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Purification and Properties of *p*-Hydroxybenzoate Hydroxylases from *Rhodococcus* Strains

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Abstract—Gram-positive bacteria of the genus *Rhodococcus* catabolize *p*-hydroxybenzoate (PHB) through the initial formation of 3,4-dihydroxybenzoate. High levels of *p*-hydroxybenzoate hydroxylase (PHBH) activity are induced in six different *Rhodococcus* species when these strains are grown on PHB as sole carbon source. The PHBH enzymes were purified to apparent homogeneity and appeared to be homodimers of about 95 kD with each subunit containing a relatively weakly bound FAD. In contrast to their counterparts from gram-negative microorganisms, the *Rhodococcus* PHBH enzymes prefer NADH to NADPH as external electron donor. All purified enzymes were inhibited by Cl^- and for five of six enzymes more pronounced substrate inhibition was observed in the presence of chloride ions.

Key words: *Rhodococcus* species, flavoprotein, *p*-hydroxybenzoate hydroxylase, substrate inhibition

4-Hydroxybenzoate-3-hydroxylase (EC 1.14.13.2) is a monooxygenase that converts *p*-hydroxybenzoate to protocatechuate for further metabolism by one of the two branches of the β-ketoadipate pathway [1]. Several *p*-hydroxybenzoate hydroxylases have been purified and characterized; these are mainly from gram-negative bacterial strains of *Pseudomonas* and *Acinetobacter* [2-6]. All of them use NADPH as the electron donor, and only one can use NADH as well [4]. The enzymes from *P. fluorescens* and *P. aeruginosa* are the best-studied enzymes of this family; they have very similar properties and structure (their amino acid sequences differ in only five amino acid residues). The three-dimension structure of oxidized enzyme complexes with different substrates [7-9], the structure of the substrate-free enzyme, reduced enzyme complexes with the substrate, and the enzyme–product complex have been elucidated [10, 11]. The three-dimension structure of enzyme–substrate complexes and some kinetic properties were studied for several mutant forms of this enzyme. PHBH from gram-positive *Rhodococcus erythropolis* S-1 strain [12] use NADH but not NADPH as the electron donor. Rather little is known about this enzyme.

We supposed that the study of 4-hydroxybenzoate-3-hydroxylases from gram-positive organisms might provide some interesting details concerning the reaction mechanisms of this type of enzyme. This article is devoted to purification and characterization of six soluble *p*-hydroxybenzoate hydroxylases from different *Rhodococcus* strains. We showed that all the purified enzymes have quite similar properties, but they have considerable differences from other known enzymes.

MATERIALS AND METHODS

Chemicals and materials. Flavin adenine dinucleotide (FAD), NADH, NADPH, Tris, dithiothreitol (DTT), citric acid, *p*-hydroxybenzoic acid (PHB), QAE-Toyopearl, Butyl-Sepharose, and Blue-Sepharose were purchased from Sigma (USA). Sephadex G-25 superfine, Superdex PG200 HR 10/30, and molecular weight markers for SDS-PAGE were from Pharmacia (Sweden). Biogel P-6DG, acrylamide, *bis*-acrylamide, and Coomassie brilliant blue R-250 were obtained from Bio-Rad (USA).

Abbreviations: PHBH) 4-hydroxybenzoate-3-hydroxylase; PHB) 4-hydroxybenzoate; PCA) protocatechuate (3,4-dihydroxybenzoate); PCADO) protocatechuate-3,4-dioxygenase; DTT) dithiothreitol; K_m, K_m^{NADH}, K_m^{NADPH}, K_m^{FAD}, K_m^{PHB}) apparent Michaelis constants for compounds marked by the upper index.

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Strains. Six strains with ability for active growth on media contained PHB as the sole source of carbon and energy were chosen after screening among several tens of *Rhodococcus* strains: *Rhodococcus opacus* 1G was isolated from soil contaminated with oil by enrichment culture technique. *Rhodococcus opacus* 420 was isolated from forest soil of South Sakhalin and *Rhodococcus opacus* 557 was isolated from the sand of the Kizil-Kum desert. *Rhodococcus rhodnii* 135 was isolated from soil contaminated with diesel oil. *Rhodococcus rhodochrous* 172 was taken from the E. L. Golovlev culture collection (Pushchino, Russia) and *Rhodococcus* sp. 400 was taken from the culture collection of the Institute of Microbiology and Virology, Ukraine (O. Nesterenko, Ukraine) [13].

Culture conditions. The microorganisms were grown at 29°C in 750 ml Ehrlenmeyer flasks containing 220-230 ml of medium. The flasks were shaken at 200 rpm. The culture medium contained per liter: 2.5 g PHB as sole carbon and energy source, 1g Na_2HPO_4, 0.5 g $NH_4H_2PO_4$, 0.75 g NH_4NO_3, 0.1 g $MgSO_4 \cdot 7H_2O$, 5 mg $MnSO_4 \cdot H_2O$, 10 mg $FeSO_4 \cdot 7H_2O$, 0.2 g $NaHCO_3$, 50 mg NaCl, 0.2 g Na_2SO_4, 0.1 g K_2SO_4 and adjusted to pH 7.4. Biomass was collected by centrifugation at 3000*g*, washed with 50 mM phosphate buffer, pH 7.0, and stored at −18°C.

Enzyme purification. All purification steps were performed in 50 mM potassium phosphate buffer, pH 7.0, with 1 mM EDTA, 0.5 mM DTT, and 10 μM FAD, referred to as buffer A. All purification steps were carried out at 2-6°C.

Frozen cell paste was suspended in an equal volume of buffer A and disrupted by Hughes-type press. After treatment with DNase I, the cell extract was clarified by centrifugation for 1 h at 31,000*g*. The supernatant was fractionated with ammonium sulfate (37-65%), and the final precipitate was dissolved in about 100 ml of buffer A and adjusted to 35% saturation with ammonium sulfate. Any precipitate left was removed by centrifugation for 30 min at 31,000*g*. The protein solution was loaded onto a Butyl-Sepharose column equilibrated in buffer A, containing 35% $(NH_4)_2SO_4$, washed with starting buffer, and eluted with a linear descending gradient of 35 to 0% $(NH_4)_2SO_4$ in buffer A. Active fractions were concentrated, centrifuged, and desalted on a Sephadex G-25 superfine column that had been pre-equilibrated with buffer A. The enzyme solution was loaded onto a QAE-Toyopearl column that had been pre-equilibrated in buffer A, washed with two column volumes of starting buffer, and eluted with a linear gradient from 0 to 0.3 M Na_2SO_4 in buffer A. Active fractions were desalted and concentrated and DTT and FAD were added concentrations of 2 mM and 20 μM, respectively, to protect the enzyme. The final enzyme preparation was divided into 0.5 ml aliquots, frozen in liquid nitrogen, and stored at −80°C. An additional purification step on a Blue-Sepharose with a linear gradient from 0 to 1 M NaCl in buffer A was needed with some *Rhodococcus* strains to obtain high-purity enzyme.

Analytical methods. PHBH activity was routinely determined spectrophotometrically by measuring the oxidation of NADH at 340 nm at room temperature (20-22°C). The assay mixture contained 300 μM NADH and 400 μM PHB in air-saturated buffer A. One unit of enzyme activity is defined as the amount that catalyses the oxidation of 1 μmol NADH/min under the assay conditions. For estimation of steady-state kinetic parameters, we used 350 μM NADH for K_m^{PHB} determination and 400 μM PHB for K_m^{NADH} and K_m^{NADPH} determination. For estimation of the pH optimum of enzyme activity and pH optimum of enzyme stability, a mixed buffer containing 20 mM citric acid, 20 mM K_2HPO_4, 20 mM Tris, and 10 μM FAD adjusted to a definite pH value with 2 M KOH was used. Protocatechuate-3,4-dioxygenase (PCADO) activities were determined spectrophotometrically as a decrease in absorbance at 290 nm. To prepare the enzymes for K_m^{FAD} determination, 0.2 ml of 0.3-0.6 mg/ml enzyme solution was loaded onto a 10 ml Biogel P-6 column equilibrated by 50 mM phosphate buffer, pH 7.3, containing 1 mM EDTA, 1 mM DTT, and 200 μM PHB. After this procedure, the enzyme was diluted by 50-100 times in the same buffer containing definite amounts of FAD in the range of 10 nM-10 μM, and activity was measured after 25-30 min by initiating the reaction with NADH (350 μM).

SDS-PAGE was carried out with 10% slab gels. Each sample contained 3-5 μg of protein. Gels were stained with Coomassie brilliant blue R250. Gel filtration analysis for enzyme molecular mass determination was performed with a Pharmacia Äkta System at room temperature (about 23°C) on a Superdex 200 HR10/30 column equilibrated with 50 mM Na_2SO_4, pH 7.0, containing 0.1 M Na_2SO_4. To determine the molecular masses of enzymes in the presence of Tween-80, the column was equilibrated with buffer containing 2% Tween-80 and 5 μM FAD and calibrated with marker proteins in the same buffer. As the standard markers for calibration, we used cytochrome *c* (12.4 kD), chymotrypsinogen A (25 kD), carbonic anhydrase (29 kD), egg albumin (45 kD), albumin, bovine serum (66 kD), alcohol dehydrogenase (150 kD), β-amylase (200 kD), catalase (232 kD), apoferritin (443 kD), thyroglobulin (669 kD), all purchased from Sigma.

RESULTS AND DISCUSSION

Growth of microorganisms. Maximal culture absorbance at the end of logarithmic phase when PHB was used as the sole source of carbon was 1.8-2.0 optical units (at wavelength 545 nm) in the range of initial substrate concentration 2-6 g/liter. An exception was for strain *Rhodococcus rhodnii* 135; these cells formed rapid-

ly precipitating aggregates, and as a result, the absorbance was considerably lower at the end of logarithmic phase (0.8-1). Though the amount of consumed substrate during logarithmic phase did not depend on initial concentration (it was 1.4-1.6 g/liter), active substrate consumption continued, but it did not lead to increased yield of biomass. The growth of the bacteria resulted in acidification of the culture media. The grow rate dropped at pH lower than 6.5.

Enzyme purification. For enzyme purification, cells were harvested near the end of the logarithmic growth phase, when the optical density of the culture of about 1.6-1.7 at 545 nm.

Table 1 summarizes the purification procedure of PHBH from *Rhodococcus opacus* 557; it is typical for all strains used. In general, the enzyme was obtained in pure form after two chromatographic steps. Results of SDS-PAGE of the purified PHBH enzymes from *Rhodococcus opacus* 557, *Rhodococcus opacus* 1G, *Rhodococcus rhodochrous* 172, *Rhodococcus* sp. 400, *Rhodococcus opacus* 420, and *Rhodococcus rhodnii* 135 are shown in Fig. 1. In all cases, a single band was observed corresponding to an apparent subunit molecular mass of 45-51 kD. Most known PHBH have similar subunit size: *P. aeruginosa* (45 kD) [3], *P. fluorescens* (44 kD) [14], *Pseudomonas* sp. CBS3 (44 kD) [4], *Acinetobacter calcoaceticus* (45 kD) [6], *Corynebacterium cyclohexanicum* (47 kD) [15].

With all *Rhodococcus* strains, the activity of PCADO—the second enzyme in the PHB degradation pathway—was observed. Spectral characteristics of reaction product and enzymes and substrate specificity indicate that the enzymes are of the intradiol type. Purified PCADO displayed a typical absorption spectrum for Fe^{3+}-containing intradiol dioxygenases with a broad band in the visible region and a λ_{max} of about 450 nm [16, 17]. Intradiol PCADOs split the aromatic ring between the third and fourth atoms while incorporating both atoms from the oxygen molecule into the substrate. PCADO from *Rhodococcus rhodnii* 135 was purified to homogeneity. As established by SDS-PAGE analysis, this enzyme consists of two different subunits with molecular mass 23 and 33 kD, but if the sample is not heated above 30°C before loading on the polyacrylamide gel, a single band was observed corresponding to molecular weight about 110-120 kD. If sample was heated for 5 min at 50°C, three bonds corresponding to molecular weight of 110, 33, and 23 kD appear. Gel filtration analysis showed 420 kD mass for this enzyme. Thus, the PCADO from *Rhodococcus rhodnii* 135 probably has $(\alpha_2\beta_2)_4$ composition, which is typical for this type of enzymes [18, 19].

Hydrodynamic properties. The native *Rhodococcus* PHBH enzymes eluted from a Superdex 200 column as symmetrical peaks with apparent molecular mass of 95-100 kD, indicating that all the purified enzymes are homodimers with approximately the same size. Minor peaks near 200 kD were observed in the case of *Rhodococcus opacus* 557, *Rhodococcus opacus* 1G, *Rhodococcus opacus* 420 (3-5% with respect to the main peak), and *Rhodococcus rhodnii* 135 (8%), this suggesting the possible ability of the enzyme to form a tetrameric structure. A similar dimeric structure was observed for PHBH from *Pseudomonas* [3, 4, 14] and *Acinetobacter calcoaceticus* [6], but PHBH from *Corynebacterium cyclohexanicum* is a monomer [15]. Therefore, all the studied PHBHs have subunit composition and mass typical for such enzymes.

The ability to dissociate into monomers in the presence of Tween-80 while retaining 50% activity was revealed for membrane-associated PHBH from *Rhodococcus erythropolis* S-1, which exists as a dimer at 30°C [20]. We checked the influence of this detergent on our enzymes and found that Tween-80 in the concentration range 0-4% had no effect on activity and stability of the isolated enzymes. Furthermore, gel filtration showed that at least two of the enzymes (from *Rhodococcus rhodnii* 135 and *Rhodococcus opacus* 557) retained their subunit structure at 2% Tween-80.

Spectral properties. All of the isolated PHBHs have absorption spectra typical for flavoproteins. The spectral characteristics of the PHBHs in the UV and visible regions are presented in Table 2. Considering the spectral properties of the enzymes, we confirmed that the active center of the PHBHs from *Rhodococcus* differs from the

Table 1. Purification scheme of *p*-hydroxybenzoate hydroxylase from *Rhodococcus opacus* 557 (120 g wet cells)

Step	Volume, ml	Total protein, mg	Total activity, U	Specific activity, U/mg	Yield, %
Cell-free extract	250	3500	1700	0.49	100
Fractionation with $(NH_4)_2SO_4$	150	2300	1750	0.76	103
Chromatography on Butyl-Sepharose	80	550	1720	3.13	101
Chromatography on QAE-Toyopearl	50	62	1550	25.0	91

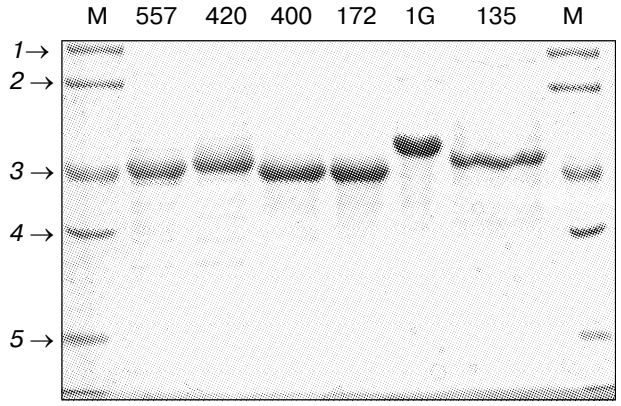

Fig. 1. SDS-PAGE of purified PHBH from strains *Rhodococcus opacus* 557 (557), *Rhodococcus opacus* 420 (420), *Rhodococcus* sp. 400 (400), *Rhodococcus rhodochrous* 172 (172), *Rhodococcus opacus* 1G (1G), *Rhodococcus rhodnii* 135 (135). Marker proteins (M): *1*) phosphorylase *b* (94 kD); *2*) bovine serum albumin (67 kD); *3*) ovalbumin (43 kD); *4*) carbonic anhydrase (30 kD); *5*) trypsin inhibitor (20.1 kD).

active centers of *Pseudomonas* enzymes. Four studied enzymes have a significant shift of maximum in the visible region to shorter wavelength in comparison with the spectrum of free FAD and the enzymes from *P. fluorescens* and *P. aeruginosa* [2, 3]. Thus, the PHBHs from *Pseudomonas* have maximum absorption at 449-450 nm (close to the maximum absorbance of free FAD). Three of our enzymes have maximum at about 440 nm, and one has maximum at 446 nm. Maximum absorbance at 440 nm was not observed before for any PHBH or its mutant forms, but Arg44-Lys mutant PHBH from *Pseudomonas fluorescens* [21] has maximum at 445 nm, this being comparable to the enzyme from *Rhodococcus rhodnii* 135.

Enzyme stability. All of the *Rhodococcus* PHBH enzymes rapidly lost activity in dilute solutions (<2 mg/ml) without stabilizers such as EDTA and DTT. In the presence of such compounds, and also in the presence of FAD, concentrated enzyme solutions (>10 mg/ml) were much more stable. The most effective stabilizer is DTT. Furthermore, addition of a considerable amount (~5 mM) of fresh DTT to enzyme solution which lost up to 30-40% of activity during storage restores a considerable part of the lost activity, this indicating the on presence of easily accessible reversibly oxidizable groups like cysteine. When stability was studied as a function of pH, most of the enzymes were found to have broad optimum stability in the range 6.5-9.5. The enzyme from *Rhodococcus opacus* 1G was an exception: it has optimum at pH 9-9.5 and a shoulder at 7-8. Also, the enzyme from *Rhodococcus* sp. 400 has optimum at 7-7.5 and a shoulder at 9-9.5. During incubation of enzymes at 50°C and pH 7.2, the PHBHs were inactivated rather fast except for the enzyme from *Rhodococcus rhodnii* 135 (Table 2), stability of this enzyme being comparable with the stability of the PHBH from *Pseudomonas fluorescens* [23]. The enzyme from *Rhodococcus opacus* 1G was the most labile, which might be explained by the fact that its optimum stability if far from 7.2. PHB considerably protected the *Rhodococcus* enzymes from heat denaturation (Table 2); such effects have been noted for similar enzymes [3, 6, 15, 22, 23].

Kinetic properties of the enzymes. HPLC analysis of product, optical absorbance measurements of NADH consumption, and measurements of oxygen consumption indicated that the PHBHs from the *Rhodococcus* strains catalyze the conversion of PHB to PCA with the consumption of stoichiometric amounts of NAD(P)H and oxygen. With all of the enzymes, NADH and NADPH were oxidized with comparable rates. With all *Rhodococcus* PHBH enzymes, no NAD(P)H oxidase activity was detectable in the absence of PHB, confirming the effector role of the aromatic substrate. Removing FAD from the active center of the enzymes leads to loss of 95-97% of the activity. The apoenzymes retain dimeric structure, and their activities are restored after addition of

Table 2. Some properties of PHBH enzymes from *Rhodococcus* strains

Strain	Subunit mass, kD	Half-inactivation time (min) at 50°C		A_{271}/A_{442}	λ_{max}, nm
		without PHB	PHB added		
R. rhodochrous 172	45	50	90		
R. opacus 1G	51	9	30	7	270; 370; 441
Rhodococcus sp. 400	45	37	65	6.2	270; 371; 440
R. opacus 420	47.5	15	40		
R. rhodnii 135	48.5	180	250	8.9	273; 370.5; 446
R. opacus 557	46	45	85	7.3	272; 370.5; 440

Table 3. Comparison of kinetic parameters of PHBH isolated from *Rhodococcus* strains with analogous enzymes from other microorganisms

Strain	pH optimum of activity	k_{cat}, min^{-1}	K_m^{PHB}, μM	K_m^{NADH}, μM	K_m^{NADPH}, μM	K_m^{FAD}, nM
R. rhodochrous 172	7.0	1500	7.2 ± 0.4	16.7 ± 0.5	22.6 ± 2.4	420 ± 32
R. opacus 1G	7.4	2700	7.9 ± 0.4	19.5 ± 1.5	23.8 ± 3.0	185 ± 25
Rhodococcus sp. 400	7.4	2200	3.3 ± 0.3	13.9 ± 1.1	23.3 ± 0.8	440 ± 42
R. opacus 420	7.2	3000	8.1 ± 0.4	20.0 ± 1.0	65.8 ± 5.4	220 ± 15
R. rhodnii 135	7.7	1300	7.3 ± 0.9	39.8 ± 1.3	155 ± 12	225 ± 25
R. opacus 557	7.2	2500	3.0 ± 0.6	12.2 ± 1.0	33.5 ± 1.4	190 ± 32
Acinetobacter calcoaceticus [6]	8.0	3340	41	—	?	?
Pseudomonas fluorescens [25]	8.0	3300	11	—	26	45 [24]
Pseudomonas aeruginosa [3]	8.0	3750	10.9	—	23.2	5*
Pseudomonas desmolytica IAM 1123 [5]	8.0	2700	25	—	40	?
Pseudomonas sp. CBS3 [4]	8.0	900	75	80	140	150
Corynebacterium cyclohexanicum [15]	7.8	1660	35	31	63	?
Rhodococcus erythropolis S-1 [12]	8.4	2140	70	130	—	?

Note: The molecular mass for the PHBH isolated from *Rhodococcus* was taken as 100 kD.
* Shown K_d value; —, no activity; ?, no data.

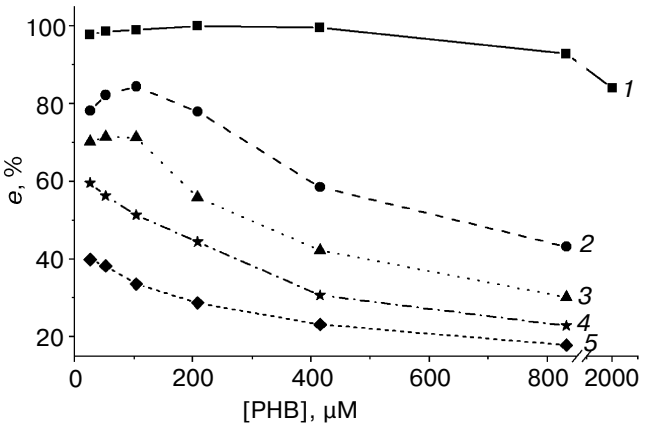

Fig. 2. Increasing of substrate inhibition in the presence of chloride illustrated with the enzyme from *Rhodococcus opacus* 557. Buffer for measurements (50 mM phosphate, pH 7.4) contained 150 μM NADH and 10 μM FAD. Concentration of KCl (mM): *1*) 0; *2*) 5.2; *3*) 10.4; *4*) 21; *5*) 42.

free FAD, but not FMN, these results indicating that the *Rhodococcus* PHBH enzymes contain FAD as a weakly bound prosthetic group. The main kinetic parameters for the purified enzymes and for PHBH from other microorganisms given for comparison are given in Table 3. The described enzymes quite similar in properties, and only the PHBH from *Rhodococcus rhodnii* 135 has notable differences from the other enzymes. For all of the enzymes the K_m^{NADPH} value was higher than the K_m^{NADH} value. Common features differentiating the isolated enzymes from other known PHBHs are somewhat lower pH values for optimum activity, lower K_m^{PHB} values, and higher K_m^{FAD} values. The PHBH from the gram-positive microorganism *Corynebacterium cyclohexanicum* has kinetic properties most similar to our enzymes. Properties of the PHBH from *Pseudomonas* sp. CBS3, an enzyme from a gram-negative microorganism, can use NADH as well as NADPH, like the PHBH from *R. rhodnii* 135, but has one order of magnitude higher K_m^{PHB} value. Interestingly, the PHBH from *Rhodococcus erythropolis* S-1, which uses only NADH, differs most significantly from our *Rhodococcus* enzymes.

Inhibition of the enzymes. The activities of all the isolated *Rhodococcus* PHBHs, like PHBHs from other strains, were inhibited by chloride. However, in contrast to the competitive type of inhibition by chloride with respect to NADPH for the PHBH enzymes from *Pseudomonas* [4, 22, 26], uncompetitive inhibition was found for the enzymes from *Acinetobacter calcoaceticus* and *Corynebacterium cyclohexanicum* [6, 15], and mixed-type inhibition was observed for the enzymes from *Rhodococcus rhodnii* 135, *R. opacus* 557, and *R. opacus* 1G strains with respect to NADH.

For all of the enzymes, substrate inhibition was observed at PHB concentrations above 600-700 μM. In the presence of chloride, substrate inhibition became more pronounced, and the inhibiting concentration of substrate was reduced for all PHBH except for the enzyme from *Rhodococcus rhodnii* 135 (Fig. 2). This property is unique for such enzymes. As known, monovalent ions can affect the binding of NAD(P)H, increasing the lifetime some flavin intermediates in catalytic cycle. Further studies of these properties may help to elucidate the mechanism of substrate inhibition of these enzymes.

Our results have shown that the PHBHs isolated from *Rhodococcus* strains have a number of properties characteristic of PHBHs. However, the spectral and some kinetic properties show significant distinctions in the structure of the active center of the PHBHs from *Rhodococcus* and enzymes from most gram-negative microorganisms. Further investigation of some enzymes presented in this paper might be useful for understanding the catalytic features of this type of enzyme.

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