{F} also showed that the
protein exists in a dimeric form. Compared to the crystal structure of \( \text{RePhaC1}_{\text{CD}} \), the reconstructed 3D model of \( \text{RePhaC1}_{\text{p}} \) exhibited an extended structure along the horizontal axis (Fig. 1F). In the 3D model, the \( \text{RePhaC1}_{\text{CD}} \) dimer is located at the center, and \( \text{RePhaC1}_{\text{ND}} \) is located opposite the dimerization subdomain of \( \text{RePhaC1}_{\text{CD}} \) (Fig. 1F). Considering that the EC-region wraps the \( \alpha/\beta \) hydrolase fold on the opposite side of the dimerization subdomain, the interaction between \( \text{RePhaC1}_{\text{ND}} \) and \( \text{RePhaC1}_{\text{CD}} \) seems to be mediated by the EC-region.

### 3.2 Localization of \( \text{RePhaC1} \) on granules requires \( \text{RePhaC1}_{\text{ND}} \)

Next, we aimed to decipher the functions of \( \text{RePhaC1}_{\text{ND}} \) in PHB polymerization. Although \( \text{RePhaC1}_{\text{CD}} \) catalyzes the PHB polymerization reaction, \( \text{RePhaC1}_{\text{CD}} \) alone showed almost no PHB polymerization activity in vitro (Fig. 2A). This result was also supported by in vivo PHB biosynthesis experiments. First, an engineered \( E. \ coli \) strain expressing either \( \text{RePhaC1}_{\text{ND}} \), \( \text{RePhaC1}_{\text{CD}} \) or \( \text{RePhaC1}_{\text{F}} \) without \( R. \ eutropha \) \( \beta \)-ketothiolase and reductase (\( \text{RePhaAB} \)) could not synthesize PHB, and all of them were located in the soluble fraction (Fig. 2B). Next, we engineered an \( E. \ coli \) strain expressing \( \text{RePhaC1}_{\text{p}} \) together with \( \text{RePhaAB} \), and showed that it was able to synthesize a large amount of PHB, as reported previously [27, 28]. In contrast, an \( E. \ coli \) strain, expressing \( \text{RePhaC1}_{\text{ND}} \) or \( \text{RePhaC1}_{\text{CD}} \), instead of \( \text{RePhaC1}_{\text{F}} \), together with \( \text{RePhaAB} \) could not synthesize PHB (Fig. 1C). These results confirm that \( \text{RePhaC1}_{\text{ND}} \) or \( \text{RePhaC1}_{\text{CD}} \) alone is not able to synthesize PHB, and \( \text{RePhaC1}_{\text{ND}} \) is also necessary for PHB polymerization. The SAXS analyses of \( \text{RePhaC1}_{\text{CD}} \) and \( \text{RePhaC1}_{\text{F}} \), showing that \( \text{RePhaC1}_{\text{ND}} \) is located opposite of the dimerization subdomain of \( \text{RePhaC1}_{\text{CD}} \) (Figs. 1E and 1F), suggest that \( \text{RePhaC1}_{\text{ND}} \) might be crucial for the binding of \( \text{RePhaC1}_{\text{F}} \) to the PHB granule. Thus, enzyme localization experiments were performed using histagged \( \text{RePhaC1}_{\text{ND}} \), \( \text{RePhaC1}_{\text{CD}} \) and \( \text{RePhaC1}_{\text{F}} \) proteins (\( h\text{RePhaC1}_{\text{ND}} \), \( h\text{RePhaC1}_{\text{CD}} \) and \( h\text{RePhaC1}_{\text{F}} \), respective-

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**Figure 2.** Functions of \( \text{RePhaC1}_{\text{ND}} \). (A) The in vitro and in vivo PHB polymerization activities of \( \text{RePhaC1}_{\text{ND}} \) and \( \text{RePhaC1}_{\text{CD}} \) compared with those of \( \text{RePhaC1}_{\text{F}}, \text{C}_{\text{ND}}, \text{and C}_{\text{CD}} \) represent \( \text{RePhaC1}_{\text{F}}, \text{RePhaC1}_{\text{ND}}, \) and \( \text{RePhaC1}_{\text{CD}}, \) respectively. (B and C) Localization of \( \text{RePhaC1} \). The domain of \( \text{RePhaC1} \) that is required for localizing the protein to the PHB granules was examined by determining the location of \( \text{RePhaC1}_{\text{ND}} \), \( \text{RePhaC1}_{\text{CD}} \) and \( \text{RePhaC1}_{\text{F}} \) in the cytosol or granule fraction (top) without (B) and with (C) coexpression of \( \text{PhaAB} \). PHB granule formation was verified by transmission electron microscopy (TEM; bottom). M, C, S, P, and E indicate the marker, whole cells, supernatant, pellet, and elution, respectively. After treating the pellet with Triton X-100, the sample was centrifuged again. PS, PP, and PE indicate the supernatant, pellet, and elution, respectively.
ly). When hRePhaC1<sub>CD</sub> was expressed in an E. coli strain expressing RePhaAB, hRePhaC1<sub>CD</sub> was localized to the soluble fraction (Fig. 2C). However, this result does not confirm its inability to bind to PHB granules because no PHB synthesis occurs when hRePhaC1<sub>CD</sub> is employed. Thus, hRePhaC<sub>1</sub>d and hRePhaC<sub>1</sub>y were co-expressed in the E. coli strain expressing RePhaAB, in which PHB was synthesized by functional hRePhaC<sub>1</sub>y and accumulated as intracellular granules. Fractionation studies revealed that hRePhaC<sub>1</sub>y was localized to the granule fraction, whereas hRePhaC1<sub>CD</sub> was localized to the soluble fraction (Fig. 2C). Interestingly, when hRePhaC1<sub>d</sub> and hRePhaC1<sub>ND</sub> were co-expressed in the E. coli strain expressing RePhaAB, both hRePhaC1<sub>ND</sub> and hRePhaC1<sub>y</sub> were localized to the granule fraction (Fig. 2C). These results suggest that RePhaC1<sub>ND</sub> is crucial for binding and localizing RePhaC1 to the PHB granule.

3.3 Truncation study of RePhaC1<sub>ND</sub>

There have been several studies showing that serial truncations of the N-terminal region of RePhaC1 influenced PHB synthesis in vivo [29, 30]. Based on our localization studies and the secondary structure prediction of the RePhaC1<sub>ND</sub>, we generated 15 truncation mutants of RePhaC1<sub>ND</sub>. Then, their soluble expression levels, activities (both cell extracts and purified proteins), and localization were examined. RePhaC1<sub>1–50</sub> and RePhaC1<sub>1–60</sub> showed characteristics similar to RePhaC1<sub>y</sub> in protein soluble expression levels, activities (both cell extracts and purified proteins), and localization (Fig. 3A), indicating that the region of amino acid 1–60 (N-α1 and N-α2) is not essential for the function of RePhaC1<sub>ND</sub>. However, the ten different truncation mutants (RePhaC1<sub>1–65</sub> – RePhaC1<sub>ND</sub>) showed no soluble expression in E. coli and no PHA synthase activity from their cell extracts (Fig. 3A). These results are slightly different from those reported previously [29, 30]. The RePhaC1 mutants having truncations for more than 80 residues did not have activities as observed in the previous study. However, the truncated mutants RePhaC1<sub>1–65</sub> – RePhaC1<sub>1–70</sub> were not expressed in soluble form and did not show any activities in cell extracts in our study, while in previous studies expressing these truncated mutants showed activities (in cell extracts) and PHB synthesis upon expression in PHA synthase gene-deleted R. eutropha strain. Although the reason is not clear, it might be due to the difference in the host strains, R. eutropha vs. E. coli, in which these truncation mutants of RePhaC1 were expressed. Based on our study, the region of amino acid 65–98, which forms N-α3, is crucial for proper folding and for the function of RePhaC1<sub>ND</sub>. Interestingly, the RePhaC1<sub>1–110</sub>, RePhaC1<sub>ND</sub>, and RePhaC1<sub>1–155</sub> mutants could be expressed in soluble form similarly to RePhaC1<sub>y</sub>. However, these three mutants showed characteristics similar to RePhaC1<sub>CD</sub> with respect to their activities and localization. These mutants showed no PHA synthase activity in both cell extract and purified protein and were localized to the soluble fraction (Fig. 3A). These results suggest that the region of amino acid 110–192 (N-α4 to N-α7) alone is not sufficient to function as RePhaC1<sub>ND</sub>. Nevertheless, the region includes several sites containing consecutive

![Figure 3. Truncation study of RePhaC1<sub>ND</sub>](https://www.biotechnology-journal.com/)

(A) Serial truncation of RePhaC1<sub>ND</sub>. Protein soluble expression, activity (cell extract), activity (protein), and localization of fifteen truncations of RePhaC1<sub>ND</sub> were compared with those of RePhaC1<sub>d</sub> and RePhaC1<sub>CD</sub>. The figure in the bottom right is the SDS-PAGE of the purified proteins that were expressed in the soluble fraction. (B) Mutations of hydrophobic residues on RePhaC1<sub>ND</sub>: Mu-1, Mu-2, and Mu-3 represent FYLL<sup>119–122</sup>EEE, FA<sup>146–148</sup>EEE, and LLI<sup>170–172</sup>EEE, respectively. The PHA synthase activities of these mutants were compared with that of the wild-type.
hydrophobic residues that could be a potential binding site for PHB polymer. To examine the potential roles of these residues, we selected 119FYLL 122, 146FAI 148, and 170LLI 172 sites and changed each site to glutamate. All three mutants showed almost complete loss of activity (Fig. 3B), suggesting that the region of amino acid 110–192 (N-α4 to N-α7) is also required for the proper function of RePhaC1 ND. Taken together, we propose that the predicted five α-helices (N-α3 to N-α7) are required for proper folding and granule binding function of RePhaC1 ND, while the first two α-helices (N-α1 and N-α2) are not.

3.4 Activation of RePhaC1 by RePhaM

Recently, PhaM from R. eutropha (RePhaM) was reported to bind to nucleoids through its C-terminal PAKKA motif and function as an activator of RePhaC1 in R. eutropha [31–34]. To elucidate the mechanism of RePhaC1 activation by RePhaM, an electrophoretic mobility shift assay (EMSA) was performed using a native RePhaM (RePhaM F) and a PAKKA motif-truncated version of RePhaM (RePhaM D). The EMSA clearly showed that the band was shifted by the addition of RePhaM F but not by the addition of RePhaM D, confirming that the C-terminal PAKKA motif is crucial for binding with nucleoids (Fig. 4A). To investigate the activating role of RePhaM, in vitro polymerization assays using RePhaC1 F and RePhaC1 CD were performed in the presence of RePhaM F or RePhaM D. Both RePhaM F and RePhaM D increased the PHB polymerization activity of RePhaC1 F, but they did not affect the activity of RePhaC1 CD (Fig. 4B). Moreover, protein pull-down assays revealed that RePhaC1 ND is required for binding to RePhaM F and RePhaM D (Fig. 4C). Thus, the formation of the RePhaC1/RePhaM complex is mediated by interaction between RePhaC1 ND and RePhaM (RePhaM F or RePhaM D), which is responsible for the enhancement of RePhaC1 activity upon binding to RePhaM. SAXS analysis and 3D structural reconstruction of the RePhaC1 F/RePhaM D complex showed that the complex formed with 2:2 stoichiometry, where one RePhaM D molecule is attached to each side of the RePhaC1 dimer and forms an extended structure with RePhaC1 ND (Fig. 4D). Based on these results, we propose that RePhaM activates RePhaC1 by extending the structure of the RePhaC1 ND, thus reinforcing the binding capacity of RePhaC1 ND to the growing PHB polymer (Fig. 5).

3.5 Regarding the lag phase of polymerization reaction by PhaC

In polymerization reaction by PhaC, the lag phase at the initial stage seems to be caused by the low affinity of the enzyme for the nucleotide moiety [35, 36]. In this study, our finding that RePhaC1 ND is responsible for binding to
Thus, reduced lag phase upon the addition of (3-HB)₃ signifies the growing PHB polymer near the active site. PHB granule but enhances PHB polymerization by localizing the growing PHB polymer to the RePhaC₁ND active site. Based on these results, we propose that RePhaC₁ND not only localizes RePhaC1 to the PHB granule but enhances PHB polymerization by localizing the growing PHB polymer chain near the active site. Thus, reduced lag phase upon the addition of (3-HB)₃ seems to be due to the reduced time to reach a certain length allowing its binding to RePhaC₁ND. Taken together, we propose that the lag phase observed in RePhaC1 might be caused by a combinatorial effect of time required for the induction of dimerization by substrates and for synthesizing PHB polymer of a certain length that can reach RePhaC₁ND.

4 Concluding remarks

In this paper together and an accompanying paper [9], we reported the crystal structure of the C-terminal domain of *Ralstonia eutropha* PHA synthase (PhaC1) and the 3D reconstructed model of full-length of *R. eutropha* PhaC1 (RePhaC₁p) determined by small angle X-ray scattering (SAXS) analysis. The locations of the catalytic C-terminal domain dimer and the N-terminal domain of RePhaC1 were determined. RePhaC₁ND is not directly involved in the enzyme catalysis, and rather plays important roles in PHA polymerization by localizing the enzyme to the PHA granules and stabilizing the growing PHA polymer near the active site of RePhaC₁CD. The serial truncation study revealed those secondary structures required for proper folding and granule binding function of RePhaC₁ND. The SAXS 3D reconstructed model of the RePhaC₁p/RePhaM complex suggested that RePhaM forms a complex with RePhaC1 by interacting with RePhaC₁ND and activates RePhaC1.

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