Investigation of the Enzymatic Cleavage of Diastereomeric Oligo(3-hydroxybutanoates) Containing Two to Eight HB Units. A Model for the Stereoselectivity of PHB Depolymerase from *Alcaligenes faecalis* T_1

Beat M. Bachmann[1] and Dieter Seebach*

Laboratorium für Organische Chemie, Eidgenössische Technische Hochschule, ETH-Zentrum, Universitätstrasse 16, CH-8092 Zürich, Switzerland

Received September 21, 1998; Revised Manuscript Received December 21, 1998

ABSTRACT: So far, it is known that various poly(3-hydroxybutanoate), PHB, depolymerases are able to degrade *all*-(*R*) chains, cyclic (*R*) oligomers, oligolides, and polymers composed of *rac*-hydroxybutanoate, HB, but not *all*-(*S*) or syndiotactic (*R*,*S*) chains. We have now studied the ability for configurational recognition (stereoselectivity) by the purified PHB depolymerase from *Alcaligenes faecalis* T_1. To this end, a titristat/HPLC method has been developed for following the degradation of oligo(3-hydroxybutanoates), OHBs, providing baseline separation for OHB methyl esters up to at least the octamers (Figure 1). With this method, we have now investigated the degradation rates and cleavage patterns of OHBs (**1**−**16**) containing up to eight HB units with given sequences of (*R*) and (*S*) configurations along the chains (Figures 2−5 and 7). Analysis of the measurements now allows us to propose more detailed structural features at the binding site of the depolymerase (Figures 6, 8, 9): (i) the enzyme is an endo esterase; (ii) it recognizes the orientation of the chain relative to its active site; (iii) the binding site contains four subsites, three of which have to be occupied by HB units for cleavage to occur at all (rate $v_{max}\beta$) and all four for cleavage to take place at the maximum rate ($v_{max}\alpha$); and (iv) the central two subsites, between which cleavage occurs, must be occupied by (*R*) HB units, whereas the terminal subunits may also be occupied by (*S*) HB units.

Introduction and Goal of Investigation

The polyester poly-(*R*)-3-hydroxybutanoate, PHB, which is accumulated by a wide variety of microorganisms as a unique intracellular storage of carbon, reducing equivalents, and energy attracts a lot of attention due mainly to its inherent biodegradability.[2] The wealth of PHB-degrading organisms and the mechanisms of action of their enzymes are the target of many investigations.[3]

A strain of *Alcaligenes faecalis* T_1, which can be isolated from activated sludge, produces and secretes an extracellular PHB depolymerase together with a "cooperating" dimer hydrolase when grown in a medium containing PHB as the sole carbon source.[4] The depolymerase, which can easily be purified, degrades PHB and its oligomers to monomers and dimers, regardless of their water solubility. The gene coding for this depolymerase (mol wt ca. 50 000) has been cloned into *Escherichia coli* which was then able to produce but not to secrete the enzyme.[5] Because of the now-facile production and purification of the enzyme (provided a sample of the *E. coli* strain described above is available), PHB depolymerase from *A. faecalis* is an ideal tool for studying the mechanism of PHB degradation.

Although earlier investigations have provided valuable information about the initial rate of cleavage, the products of degradation of *all*-(*R*)-oligo(3-hydroxybutanoates), OHBs, by PHB depolymerase from *A. faecalis*,[6] and the enzyme's substrate specificity,[4] its stereoselectivity has hitherto not been studied.[7]

The development of a method for the synthesis of pure OHBs with well-defined sequences of (*R*)-and (*S*)-3-hydroxybutanoate, HB, units[8] now enables us to undertake an in-depth investigation on the stereoselectivity of this PHB depolymerase. To save material and to take into account the fact that some of the OHBs are water-soluble and others are not, we chose the highly sensitive titristat method[6,9,10] for monitoring the degradation of our compounds. This method, which proved to be highly reproducible, was combined with an improved normal-phase HPLC analysis for the quantitative determination of the degradation product fragments.

Results and Discussion

Analysis and Degradation of *all*-(*R*)-OHBs. In a first stage we developed a method to determine the content of mixtures of OHBs **1**−**8** consisting of two to eight HB units. To this end, we treated such mixtures with diazomethane (to convert the CO_2H into a CO_2Me terminus) and injected a CH_2Cl_2 solution of the resulting hydroxyesters onto a LiChrosorb Si-60 HPLC column. As can be seen from Figure 1, baseline separation of the monomer and each oligomer is achieved. Fortunately, at least for the present investigation, diastereomeric oligomers are not separated, so that this analysis

* Telephone: 0041-1-632 29 90. Fax: 0041-1-632 11 44. E-mail: seebach@org.chem.ethz.ch.

10.1021/ma981496w CCC: $18.00
Published on Web 02/24/1999

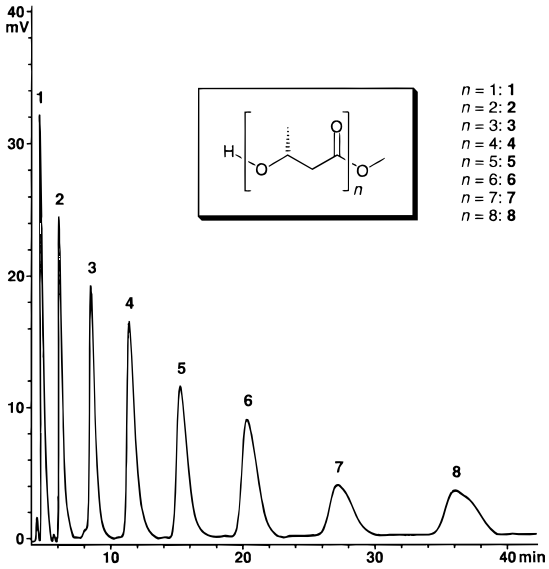

Figure 1. HPLC chromatogram of an equimolar mixture of the methylesters of OHBs **1**−**8** (column: LiChrosorb Si-60).

is ideally suitable for following the rate of enzymatic degradation of various OHBs containing (*R*) and (*S*) residues. The acidimetric titration in the titristat (measuring the equivalents of ester groups cleaved by the enzyme) and the subsequent esterification and HPLC analysis of oligomer distribution thus allowed us to follow the cleavage events and the cleavage sites with time.

The degradation experiments with the *all*-(*R*)-OHBs showed that, except for the dimer **2**, all substrates are being cleaved by the depolymerase and that the monomer or dimer or both are the exclusive final degradation products. The trimer **3** was cleaved only once, yielding the monomer and dimer in a ratio of 1:1 (Figure 2). The tetramer **4** was also cleaved only once, to give the dimer as sole degradation product (Figure 3). Interestingly, in the initial phase of degradation, the trimer **3** was degraded about 20 times more slowly (∼30 U mg^{-1}) than were the tetramer **4** (∼600 U mg^{-1}) and all other OHBs! In all our experiments, we only observed these two discrete rates for the OHB degradation. Therefore we will call the faster rate (>500 U mg^{-1}) $v_{max}\alpha$ and the slower rate (<40 U mg^{-1}) $v_{max}\beta$ throughout this paper.

The pentamer **5** was cleaved twice in a two-stage fashion, with the first cleavage being approximately as fast as that of the tetramer cleavage and the second one as slow as the one observed for the trimer (final products, monomer and dimer in a ratio of 2:1). When the degradation was terminated after the fast first "cut" (1 equiv of acid formed), a 1:1 dimer/trimer mixture was detected (Figure 4).

The experiments with the hexa-, hepta-, and octamer **6**−**8** showed similar, discrete two-stage degradation behavior, with the dimer being the main product, together with small amounts of monomer, and with a dimer monomer ratio which increased with increasing chain length of the substrate. Termination experiments with the octamer **8** showed that, at an early stage (on average, one-third of 1 ester bond/molecule cleaved), oligomers of all chain lengths are present in the mixture

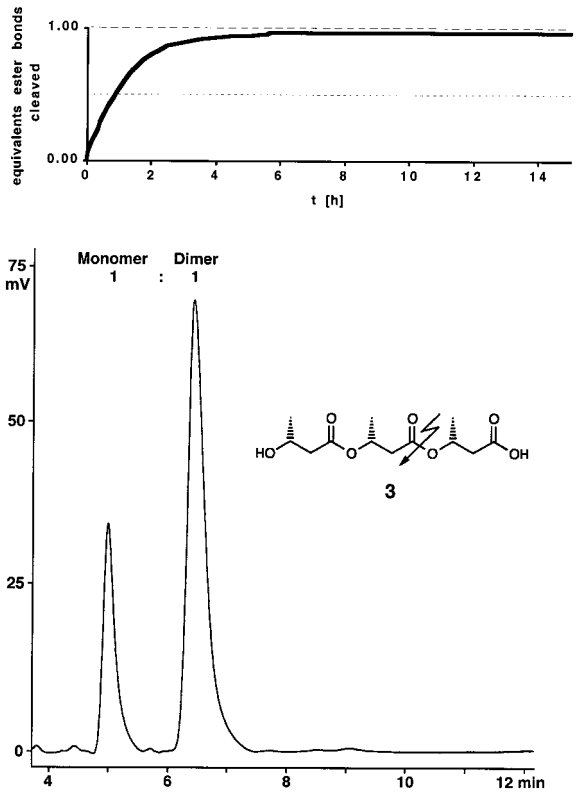

Figure 2. Degradation of *all*-(*R*)-trimer **3**. Top: titristat curve. Bottom: HPLC chromatogram of the degradation mixture.

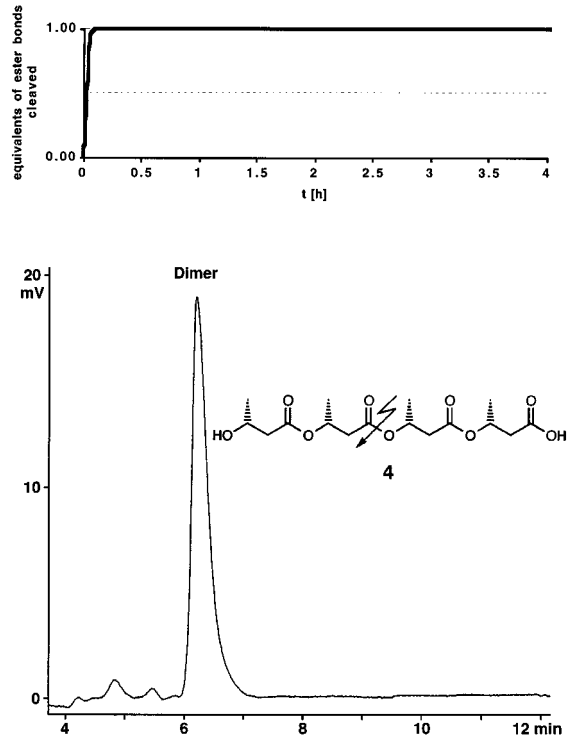

Figure 3. Degradation of *all*-(*R*)-tetramer **4**. Top: titristat curve. Bottom: HPLC chromatogram of the degradation mixture.

of degradation products. The longer the degradation is allowed to proceed, the more dimer (with traces of monomer) can be found, while the longer oligomers gradually disappear (Figure 5).

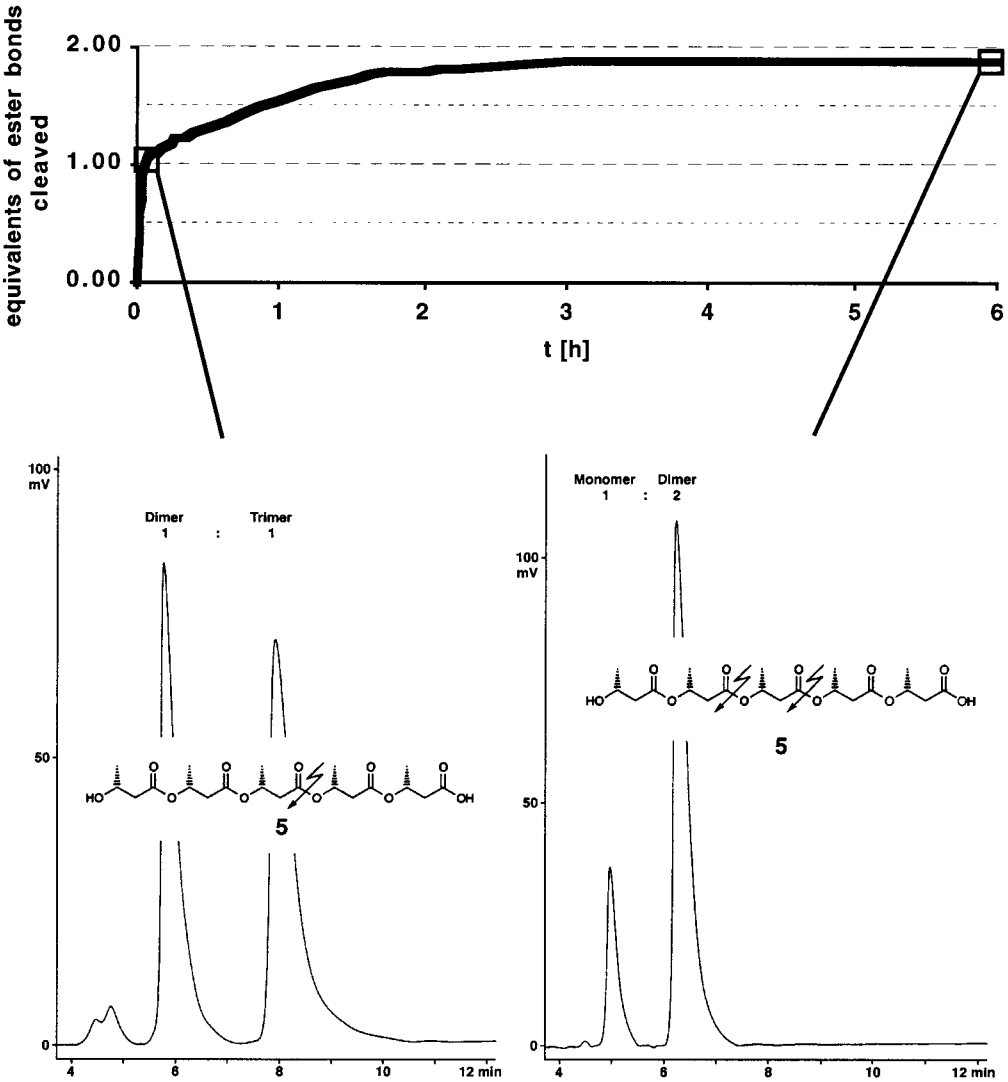

Figure 4. Degradation of *all*-(*R*)-pentamer **5**. Top: titristat curve. Bottom, left: HPLC chromatogram of the degradation mixture after 1 equiv of ester bonds had been cleaved. Bottom, right: after complete degradation.

If the enzyme attacked terminal ester bonds preferentially (exo fashion), a much higher proportion of hepta- and hexamer should be found in the product mixture at an early stage of degradation. Therefore, these measurements strongly support an endo attack, at least by the PHB depolymerase from *A. faecalis*.[11] From the results described, a first model (not considering stereoselectivity) for the PHB depolymerase from *A. faecalis* may be derived (Figure 6); because the trimer **3** is the shortest OHB to be cleaved at all and a drastic cleaving rate increase can be observed when going from the trimer **3** to the tetramer **4** (but no further increase for the longer OHBs), it can be concluded that the enzyme has four binding subsites of which at least three must be occupied by HB units of the substrate for a cleavage to take place. The cut occurs in the middle of the four subsites, and according to findings by Shirakura et al. (experiments with radioactive OHBs),[6] the enzyme is able to recognize the orientation of the oligomer chain relative to its active site: it cleaves only when the substrate chain has the "correct" orientation in the binding site (Figure 6). Our observation of a cleavage-rate increase by a factor of ca. 20 (from $v_{max}\alpha$ to $v_{max}\beta$, see Table 1) when going from the tetramer to the trimer (four versus three subsites occupied) suggests that the enzyme exhibits cooperative binding kinetics.

Finally, an endo-type attack of the enzyme is responsible for the traces of monomer that can be found in every degradation-product mixture: Whenever the stochastic endo cleavage of an OHB produces a trimer, the subsequent slow ($v_{max}\beta$) cleavage of this trimer yields a dimer and a monomer as the final products.

The surprisingly slow degradation of the polymer by the depolymerase of *A. faecalis* can be attributed to the high degree of crystallinity of PHB that has an unfavorable effect on the rate of degradation[12] (the measurements with all OHBs were done in solution; solubility of **8**, ca. 0.8 g/l H_2O).[13]

Stereoselective Degradation of HB Octamers with Various Configurational Patterns. So far, our studies with *all*-(*R*) OHBs provided us with information about the number and cooperation of binding subsites in the enzyme and about the cleavage sites in the substrate. We then proceeded with the investigation on the stereoselectivity of the depolymerase. For this purpose, we subjected a set of octamers with one to four terminal (*S*) HB units, **9**−**15** and *ent*-**12**, the *all*-(*S*)-octamer *ent*-**8**, and the syndiotactic octamer **16** to our degradative conditions (see Chart 1).

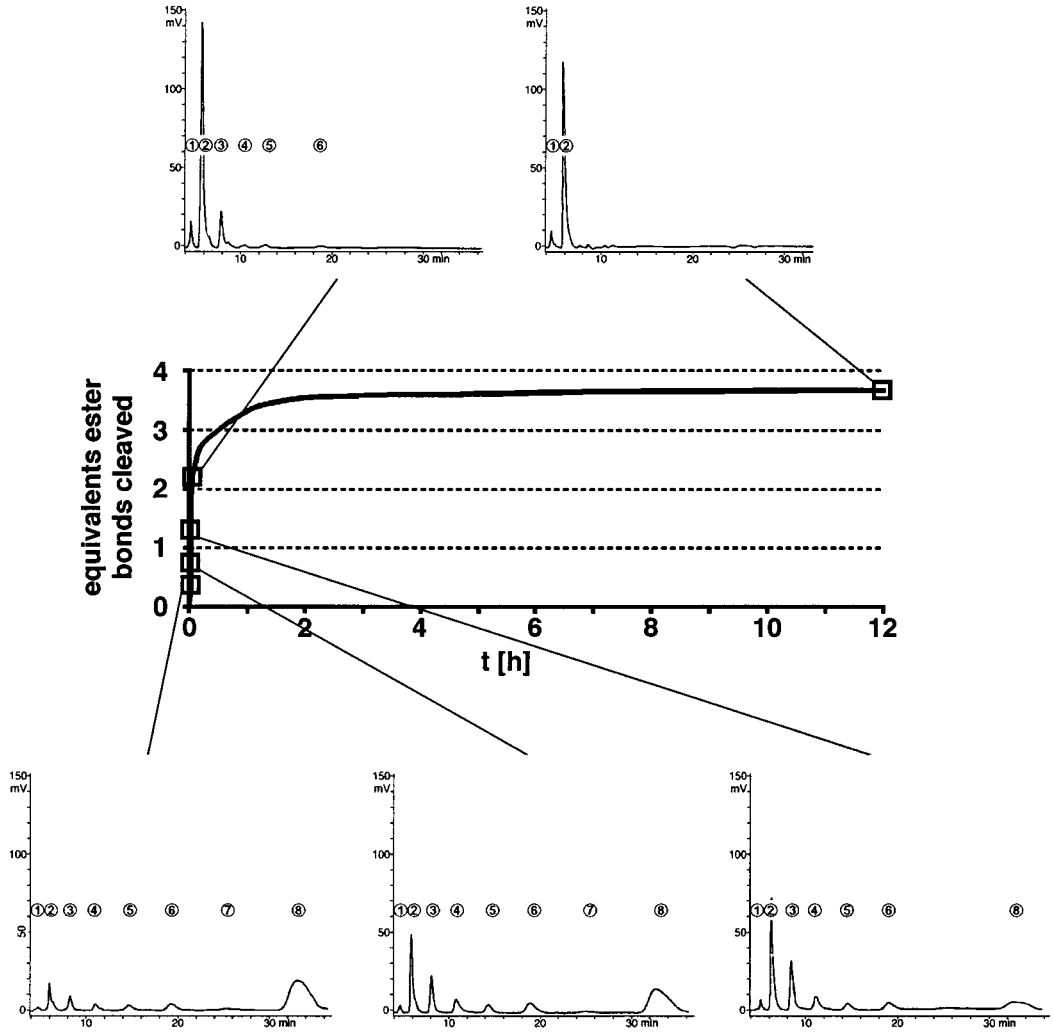

Figure 5. Degradation of *all*-(*R*)-octamer **8**. Titristat curve with HPLC chromatograms of the degradation mixtures at different stages of the reaction: ① monomer, ② dimer, ③ trimer, ④ tetramer, ⑤ pentamer, ⑥ hexamer, ⑦ heptamer, and ⑧ octamer.

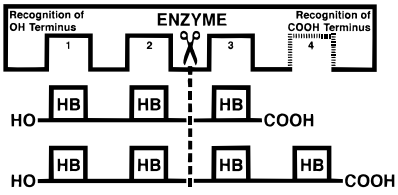

Figure 6. Schematic model of the PHB depolymerase from *A. faecalis* T_1 without consideration of its stereoselectivity. Cleavage rate of the trimer $v_{max}\beta$ (only subsites 1−3 occupied), of the tetramer $v_{max}\alpha$ (all subsites occupied), with $v_{max}\alpha/v_{max}\beta$ being ca. 20.

As could be expected from previous studies of "unnatural" PHB,[12,14] the *all*-(*S*) and the syndiotactic octamer were not at all degraded by the enzyme. Because all other substrates were cleaved (Table 2), it became obvious that at least two neighboring (*R*)-HB units are necessary for a degradation to occur.

An analysis of the degradation mixtures' compositions showed that the largest fragment that could be found was always one HB unit longer than the length of the (*S*)-HB sequence in the substrate (Figure 7). The remaining sequence of (*R*)-HB units was then degraded according to the model described above, as could be derived from the number of cuts in the molecule. Because no difference of either cleavage frequency or degradation products could be detected between substrate pairs with the same number of (*S*)-HB units on the C- or the O-terminus, we conclude that a symmetrical arrangement (in terms of stereoselectivity) must exist for the binding subsites.

Table 1. Results of the Titristat Degradation Experiments of (*R*)-3-HB Oligomers 2−8

compound	equiv of ester bonds cleaved	$v_{max}\alpha$; $v_{max}\beta$ [U mg^{-1}]	v_{max}-(norm)	degradation products
2 (dimer)	0	−; −	−	(no degradation)
3 (trimer)	1.0	−; 33.2	0.05	monomer/dimer (1:1)
4 (tetramer)	1.0	635; −	1.0	dimer (exclusively)
5 (pentamer)	1.9	707; 21.6	1.1	monomer/dimer (1:2)
6 (hexamer)	2.9	654; 13.5	1.0	dimer (traces of monomer)
7 (heptamer)	3.4	572; 12.5	0.9	dimer (traces of monomer)
8 (octamer)	3.7	712; 19.9	1.1	dimer (traces of monomer)
polymer	n/a	−; 13.7	0.02	dimer (traces of monomer)

The arrangement and stereoselectivity of four HB-binding subsites that are able to rationalize the results from our experiments is shown in Figure 8: Although the two peripheric subsites are not stereospecific, the two in the center, between which the cleavage takes place, only bind to (*R*)-HB units and therefore are

Chart 1

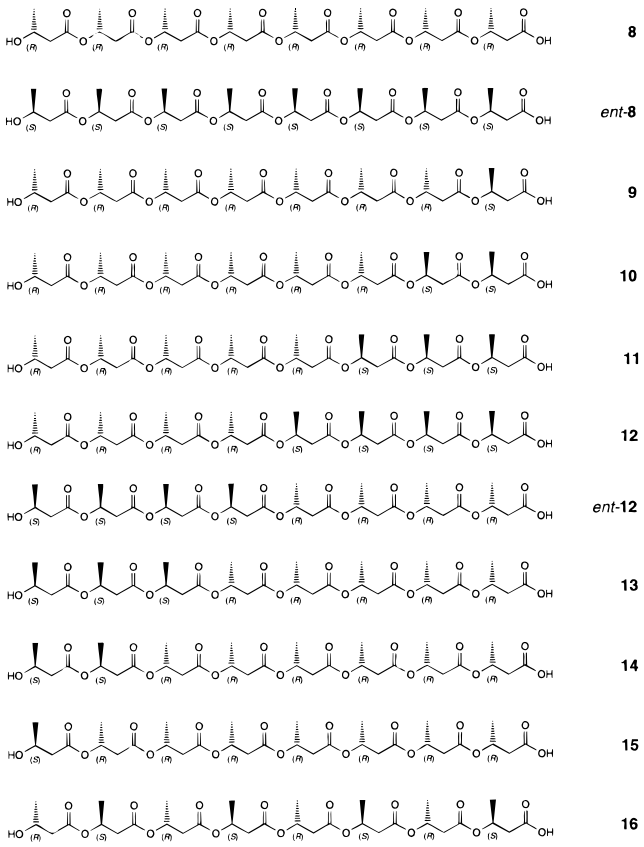

Table 2. Results of the Titristat Degradation Experiments of 3-HB Octamers 8−16

compound	equiv of ester bonds cleaved	$v_{max}\alpha$; $v_{max}\beta$ [U mg^{-1}]	degradation products
8 (*R*8)	3.7	712; 19.9	dimer (little monomer)
ent-**8** (*S*8)	–	–; –	(no degradation)
9 (*R*7*S*1)	3.0	635; 5.8	dimer (little monomer)
10 (*R*6*S*2)	2.4	673; 4.5	monomer/dimer/trimer (1:2:1)
11 (*R*5*S*3)	1.9	615; 6.0	dimer/tetramer (2:1)
12 (*R*4*S*4)	1.8	558; 6.0	monomer/dimer/pentamer (1:1:1)
ent-**12** (*S*4*R*4)	1.8	539; 7.2	monomer/dimer/pentamer (1:1:1)
13 (*S*3*R*5)	2.0	673; 6.4	dimer/tetramer (2:1)
14 (*S*2*R*6)	2.7	692; 5.8	monomer/dimer/trimer (1:2:1)
15 (*S*1*R*7)	2.9	625; 7.7	dimer (little monomer)
16 ((*RS*)4)	–	–; –	(no degradation)
(polymer)	n/a	–; 13.7	dimer (little monomer)

responsible for the overall stereoselectivity of the enzyme.

As an example, the degradation of octamer **14**, containing two HB units with (*S*) configuration on the C-terminus, is discussed in more detail. The first possible cut on the C-terminus of the molecule can take place between the third and the fourth HB unit (Figure 9, top). The (*S*,*S*,*R*)-trimer that is cleaved off by such a cut cannot be degraded any further, because two neighboring HB units with (*R*) configuration are not present in the fragment. The other fragment, the *all*-(*R*)-pentamer, can be degraded according to the scheme shown in Figure 4. Of course, the first cut can, with the same probability and cleavage rate as the one described first, take place between HB units 4 and 5, 5 and 6, or 6 and 7. With a first cut also possible between units 7 and 8, the cleavage rate does (due to the fact that only three subunits are occupied) decrease from $v_{max}\alpha$ to $v_{max}\beta$. If the first cleavage actually takes place between units 4 and 5 (Figure 9, bottom), the resulting (*S*,*S*,*R*,*R*)-tetramer can be cut once more ($v_{max}\beta$) to yield the (*S*,*S*,*R*)-trimer and an (*R*)-monomer. The other fragment, the *all*-(*R*)-tetramer, then is cleaved according to Figure 3. The sum of all possible degradation paths for octamer **14** finally adds up to what can be observed in the titristat/HPLC analysis: The degradation starts with a high cleavage rate ($v_{max}\alpha$) until an average of two ester bonds per molecule are cleaved, which is followed by a slow degradation ($v_{max}\beta$) of the intermediate *all*-(*R*) or (*S*,*R*,*R*)-trimers and (*S*,*S*,*R*,*R*)-tetramers. In the final degradation mixture, only monomer, dimer, and trimer (ratio 1:2:1) are found.

Conclusion

In this paper, we propose a model for the stereoselectivity of PHB depolymerase from *A. faecalis* T_1. It was shown that the prerequisite for a degradation to take place is an OHB stretch consisting of at least three HB units of which two neighboring ones must be of (*R*) configuration and cannot be situated on the O-terminus of the chain. The degradation of OHBs can then be explained as a combination of all possible cleavages with $v_{max}\alpha$ (all four binding subsites of the enzyme occupied) or with $v_{max}\beta$ (only three binding subsites occupied). The model described here is in accordance with previous degradation studies of PHB with different tacticities,[15] although it does not take into account the effects of crystallinity on the rate of degradation.

Experimental Section

Materials and Reagents. The materials and reagents used were from the following sources. *E. coli* JM 109/pDP14: METABOLIX Inc. (USA). LB broth, ampicillin, and CH_2Cl_2 (puriss. p.a. quality and distilled three times prior to use): Fluka (Switzerland). DEAE-cellulose: Pharmacia (USA). Butyl-Toyopearl: TosOH (Japan). Titristat (Dosimat 665 with exchange unit 10 mL, Impulsomat 614, pH meter 632, pH electrode 6.0236.100, and all parts): Metrohm (Switzerland). HPLC (pump 64; degasser; variable wavelength monitor; HPLC interface; software, Eurochrom version 1.57; column, LiChrosorb Si-60, 7 μm, 25 × 0.4 cm, injection volume 20 μL, solvent hexane/2-propanol (97:3), flow 3 mL min^{-1}, detection at 210 nm): Knauer. The 3-hydroxybutanoate derivatives were synthesized in our laboratories (most of them by B.M.B.).[1]

PHB Depolymerase from *E. coli* JM 109/pDP14. Solutions of PHB depolymerase from *A. faecalis* were obtained by growing a 12 L LB broth culture (100 mg ampicillin L^{-1}) of *E. coli* JM 109/pDP14 at 37 °C for 6 h (late stationary phase). The culture was centrifuged at 3 500 rpm for 30 min at 4 °C, and the pellets obtained were suspended in 150 mL Tris−HCl/Ca^{2+} buffer. After centrifugation (20 min, 7 000 rpm, 4 °C) 68 g of wet cell mass was obtained. The cells were suspended again in 150 mL Tris−HCl/Ca^{2+} buffer and passed twice through a "french press" (500−1 000 psi, 4 °C). From the resulting 200 mL suspension, the depolymerase was isolated and purified following a slightly modified procedure (DEAE-cellulose instead of TEAE-cellulose column) described by Shirakura et al.[6] The final protein concentration was 0.14 mg mL^{-1} and according to Tanio et al.[4] and Shirakura et al.,[6] the depolymerase activity was 1.7 U mL^{-1} with no dimer hydrolase activity detectable. SDS−polyacrylamide gel electrophoresis (Coomassie brilliant blue R-250) showed only one protein band at ca. 50 kDa.

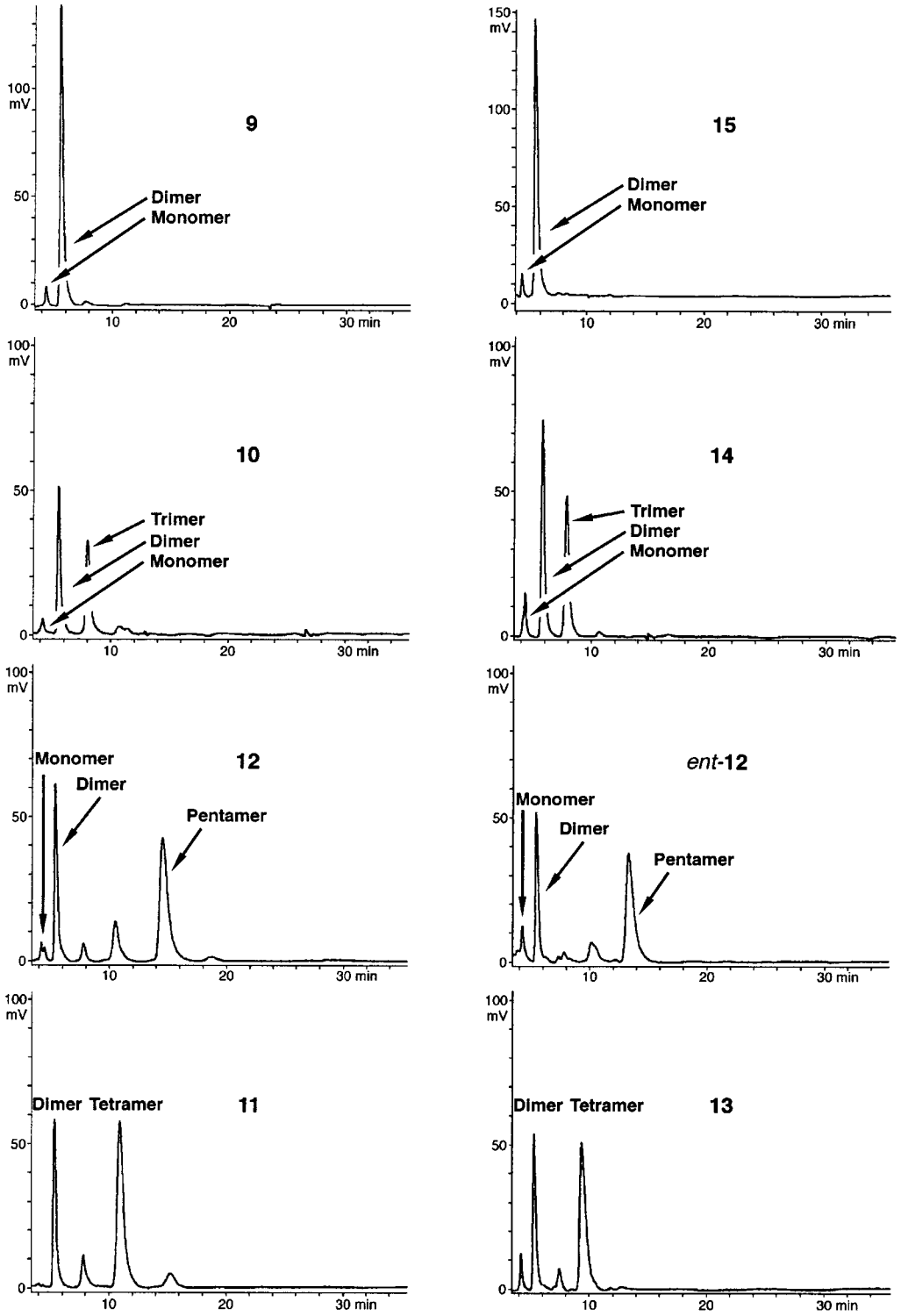

Figure 7. HPLC chromatograms of the resulting final degradation products of octamers **9**−**15** and of *ent*-**12**.

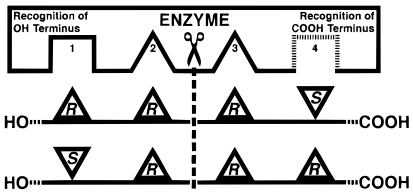

Figure 8. Schematic model of the PHB depolymerase from *A. faecalis* T_1 with consideration of its stereoselectivity. Subsites 1 and 4 may be occupied by (*S*) units.

Degradation Experiments. Titristat Degradation Experiments. Of each compound tested, 20.0 mg was dissolved in 2.0 mL CH_2Cl_2, and 100 μL of the resulting solution was injected into a titristat cell (diameter 2 cm; height 4 cm). The cell was then pivoted and swung so that the solvent slowly evaporated and the inner surface of the cell wall was coated evenly up to a level of ca. 20 mm. After the solvent had been evaporated completely, 4 mL of H_2O and a magnetic stirring plate were added. At 30 °C and under N_2, the pH of the solution was adjusted to 7.5 using 5 mM NaOH. This procedure caused the substrates to dissolve in the water. The measurement was started by addition of 10 μL of enzyme solution. The free acid released by the degradation was monitored as a function of time by measuring the quantity

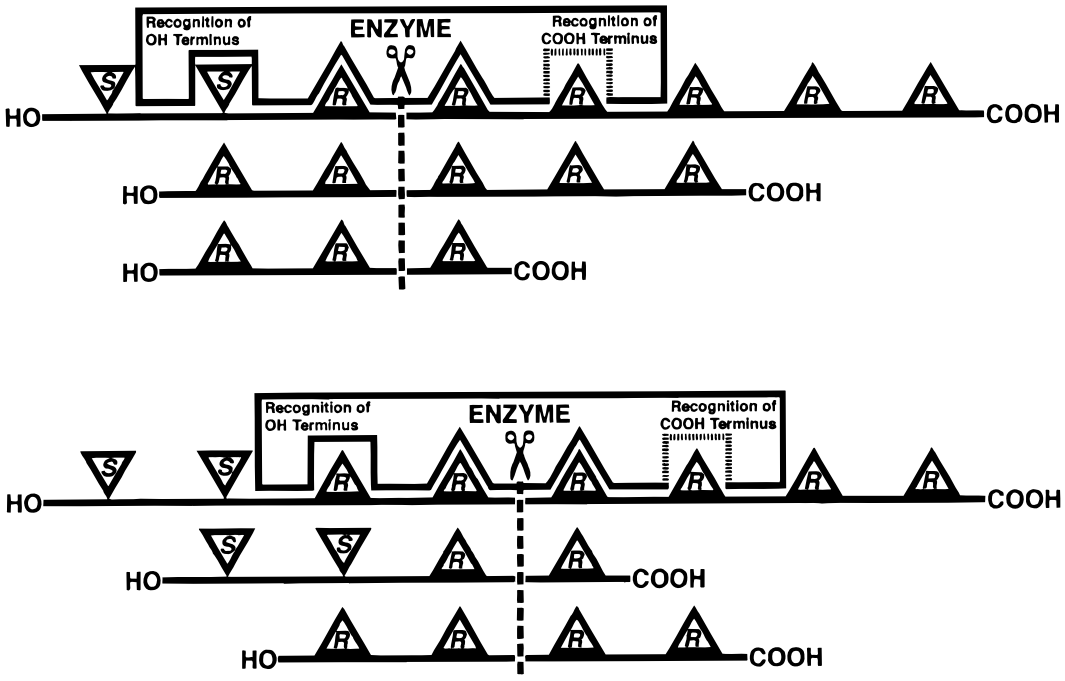

Figure 9. Two different degradation pathways for the cleavage of octamer **14** by PHB depolymerase from *A. faecalis* T_1.

and rate of the 5 mM NaOH addition required to maintain pH 7.5 (pH-stat method).

In the termination experiments described above, ca. 2 mL of acetone was injected into the degradation solution when the reaction was to be terminated. The enzyme was thus denatured, and the reaction stopped within 5−10 s.

Test Tube Degradation Experiments. To obtain enough samples for the HPLC analysis in a reasonable period of time, the titristat degradation experiment was modified as follows. Instead of a titristat cell, a test tube (180 × 20 mm) was coated with 1 mg of the compound as described above. After 4 mL of H_2O and 10 μL of enzyme solution had been added, the test tube was shaken and then sealed with aluminum foil. The test tube then was stored at 30 °C, and after 12 and 24 h (termination of the experiment), the pH of the solution was adjusted to 7.5 using 5 mM NaOH.

HPLC Analysis of the Degradation Products. The clear solutions obtained from titristat or test tube degradation experiments were poured into a 100 mL round-bottomed flask, and the water was removed completely during 30−45 min at room temperature with a rotary evaporator (14−17 mbar). The residue was extracted twice with a solution of 0.1% AcOH in CH_2Cl_2 and transferred into a 5 mL flask with a pointed bottom. The solution was then concentrated in a rotary evaporator at room temperature (300 mbar) to approximately 100 μL. To this solution, single drops of a diazomethane solution were added until the yellow color persisted. After the solvent had been evaporated, the flask was stored upside down for some minutes in order for the solvent vapors to evade completely. Then approximately 50 μL of CH_2Cl_2 was added, and the resulting solution was injected directly on the HPLC column.

Acknowledgments. The authors gratefully acknowledge the donation of a strain of *E. coli* JM 109/pDP14 by METABOLIX Inc., Cambridge, MA. We also thank Prof. R. W. Lenz and T. Scherer for the generous help in growing the *E. coli* JM 109/pDP14 and the isolating and purifying of the PHB depolymerase. This part of the work was done in the laboratories of the University of Massachusetts at Amherst. We also thank Dr. B. Martinoni (ETH Zürich) for his valuable comments and suggestions.

References and Notes

(1) Bachman, B. M. Ph.D. Thesis, ETH Zürich, 1997, # 12074.

(2) Doi, Y. *Microbial Polyesters*; VCH: Weinheim, 1990. Steinbüchel, A. *Nachr. Chem. Techn. Lab.* **1991**, *39*, 1112. Müller, H.-M.; Seebach, D. *Angew. Chem., Int. Ed. Engl.* **1993**, *32* (4), 477−502. Seebach, D.; Brunner, A.; Bachmann, B. M.; Hoffmann, T.; Kühnle, F. N. M.; Lengweiler, U. D. *Biopolymers and -oligomers of* (R)*-3-Hydroxyalkanoic Acids − Contributions of Organic Chemists*; Ernst Schering Research Foundation: Berlin, 1995. See also: the Congress volumes of the symposia on PHB in Sitges 1990, Montpellier 1991, Göttingen 1992, Montreal 1994, and Davos 1996.

(3) Jendrossek, D.; Schirmer, A.; Schlegel, H. G. *Appl. Microbiol. Biotechnol.* **1996**, *46*, 451−463. Brandl, H.; Bachofen, R.; Mayer, J.; Wintermantel, E. *Can. J. Microbiol.* **1995**, *41* (suppl.), 143−153.

(4) Tanio, T.; Fukui, T.; Shirakura, Y.; Saito, T.; Tomita, K.; Kaiho, T.; Masamune, S. *Eur. J. Biochem.* **1982**, *124*, 71−77.

(5) Saito, T.; Suzuki, K.; Yamamoto, J.; Fukui, T.; Miwa, K.; Tomita, K.; Nakanishi, S.; Odani, S.; Suzuki, J.-I.; Ishikawa, K. *J. Bacteriol.* **1989**, *171*, 184−189.

(6) Shirakura, Y.; Fukui, T.; Saito, T.; Okamoto, Y.; Narikawa, T.; Koide, K.; Tomita, K.; Takemasa, T.; Masamune, S. *Biochim. Biophys. Acta* **1986**, *880*, 46−53.

(7) For our previous investigation of *all*-(*R*)-OHB degradation by a whole-cell preparation from *Acidovorax delafieldii*, see: Brandl, H.; Aeberli, B.; Bachofen, R.; Schwegler, I.; Müller, H.-M.; Bürger, M. H.; Hoffmann, T.; Lengweiler, U. D.; Seebach, D. *Can. J. Microbiol.* **1995**, *41* (suppl.), 180−186.

(8) Bachmann, B. M.; Seebach, D. *Helv. Chim. Acta* **1998**, *81*, 2430−2461.

(9) Hocking, P. J.; Marchessault, R. H.; Timmins, M. R.; Lenz, R. W.; Fuller, R. C. *Macromolecules* **1996**, *29*, 2472−2478.

(10) Walter, T.; Augusta, J.; Müller, R.-J.; Widdecke, H.; Klein, J. *Enzyme Microb. Technol.* **1995**, *17*, 218−224.

(11) The ability of PHB depolymerases from *A. faecalis*[6] and *A. delafieldii*[7] to attack OHBs in an endo fashion was shown earlier by the degradation of cyclic *all*-(*R*)-OHBs.

(12) For a review article about the influence of substrate crystallinity on the rate of PHB degradation, see: Kemnitzer, J. E.; McCarthy, S. P.; Gross, R. A. *Macromolecules* **1992**, *25*, 1221.

(13) Shirakura, Y.; Fukui, T.; Tanio, T.; Nakayama, K.; Matsuno, R.; Tomita, K. *Biochim. Biophys. Acta* **1983**, *748*, 331−339.
(14) Abe, H.; Matsubara, I.; Doi, Y.; Hori, Y.; Yamaguchi, A. *Macromolecules* **1994**, *24*, 6018−6025.
(15) Hocking, P. J.; Marchessault, R. H. *Macromol. Rapid Commun.* **1994**, *15*, 447−452.

MA981496W