Morphological Change in Cellular Granule Formation of Poly[(R)-3-hydroxybutyrate] Caused by DNA-Binding-Related Mutations of an Autoregulated Repressor PhaR

Miwá YAMADA, Akiko WAKUDA, and Seiichi TAGUCHI

Division of Biotechnology and Macromolecular Chemistry, Graduate School of Engineering, Hokkaido University, N13W8, Kita-ku, Sapporo, Hokkaido 060-8628, Japan

Received January 17, 2007; Accepted February 22, 2007; Online Publication, June 7, 2007
[doi:10.1271/bbb.70038]

PhaP is a major poly[(R)-3-hydroxybutyrate] [P(3HB)]-granule-associated protein. Its gene expression is controlled by an autoregulated repressor, PhaR, in Paracoccus denitrificans. The packing force of the P(3HB) granule by PhaP is greatly influenced by the number of PhaP molecules. In this study, the effects of DNA-binding-ability-reduced mutations of PhaR on morphological change in the cellular granule formation of P(3HB) were examined under a transmission electron microscope using an Escherichia coli recombinant system. Microscopic observation indicated that stronger packing of P(3HB) granules took place when the number of PhaP molecules was increased by reduction in the DNA-binding ability of PhaR.

Key words: polyhydroxybutyrate; granule-associated protein; autoregulated repressor; DNA-binding related mutation; microscopic observation

Bacterial polyesters, polyhydroxyalkanoates (PHAs), have been drawing much attention as new environmentally compatible materials that can be used in place of conventional petrochemical-based plastics.1,2) Paracoccus denitrificans is a facultative methylotrophic bacterium that can synthesize a typical PHA, poly[(R)-3-hydroxybutyrate] [P(3HB)], from several alcohols.3) In our proposed mechanistic model of P(3HB) biosynthesis (Fig. 1A), granule formation of P(3HB) is coordinately managed in the cells by granule-associated proteins, synthase (PhaC), phasin (PhaP), and repressor (PhaR). In particular, PhaP forms a boundary layer on the P(3HB) surface, perhaps to prevent individual granules from coalescing or to sequester hydrophobic P(3HB) from the cytoplasmic substances.4) Gene expression of PhaP is negatively controlled by an autoregulated repressor, PhaR, in P. denitrificans.5–7) Most recently, the proposed regulation model has been further supported by direct evidence that PhaR has two separate domains binding to the target DNA (including the phaP promoter region) and P(3HB), as was discovered using a quartz crystal microbalance (QCM).8,9) In addition, amino acid residues responsible for DNA binding of PhaR were addressed by deletion and polymerase chain reaction-mediated random point mutation experiments coupled with a green fluorescent protein reporter-based monitoring assay system.9)

In this study, the reduced effect of the DNA-binding ability of PhaR on phaP expression was analyzed by microscopic observation of differences in the morphogenesis of P(3HB) granules caused by changes in granule-packing intensity associated with the number of PhaP molecules present. The dissociation constant for wild type PhaR to the target DNA was calculated to be $1.82 \times 10^{-7}$ M by a previous surface plasmon resonance analysis.9) Here, we used three PhaR single mutants, S17R, R18C, and R18H, which had decreasing degrees of DNA-binding ability (down to 3.4% to 16.4% in dissociation constant relative to the wild type).9)

Recombinants of Escherichia coli DH5α were prepared for microscopic study by the introduction of two plasmid vector constructs, pBBRKmAB (to supply the 3HB-CoA monomer) and pTVCPR (for regulatory synthesis of P(3HB)), as shown in Fig. 1B. Expression plasmids, a series of pTVCPRs, that carry phaR (wild type and single mutants) with phaC and phaP, were constructed by inserting 0.7-kb NcoI-XbaI fragments containing mutated phaR into the same restriction sites of pTVCPR-WT carrying wild type phaR.3) DNA sequencing to confirm the new plasmid constructs was carried out with a Prism 310 Genetic Analyzer (Applied Biosystems, CA) using a BigDye terminator cycle sequencing ready reaction kit (Applied Biosystems). The nucleotide sequence was analyzed with GENETYX genetic information processing software (Software Development, Tokyo). Recombinant cells carrying pBBRKmAB and pTVCPR were grown in Luria-Bertani...
medium supplemented with ampicillin (100μg/ml) and kanamycin (50μg/ml) at 37°C. As shown in Fig. 2A, Western blot analysis revealed that the gene expression levels of the mutants and wild type of PhaR were nearly identical when culture condition for P(3HB) accumulation was employed in the presence of 2% glucose. This suggests that there is no distinguishable difference in binding ability to the target DNA including the \( \text{phaR} \) promoter as between the wild type and mutants of PhaR. In contrast, a large amount of the translated product of \( \text{phaP} \) was detected by SDS–PAGE in both the whole-cell extract (Fig. 2B, lane 2) and the P(3HB)-bound fraction (Fig. 2C, lane 2) for the pTVCP construct that contained no \( \text{phaR} \). On the other hand, pTVCP-WT carrying wild type \( \text{phaR} \) showed a significant reduction in the expression of \( \text{phaP} \) (Fig. 2B and C, lane 3). This strongly suggests that \( \text{phaP} \) expression was repressed by specific binding of the translated product of \( \text{phaR} \) to the target DNA, including the \( \text{phaP} \) promoter. Furthermore, enhanced expression of \( \text{phaP} \) was detected by replacement of wild type \( \text{phaR} \) (pTVCP-WT) with other pTVCP constructs carrying genes for PhaR mutants (S17R, R18C, or R18H) (Fig. 2B and C, lanes 4–6). This result is reasonable in view of derepression of \( \text{phaP} \) expression, which was mainly caused by the reduced DNA-binding abilities of the mutated \( \text{phaR} \) products to the target DNA.

Previously, to examine the localization of PhaR in vivo, SDS–PAGE and Western blot analysis were carried out for P(3HB) granule-associated protein from \( \text{Paracoccus denitrificans} \) and a recombinant \( \text{E. coli} \) strain carrying pBBRKmAB and pTVCPR, and the results showed that PhaP is a predominant P(3HB) granule-associated protein. The ratios of PhaR and PhaP to total P(3HB) granule-associated proteins were estimated to be 0.3% ± 0.1% and 96%, respectively, by densitometric analysis. Accordingly, PhaP is much more prominent than PhaR (negligible) in terms of its contribution to the granule packing effect in this recombinant system.

Using the recombinant \( \text{E. coli} \) system established in
this study, the granule formation of P(3HB) was observed with a transmission electron microscope (TEM). Cells were prefixed in the presence of 2% (v/v) glutaraldehyde in 50 mM sodium phosphate buffer (pH 7.2) and fixed with 1% osmium tetroxide. After dehydration with a graded ethanol series, the cells were embedded in Supr’s low viscosity resin. Ultrathin sections were mounted on copper grids treated with neoprene, stained with uranyl acetate and lead citrate, and examined with the TEM (H-800, Hitachi, Tokyo). The differences in granule structure were microscopically visualized in recombinant bacterial cells harboring genes encoding wild type or mutants of PhaR (Fig. 3). As shown in Fig. 3A and B, the difference between the two constructs in the sizes of granules formed within the bacterial cells was clearly evident (pTVCP without phaR, 0.6–0.8 μm in diameter; pTVCPR-WT with wild type phaR, 1.2–1.5 μm in diameter). As expected, PhaP-mediated P(3HB) granule packing effects similar to that of the phaR-deficient construct (pTVCP), were evident for all the recombinants carrying the three mutant constructs of pTVCPR (S17R, R18C, and R18H), as shown in Fig. 3C to E. Hence, changes in the morphology and size of P(3HB) granules can be attributed to the degree of DNA binding to the target DNA fragment including the phaP promoter of PhaR. In other words, DNA-binding-ability-dependent repression of PhaR can be evaluated microscopically by monitoring cellular granule formation of P(3HB) through repression of phaP expression that is directly mediated by phaR expression. In conclusion, the feasibility of our regulation model of P(3HB) biosynthesis was confirmed by in vivo analysis as well as the previous in vitro analyses. At the next stage, an in vitro system such as the QCM system can be reconstructed for quantitative analysis of intermolecular interactions by mimicking the in vivo system consisting of four molecules (PhaR, the target DNA, P(3HB), and PhaP).

Acknowledgments

We are grateful to T. Ito (Hokkaido University) for preparation of electron micrographs. We would also like to thank A. Maehara (Mitsubishi Gas Chemical Company), K. Tajima (Hokkaido University), Y. Satoh (Hokkaido University), and M. Maeda (Meiji University) for useful discussion. The work described here was partly supported by a Grant-in-aid for Scientific Research (no. 70216828) from the Ministry of Education, Culture, Sports, Science and Technology of Japan (to S.T.) and a Grant-in-aid from the Industrial Technology Research Grant Program for 2003 from the New Energy and Industrial Technology Development Organization (NEDO) of Japan.
References


Fig. 3. Microscopic Observation of Cellular Granule Formation of P(3HB).

Recombinant cells of E. coli DH5α were cultivated at 37 °C for 30 h in Luria-Bertani medium with ampicillin and kanamycin. The plasmid vectors used in the microscopic analysis were pTVCP (A), pTVCPR-WT (wild type) (B), pTVCPR-S17R (C), pTVCPR-R18C (D), and pTVCPR-R18H (E). Bars, 5 μm. Schematic illustrations are presented near the corresponding micrographs to explain the cellular granule formation of P(3HB).
