A structural study of the crystalline state of the bacterial copolyester poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate)

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Poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate)s (P(3HB-*co*-4HB)s) with a wide range of 4HB contents were biosynthesized, and their crystalline states were studied by differential scanning calorimetry (d.s.c.) and compared with those of the cold-stretched copolyesters of poly(3HB-*co*-3-hydroxyvalerate) (P(3HB-*co*-3HV)). A structural study was also carried out by the use of molecular mechanics calculations. It was demonstrated that P(3HB-*co*-4HB) cannot form isomorphic crystals because of the extent of the structural difference between the 3HB and 4HB comonomer components.

(Keywords: poly(3-hydroxybutyrate); hydroxyalkanoate copolyesters; molecular mechanics)

INTRODUCTION

It is known that many kinds of bacteria accumulate amounts of poly(3-hydroxybutyrate), P(3HB), as an intracellular storage polymer for both energy and carbon sources[1,2]. *Alcaligenes eutrophus* is a representative bacterium for synthesizing various copolyesters of this family, namely the poly(hydroxyalkanoates) (PHAs)[3]. Among these, the physical, thermal and mechanical properties of the copolyesters of 3HB and 3-hydroxyvalerate (3HV), i.e. P(3HB-*co*-3HV)s, have been widely studied, and it has been shown that these properties depend significantly on the copolymer composition[4–10]. Results based on both experimental and theoretical approaches[4,8–10] indicate that P(3HB-*co*-3HV)s form isomorphic crystals, in which some of the minor comonomer components co-crystallize with the major comonomer components. On the other hand, it had been believed until recently that the degree of crystallinity of the copolyester of 3HB and 4-hydroxybutyrate (4HB), i.e. P(3HB-*co*-4HB), decreased with an increase in the amount of the 4HB component and that P(3HB-*co*-4HB) with a 4HB content of more than 50 mol% showed no crystalline properties[11,12]. However, Scandola *et al.*[13] in 1990 revealed that P(3HB-*co*-4HB) containing 82 mol% 4HB units, i.e. P(3HB-*co*-82%4HB), shows a sharp melting endotherm at 44°C that is close to the melting point of the P(4HB) homopolymer (54°C)[14]. Recently, Nakamura *et al.*[14] reported that P(3HB-*co*-4HB)s with 4HB contents over the range 85–100 mol% were synthesized by *A. eutrophus* and these samples had the crystalline lattice of a non-P(3HB) type with a quasicrystalline character (degree of crystallinity of 30–40%). Nevertheless, no structural study has yet been reported of the solid-state properties of 4HB-dominant P(3HB-*co*-4HB)s, probably due to their relatively low crystallinities.

In this study, P(3HB-*co*-4HB) copolymers with 4HB contents of 17, 63, and 82 mol%, synthesized by the bacterium *A. eutrophus* H16, have been characterized by ^{1}H n.m.r. and d.s.c., and a structural comparison made with the cold-stretched P(3HB-*co*-3HV) material in its β-form. Furthermore, the conformations of the molecular chains of the crystalline P(3HB-*co*-4HB) material with a high 4HB content have been investigated by molecular mechanics (MM) simulations using the MM2 method[15].

EXPERIMENTAL

P(3HB-*co*-4HB) copolymers with mole fractions of 4HB of 13, 17, 63, and 82 mol% were biosynthesized using *A. eutrophus* H16 in a two-stage fermentation process. Cells were incubated in the first stage at 30°C for 24 h in a medium containing 10 g of yeast extract, 10 g of polypeptone, 5 g of meat extract and 5 g of $(NH_4)_2SO_4$ per litre of distilled water. The cells were then transferred to a nitrogen-free medium[16] containing an appropriate amount of the carbon source and incubated at 30°C for 48 h to promote the synthesis of P(3HB-*co*-4HB). 4-Hydroxybutyrate and a 3-hydroxybutyrate/4-hydroxybutyrate mixture were used as carbon sources. After fermentation, the cells were washed and dried, and the polyesters were extracted into chloroform and reprecipitated with hexane. P(3HB-*co*-3HV) containing 18 mol% 3HV units, hereafter abbreviated as P(3HB-*co*-18%3HV), was purchased from the Aldrich Chemical Co., Ltd, and was used without further purification.

Polyester films were prepared by a solution casting technique using chloroform as the solvent, with samples left for a week at room temperature to promote crystallization.

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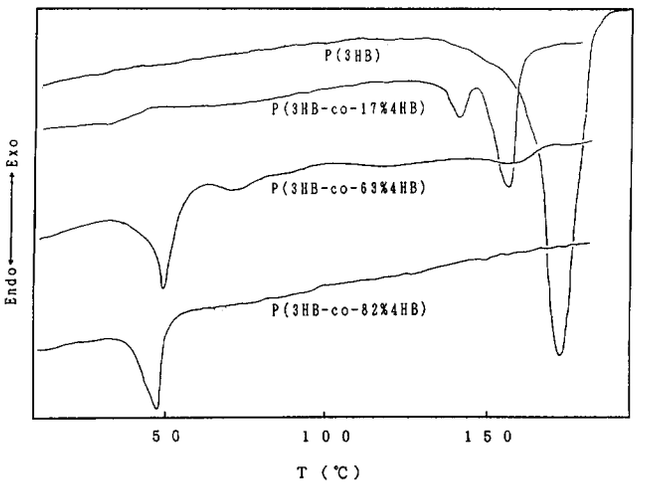


Figure 1 D.s.c. thermograms of various P(3HB-*co*-4HB) copolymers

The compositions of the synthesized PHA copolymers were determined by ^{1}H n.m.r. spectroscopy, with spectra recorded in $CDCl_3$ at 30°C, using a JEOL GX-270 *FT*n.m.r. spectrometer operated at 270 MHz.

The melting temperature (T_m) and the enthalpy of fusion (ΔH) of the P(3HB-*co*-4HB) copolymers were determined from the d.s.c. endotherms recorded on a Seiko DSC-20 equipped with a SSC-580 thermal controller. 5 mg of sample were encapsulated in an aluminium d.s.c. pan and heated at a rate of 10°C min^{-1}.

The molecular mechanics study was performed on a SUN4-workstation using the MM2 program[15], which had been slightly modified by us to allow the calculation of the conformational energy of a molecule consisting of up to 300 atoms[17].

RESULTS AND DISCUSSION

D.s.c. melting curves of P(3HB-co-4HB)

In order to confirm that the P(3HB-*co*-4HB) copolymers form different types of crystals depending on their comonomer composition, d.s.c. melting curves were obtained for P(3HB-*co*-4HB) films with 4HB fractions ranging from 17 to 82 mol%, as shown in *Figure 1*. The d.s.c. data are identical to those reported by Scandola *et al.*[13], showing that P(3HB-*co*-4HB) forms the P(3HB)-type of crystals (T_m = 140–170°C) at a low 4HB content, while the P(4HB)-type of crystals (T_m = 45–54°C) are formed at a high 4HB content.

Figure 2 shows the d.s.c. melting endotherms of film samples of P(3HB-*co*-13%4HB), P(3HB-*co*-18%3HV) and P(3HB-*co*-82%4HB) after the cold-stretching process[18]. In each melting curve, a new melting endotherm emerged at 46–49°C, in addition to the original ones. In the case of P(3HB-*co*-82%4HB), the melting point shifted from 46 to 46.7°C, and the enthalpy of fusion of the original film increased by a factor of 1.7 as a result of the stretching process. Orts *et al.*[18] have revealed by X-ray diffraction measurements and MM calculations that P(3HB-*co*-21%3HV) adopts an almost planar-zigzag conformation when the film sample is subjected to cold-stretching. Recently, analogous results have been reported for PHAs with long side chains (l.s.c. PHAs) in which an additional endothermal peak at 45°C was observed when films of these PHAs were stretched[19]. The structure of the crystals formed by this stress-induced crystallization treatment is likely to be the same as that reported by Orts *et al.*[18], namely the distorted β-form, and it should be noted that the melting range of the crystals is almost the same as that reported for P(3HB-*co*-82%4HB). These experimental results strongly suggest that the crystalline structures of both the PHA crystals formed by stress-induced crystallization and the 4HB-dominant P(3HB-*co*-4HB) systems share some common structural features.

COMPUTATIONAL ANALYSIS OF THE CONFORMATION OF P(3HB-*co*-4HB)

In order to simulate the structure of the molecular chains in the crystalline lattice of P(3HB-*co*-4HB), model compounds must be selected for the two parent homopolymers, namely P(3HB) and P(4HB). In practice, model compounds with finite chain lengths were used to represent the isolated molecular chains present in real polyester crystals. In order to determine the appropriate chain length for this present purpose, the dependence of the geometry on the number of included monomer units was examined. In this case of P(3HB), three different X-ray crystal structures have been reported[20–22]. We have reported in a previous paper[23] that the structure of P(3HB) determined by Yokouchi *et al.*[20] using X-ray diffraction led to geometrically the most reproducible and energetically the most stable structure. The deviation in the dihedral angles after optimization was less than 5 degrees over 10 successive units from the 3rd to the 12th unit of a 15-mer P(3HB) model compound, when the structural parameters reported by Yokouchi *et al.*[20] were chosen for the initial structure. Therefore, the geometrically homogenous region from the 3rd to the 12th unit of this chain compound was employed as the P(3HB) model compound.

On the other hand, no structural data obtained by X-ray diffraction measurements has been reported so far for P(4HB), although Nakamura *et al.*[14] have shown by X-ray diffraction that P(3HB-*co*-4HB) copolymers with high 4HB contents form crystals that are quite different from that of P(3HB). Two related linear polyesters, namely poly(propiolactone) (PPL) and poly(ε-caprolactone) (PCL) consist of three and six methylene groups with one carboxyl group per monomer unit,

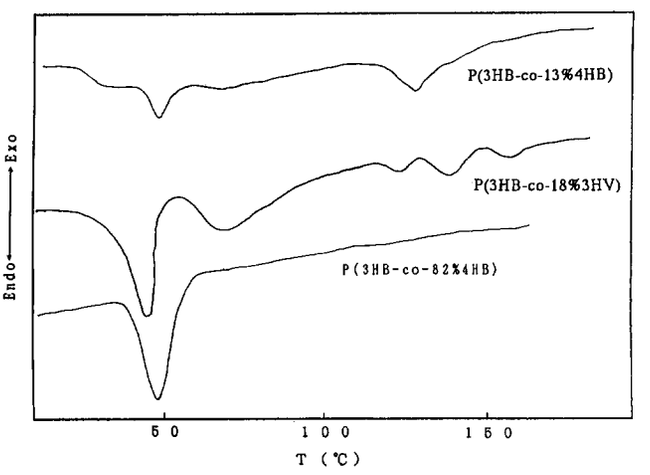


Figure 2 D.s.c. thermograms of film samples of P(3HB-*co*-13%4HB), P(3HB-*co*-18%3HV) and P(3HB-*co*-82%4HB) recorded after cold-stretching treatment

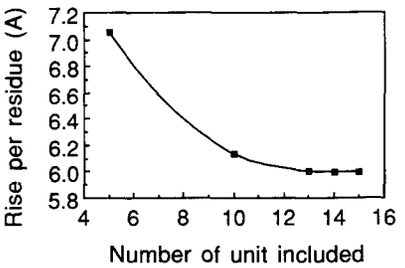


Figure 3 'Rise per residue' *versus* the number of monomer units included in the P(4HB) model compound (see text for details)

respectively, while the 4HB units are composed of four methylene groups with one carboxyl group (per unit). On the basis of X-ray analysis, the chain conformation of both PPL[24] and PCL[25] has been reported to be the all-*trans* planar-zigzag type. By structural analogy with PPL and PCL, as well as the experimental results shown in *Figures 1* and *2*, the 4HB crystalline lattice is reasonably assumed to be composed of chains with an all-*trans* conformation. This assumption is based on a simulation reported by Liau and Boyd[26], in which they describe the structures of aliphatic polyesters by considering both intra- and intermolecular interactions, and also showed that these polymers tend to take the planar-zigzag conformation, although some include kinks made up of pairs of *gauche* bonds. Thus the initial structure of the P(4HB) model compound may be fixed in an all-*trans* conformation. The validity of this model compound was examined by comparing one of the calculated geometrical values, namely the interval between one monomer unit and the next neighbouring unit along the fibre axis (hereafter referred to as the 'rise per residue') with that obtained from the crude X-ray diffraction measurements. Finally, the model compound for P(3HB-*co*-4HB) was constructed on the assumption of the three initial structures.

By analysing the MM2-optimized structures of the model compounds from both energetic and structural viewpoints, the conformational aspect of P(3HB-*co*-4HB) over various comonomer ranges can be described as follows.

Construction of model compounds for parent homopolymers

As discussed above, we have reported in a previous paper[23] that the 15-mer 3HB model compound includes a geometrically homogenous region when optimized from the structure determined using X-ray diffraction (Yokouchi *et al.*[20]), and that the resulting structure can be approximated to the polymer chain of P(3HB). In this study, the same approach as that used for P(3HB) was employed for construction of the P(4HB) crystalline structure by assuming its initial structure to be an all-*trans* conformation. *Figure 3* shows the dependence of the value of the 'rise per residue' on the number of monomer units included in the P(4HB) model compound. This value, taken as the 'rise per residue' of the central unit of each model compound, becomes independent of the number of monomer units (at a value of 5.99 Å) when the number of monomer units exceeds 13. The deviation of the dihedral angles along the main chain of the model compound composed of 15 monomer units (compound 4HB15) is $\leqslant 5°$ over the range from the 4th to the 13th units. Thus we chose compound 4HB15 with an all-*trans* conformation as an appropriate model compound for P(4HB).

Nakamura *et al.*[14] have reported that 4HB-dominant P(3HB-*co*-4HB) (up to 97 mol% 4HB) gives an X-ray diffraction pattern which is quite different from that of either P(3HB) or P(3HB-*co*-4HB) with a low 4HB content. However, for the P(3HB-*co*-82%4HB) sample, almost the same X-ray diffraction profile was observed in the equatorial scan, followed by a diffraction maximum at $2\theta = 14.6°$ (corresponding to a 'rise per residue' of 6.06 Å) in the meridional scan (data not shown). This value is in good agreement with the calculated value (i.e. 5.99 Å) for the 4HB15 model compound. Consequently, it is confirmed that we can suitably select the structure of 4HB15 as a P(4HB) model compound and employ the chain sequence ranging from the 4th to the 13th monomer unit in our subsequent study concerning the copolymer conformations in crystals with different compositions. The dihedral angles of the 8th 4HB unit of 4HB15 after optimization are given in *Table 1*.

Conformational analysis of copolymers

The model compound, (3HB14–4HB1), for P(3HB-*co*-16%4HB) was constructed by substituting one 4HB unit for the 8th 3HB unit. Since P(3HB-*co*-4HB) with such a comonomer composition should form the P(3HB) type of crystal, the geometrical parameters obtained from the X-ray analysis of P(3HB) were selected to determine the initial structure of the 3HB moieties; the structure of the 4HB moiety at the 8th position, however, is difficult to predict empirically. Since the 4HB unit includes one methylene unit more than the 3HB unit, the former unit cannot form the 2_1 helical structure. Here, an all-*trans* conformation was tentatively assumed as the initial structure of the 4HB unit. The molecular chain of 3HB14–4HB1 no longer maintained the linear structure at the 8th 4HB unit after optimization. Some other initial structures for the 4HB moieties, such as a 2_1 helix-like structure, were examined, but these again resulted in non-linear structures (data not shown). Therefore, it was predicted that 4HB units cannot be incorporated into the P(3HB) type of crystalline lattice, in contrast to the previously studied case of P(3HB-*co*-3HV).

The d.s.c. results confirmed that P(3HB-*co*-4HB)s with high 4HB contents form the P(4HB) type of crystals. It was assumed in the previous section that P(4HB) forms crystals with the polymer chains in an all-*trans* conformation. Since Orts *et al.*[18] have reported that the

Table 1 Dihedral angles[a] and total steric energy of 4HB15, the model compound of P(4HB), with values obtained both before and after optimization

Structure	Angle (°)					Energy (kJ)
	ψ	ϕ	ω	θ	ε	
Initial	180.0	180.0	180.0	180.0	180.0	163.0
Optimized	−171.9	173.7	172.1	−160.7	168.7	93.4

[a] Angles of the bonds between the ether oxygen atoms and the methylene carbon atoms are defined as ψ, with the remaining angles defined as follows:

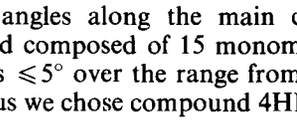


Table 2 Values of the dihedral angles (°) and steric energies (kJ) of the backbone of each model compound of P(3HB-*co*-4HB)

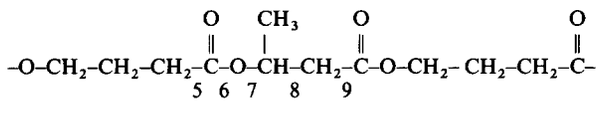
	Model 1		Model 2	
Dihedral angles[a]	Initial	Optimized	Initial	Optimized
5	180	−93	180	−179
6	180	−178	172	176
7	180	74	113	79
8	180	178	−169	−166
9	180	−136	−126	−101
Steric energy	180.4	92.1	310.4	91.6

[a] See text for definition of the dihedral angles

3HB units can also form crystals in the β-form when P(3HB-*co*-21%3HV) films are stretched to eight-fold their original length, there might be some possibility that the 3HB units can be incorporated into the P(4HB) lattice by forming the β-form. We have made attempts to simulate the structural changes that might occur when the 3HB units are incorporated into the P(4HB) crystalline lattice after forming the β-form. A model compound for such a case was made by substituting one 3HB unit for the 8th 4HB unit in 4HB15 (4HB14–3HB1). The initial structure for the 4HB units of the 4HB14–3HB1 compound was set in an all-*trans* conformation, while those for the 3HB unit were as follows:

(a) all-*trans* (completely planar-zigzag) (Model 1) and;
(b) β-form, calculated by Orts *et al.*[18], using the POLYGRAF program for a stretched P(3HB) system (Model 2).

Table 2 summarizes the drastic changes in the dihedral angles that occur after optimization and the steric energy of each of the model compounds, with the angle numbers in this table defined as follows:



Considering Model 1, the dihedral angles 5, 7 and 9 changed drastically while the changes in the other two angles namely 6 and 8, were small. The changes in the remaining dihedral angles were negligibly small (not shown in the table). The three angles 5, 7, and 9 should determine the orientation of the two oxygen atoms of the carbonyl groups adjacent to the methyl group of the 8th 3HB unit, namely the 7th 4HB and the 8th 3HB carbonyl oxygens. Therefore, these deviations from the initial values strongly suggest the existence of steric hindrance between the methyl side chain and the neighbouring carbonyl oxygen atoms. The final structure of Model 1 included the disordered segment induced by this distortion. On the other hand, the structural optimization caused only slight changes in the dihedral angles of Model 2. This optimized structure possessed a steric energy value which was slightly lower than that of the model compound 4HB15 (composed of the same number of C, O, and H atoms). It can be easily understood from a geometrical viewpoint that such a structure should minimize the steric hindrance of the three groups mentioned in Model 1, namely the 8th methyl group and the adjacent two carbonyl oxygen atoms. The structure of Model 2 possessed two regions consisting of linear planar-zigzag chains, but yet did not match the P(4HB) lattice.

CONCLUSIONS

D.s.c. measurements of 4HB-dominant P(3HB-*co*-4HB) and stretched film samples of P(3HB-*co*-4HB) and P(3HB-*co*-3HV) revealed that they have a common melting range of 45–50°C. These results indicate that 4HB-dominant P(3HB-*co*-4HB) forms crystals that resemble crystals of the cold-stretched samples of P(3HB-*co*-3HV) or PHAs with long side chains.

Conformational analysis by the MM2 method of P(3HB-*co*-4HB) model compounds revealed that the 4HB units cannot be included within the P(3HB) crystal lattice because of the difference in the length of the main chains of the repeating monomer units. It was also revealed that the 3HB units included within the P(4HB)-type of lattice cannot form a complete planar-zigzag conformation but may be deformed into other structures in order to relax the steric hindrance between the methyl side chains of the 3HB units and the adjacent carbonyl oxygen atoms. It can be predicted from such simulation studies that the structural difference between the two comonomer units mentioned above makes it impossible to form isomorphic crystals in both of the P(3HB) and P(4HB) types of crystalline lattice.

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