Biocatalytic Potential of *p*-Hydroxybenzoate Hydroxylase from *Rhodococcus rhodnii* 135 and *Rhodococcus opacus* 557

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Received: September 9, 2003; Accepted: January 9, 2004

Abstract: The biocatalytic potential of the NADH-dependent *p*-hydroxybenzoate hydroxylases (PHBH) from *Rhodococcus rhodnii* 135 and *Rhodococcus opacus* 557 was investigated. Monofluorinated 4-hydroxybenzoates were efficiently hydroxylated, albeit at different rates. 2-Fluoro-4-hydroxybenzoate was a true substrate for PHBH from *R. rhodnii* 135 but a substrate inhibitor for PHBH from *R. opacus* 557. Monochlorinated 4-hydroxybenzoates also acted as PHBH substrates, but with these compounds strong uncoupling of hydroxylation (formation of hydrogen peroxide) occurred. PHBH from *R. rhodnii* 135 preferred the 5′-hydroxylation of 2-chloro-4-hydroxybenzoate but the enzyme from *R. opacus* 557 favored the formation of 2-chloro-3,4-dihydroxybenzoate. Conversely, PHBH from *R. rhodnii* 135 regioselectively hydroxylated 2-fluoro-4-hydroxybenzoate to 2-fluoro-3,4-dihydroxybenzoate whereas the enzyme from *R. opacus* 557 also produced significant amounts of 2-fluoro-4,5-dihydroxybenzoate. At high NADH/substrate ratio, both 2-fluorodihydroxybenzoate products were further converted to 2-fluoro-3,4,5-trihydroxybenzoate. PHBH from *R. rhodnii* 135 and *R. opacus* 557 preferred the 5′-hydroxylation of 3-chloro-4-hydroxybenzoate. However, conversion of 3-fluoro-4-hydroxybenzoate involved considerable dehalogenation affording nearly equal amounts of 3,4-dihydroxybenzoate and 5-fluoro-3,4-dihydroxybenzoate. At high NADH/substrate ratio, the latter compound was further converted to 3,4,5-trihydroxybenzoate. The results are discussed in relation to the properties of the NADPH-specific PHBH from *Pseudomonas fluorescens.*

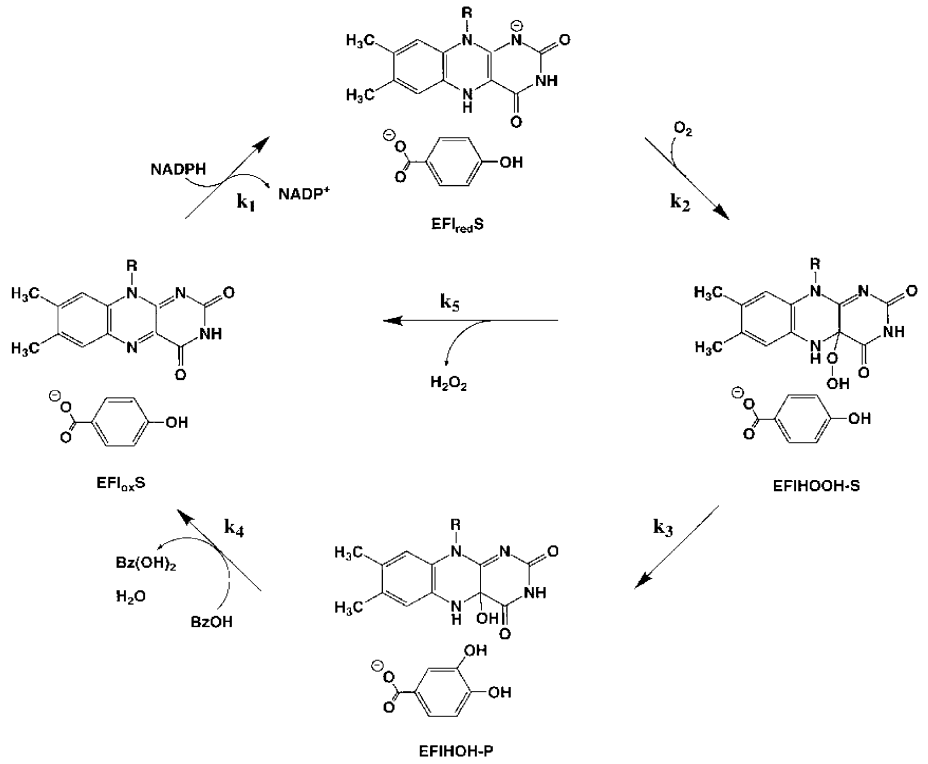
Keywords: coenzyme specificity; flavoprotein monooxygenase; ^{19}F NMR; *p*-hydroxybenzoate hydroxylase; oxidative dehalogenation; regioselectivity

Introduction

p-Hydroxybenzoate 3-hydroxylase (PHBH) (EC 1.14.13.2) is a flavoprotein monooxygenase that catalyses the conversion of 4-hydroxybenzoate into 3,4-dihydroxybenzoate in the presence of NAD(P)H and molecular oxygen.[1,2] The inducible enzyme is responsible for channeling 4-hydroxybenzoate *via* 3,4-dihydroxybenzoate into the β-ketoadipate pathway of microbial aromatic degradation.[3,4] PHBH and related monooxygenases are of potential interest for biocatalytic applications, since these enzymes are able to perform regioselective and stereoselective oxidations under mild and environmentally friendly conditions.[5,6] PHBH has been isolated from several microorganisms, but the strictly NADPH-dependent enzymes from *Pseudomonas fluorescens* and *P. aeruginosa* have received most attention.[1,7,8] The crystal structure of *Pseudomonas* PHBH is known in atomic detail[9–12] and the mechanism of enzyme catalysis has been thoroughly explored.[1,8,13]

Scheme 1 shows the reaction cycle of PHBH. In the reductive half-reaction, the protein-bound FAD is reduced by NADPH. This reaction is highly stimulated in the presence of the aromatic substrate, which acts as an effector. In the oxidative half-reaction, the reduced flavin reacts with oxygen resulting in the formation of a transiently stable flavin hydroperoxide oxygenating species.[13] This electrophilic flavin adduct is responsible for the regioselective hydroxylation of aromatic substrates, a reaction difficult to achieve by chemical means.[14] In the absence of substrate or in the presence of non-substrate effectors, the flavin hydroperoxide decays to oxidized enzyme with release of hydrogen peroxide.[1] Studies of PHBH mutant enzymes showed that the replacement of tyrosine residues involved in substrate activation leads to strong uncoupling of

 DOI: 10.1002/adsc.200303146



Scheme 1. Reaction cycle of *p*-hydroxybenzoate hydroxylase from *P. fluorescens*.[1] BzOH: 4-hydroxybenzoate; $Bz(OH)_2$: 3,4-dihydroxybenzoate; k_1: reduction of enzyme-substrate complex; k_2: formation of flavin hydroperoxide; k_3: substrate hydroxylation; k_4: dehydration of flavin hydroxide and product release; k_5: uncoupling of hydroxylation.

hydroxylation and the formation of hydrogen peroxide.[15,16] Furthermore, it was established that the regioselectivity of hydroxylation of PHBH substrates can be modulated by amino acid substitutions in the substrate binding pocket.[1,8,17–19]

So far, only a few studies have been devoted to PHBH enzymes from Gram-positive microorganisms. Without exception, these enzymes are more specific for NADH,[20,21] a property of considerable interest for biotechnological applications.[5,7,22–26] Studies on PHBH from *Rhodococcus erythropolis* mainly concentrated on protein stability and oligomerization properties, rather than biocatalytic features.[21,27,28] In order to provide more insight into the biocatalytic potential of PHBH from Gram-positive microorganisms, we recently purified several new PHBH enzymes from *Rhodococcus* strains.[29] Here we report on the substrate specificity and regioselectivity of hydroxylation of PHBH from *Rhodococcus rhodnii* 135 and *Rhodococcus opacus* 557. It is shown that these actinobacterial enzymes differ in several catalytic features from each other and from their proteobacterial counterparts.

Results and Discussion

Substrate Specificity

PHBH from *R. rhodnii* 135 and *R. opacus* 557 showed hardly any NADH oxidase activity when assayed in the absence of aromatic compounds. A limited number of substituted 4-hydroxybenzoates strongly stimulated the rate of NADH oxidation. Table 1 shows that the highest rates of catalysis occurred with the parent substrate 4-hydroxybenzoate and with monofluorinated 4-hydroxybenzoates. Lower turnover rates were observed for the reactions with monochlorinated substrate analogues and with 4-hydroxybenzoates bearing an additional hydroxy group (Table 1). It should be stressed here that the estimated turnover rates do not necessarily reflect the rate of the actual substrate hydroxylation step. For PHBH from *P. fluorescens* it was established that with several substrate analogues, among which was 2,4-dihydroxybenzoate, the rate of flavin reduction is rate-limiting in overall catalysis.[13,16] Nevertheless, the steady state kinetic data presented here suggest that both electronic and steric effects are involved in determining the turnover rate. In this respect, it is interesting to note that both *Rhodococcus* enzymes have lower Michaelis constants (K_m values) for *ortho*-substituted 4-hydroxy-

Table 1. Kinetic parameters of PHBH from *R. rhodnii* 135 and *R. opacus* 557. All kinetic constants have a maximal error of 10%.

Substrate/effector	*Rhodococcus rhodnii 135*			*Rhodococcus opacus 557*		
	k_{cat} [min^{-1}][a]	K_m [μM]	k_{cat}/K_m [min^{-1} μM^{-1}]	k_{cat} [min^{-1}][a]	K_m [μM]	k_{cat}/K_m [min^{-1} μM^{-1}]
4-hydroxybenzoate	1300	7	186	2500	3	833
2-fluoro-4-hydroxybenzoate	1050	5	210	1100	0.7	1571
3-fluoro-4-hydroxybenzoate	1100	10	110	1600	8	200
2-chloro-4-hydroxybenzoate	190	3.5	54	510	0.9	567
3-chloro-4-hydroxybenzoate	47	70	0.7	380	14	27
2,4-dihydroxybenzoate	15	~1	15	200	8	25
3,4-dihydroxybenzoate	26	32	0.8	280	215	1.3

[a] NADH oxidation rate.

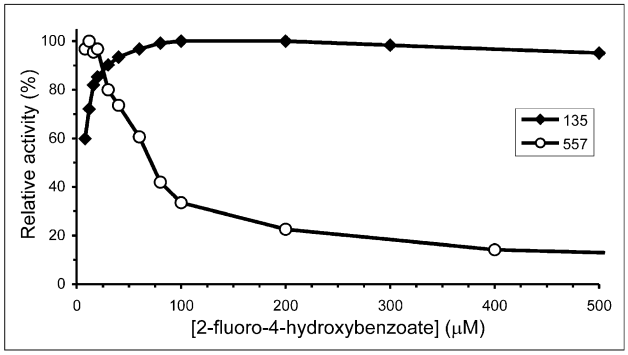


Figure 1. Relative activity of PHBH from *R. rhodnii* 135 (135) and *R. opacus* 557 (557) with 2-fluoro-4-hydroxybenzoate in dependence on the concentration of haloaromatic substrate.

benzoates than for *meta*-substituted 4-hydroxybenzoates.

A main difference in kinetic behavior of the *Rhodococcus* enzymes was found in the reaction with 2-fluoro-4-hydroxybenzoate. In analogy with the properties of PHBH from *P. fluorescens*,[30] this compound appeared to be a strong substrate inhibitor of PHBH from *R. opacus* 557. As can be seen from Figure 1, inhibition of the *R. opacus* 557 enzyme occurred at substrate concentrations as low as 20–30 μM and is most likely due to the stabilization of a complex between the fluorinated substrate and the reduced enzyme-hydroxyflavin intermediate.[5,30] Intriguingly, with PHBH from *R. rhodnii* 135, hardly any substrate inhibition with 2-fluoro-4-hydroxybenzoate occurred (Fig. 1), pointing at a rapid release of water from the hydroxyflavin with regeneration of the oxidized enzyme.

Possible uncoupling of substrate hydroxylation (formation of hydrogen peroxide) was analyzed by oxygen consumption experiments, performed either in the absence and presence of catalase. These experiments revealed that 4-hydroxybenzoate, 2-fluoro-4-hydroxybenzoate, 3-fluoro-4-hydroxybenzoate and 2,4-dihydroxybenzoate were true substrates, showing tight coupling of NADH reduction to substrate hydroxylation (Table 2). 2-Chloro-4-hydroxybenzoate and 3-chloro-4-hydroxybenzoate were also converted by *Rhodococcus* PHBH, but with both these chlorinated compounds the efficiency of hydroxylation was rather low (Table 2). 3,4-Dihydroxybenzoate, the product of the enzymatic reaction with the parent substrate, was a competent non-substrate effector, especially for PHBH from *R. opacus* 557 (Tables 1 and 2). The substrate and effector specificities of the *Rhodococcus* PHBH enzymes are in line with the corresponding properties of PHBH from *P. fluorescens*[1,18] and suggest that the active sites of these actinobacterial and proteobacterial enzymes are highly conserved.

Regioselectivity of Hydroxylation of 2,4-Dihydroxybenzoate

HPLC analysis confirmed that both *Rhodococcus* enzymes catalyzed the hydroxylation of 2,4-dihydroxybenzoate with more than 95% efficiency (Table 2). Moreover, one major product with the same retention time as 2,3,4-trihydroxybenzoate was observed (Fig. 2; Table 2). The identity of this trihydroxylated product was confirmed by ^{1}H NMR and checked with the reference spectrum of 2,3,4-trihydroxybenzoic acid at http://www.sigmaaldrich.com. Formation of 2,3,4-trihydroxybenzoate from 2,4-dihydroxybenzoate was also reported for wild-type PHBH from *P. fluorescens*.[13] However, for the Ser212Ala variant of this enzyme, the reaction with 2,4-dihydroxybenzoate was less regioselective and about equal amounts of 2,3,4-trihydroxybenzoate and 2,4,5-trihydroxybenzoate were formed.[17,18] From this it was concluded that the removal of Ser212, involved in binding the carboxylic moiety of the substrate (Fig. 3), creates a favorable hole to accommodate the 2′-hydroxy group of 2,4-dihydroxybenzoate in the flipped orientation with the 2′-substituent pointing away from the isoalloxazine ring of the FAD.

Table 2. Efficiency and regioselectivity of hydroxylation of PHBH from *R. rhodnii* 135 and *R. opacus* 557.

Substrate	*Rhodococcus rhodnii* 135		*Rhodococcus opacus* 557	
	Product [%][a]	C3:C5 ratio[b]	Product [%][a]	C3:C5 ratio[b]
4-hydroxybenzoate	98 ± 2	–	98 ± 2	–
2-fluoro-4-hydroxybenzoate	92 ± 2	100 : 0	92 ± 2	85 : 15
3-fluoro-4-hydroxybenzoate	92 ± 3	46 : 54	93 ± 2	52 : 48
2-chloro-4-hydroxybenzoate	35 ± 1	40 : 60	20 ± 2	77 : 23
3-chloro-4-hydroxybenzoate	8 ± 1	28 : 72	4 ± 1	6 : 94
2,4-dihydroxybenzoate	97 ± 2	100 : 0	97 ± 2	100 : 0
3,4-dihydroxybenzoate	< 1	–	< 1	–

[a] Average hydroxylation efficiency measured by three methods (see Experimental Section).
[b] Regiospecificity of hydroxylation, C3/C5 ratio.

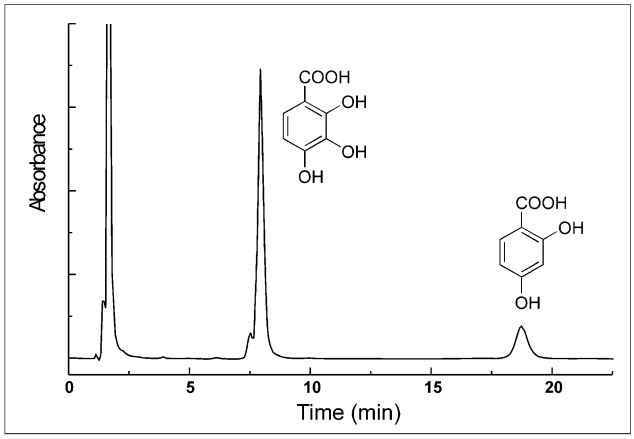


Figure 2. HPLC product analysis of the reaction of PHBH from *R. opacus* 557 with 2,4-dihydroxybenzoate. The concentrations of 2,4-dihydroxybenzoate and NADH were 300 μM. Detection was at 260 nm. The product was identified as 2,3,4-trihydroxybenzoate from running reference compounds in parallel and ^{1}H NMR (see Experimental Section).

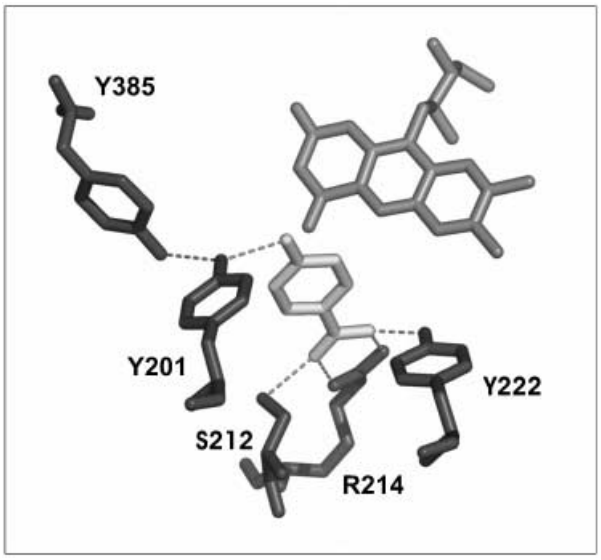


Figure 3. Substrate binding pocket of wild-type PHBH from *P. fluorescens*.[10] The aromatic substrate is light gray, the isoalloxazine ring of FAD is dark gray and the protein residues are black.

Regioselectivity of Hydroxylation of Monochlorinated Substrates

PHBH from *R. rhodnii* 135 and *R. opacus* 557 showed significant differences with respect to the regioselectivity of hydroxylation of 2-chloro-4-hydroxybenzoate. HPLC and MS analysis revealed that PHBH from *R. rhodnii* 135 mainly converted this compound to 2-chloro-4,5-dihydroxybenzoate, whereas the enzyme from *R. opacus* 557 was more specific for the production of 2-chloro-3,4-dihydroxybenzoate (Fig. 4, Table 2). In MS analysis, both aromatic products showed main peaks at m/z $(M^+) = 188$ and m/z $(M-OH)^+ = 171$, containing 1 chlorine atom. Different regioselectivities for the hydroxylation of 2-chloro-4-hydroxybenzoate were also observed for wild-type and several mutant enzymes of PHBH from *P. fluorescens*.[18] Again, as for the conversion of 2,4-dihydroxybenzoate, it was noted that the ratio of C3:C5 hydroxylation is linked to the interactions between the protein and the carboxylic moiety of the substrate. Experiments performed at high NADH/substrate ratio (Fig. 5B) revealed that 2-chloro-3,4-dihydroxybenzoate and 2-chloro-4,5-dihydroxybenzoate were not further converted to 2-chloro-3,4,5-trihydroxybenzoate.

Conversion of 3-chloro-4-hydroxybenzoate by *Rhodococcus* PHBH resulted in the formation of 3-chloro-4,5-dihydroxybenzoate (Fig. 5A). As can be seen from Figure 5B, with both *Rhodococcus* enzymes some 3,4-dihydroxybenzoate was produced as well. Figure 5B also shows that PHBH from *R. rhodnii* 135 was more effective than the enzyme from *R. opacus* 557 in converting 3-chloro-4-hydroxybenzoate. Because 3,4-dihydroxybenzoate is a rather strong effector for PHBH from *R. opacus* 557 (cf. Table 1), the incomplete conversion of 3-chloro-4-hydroxybenzoate by this enzyme at high NADH/substrate ratio (Fig. 5B) is partly due to the competing NADH oxidase activity with the

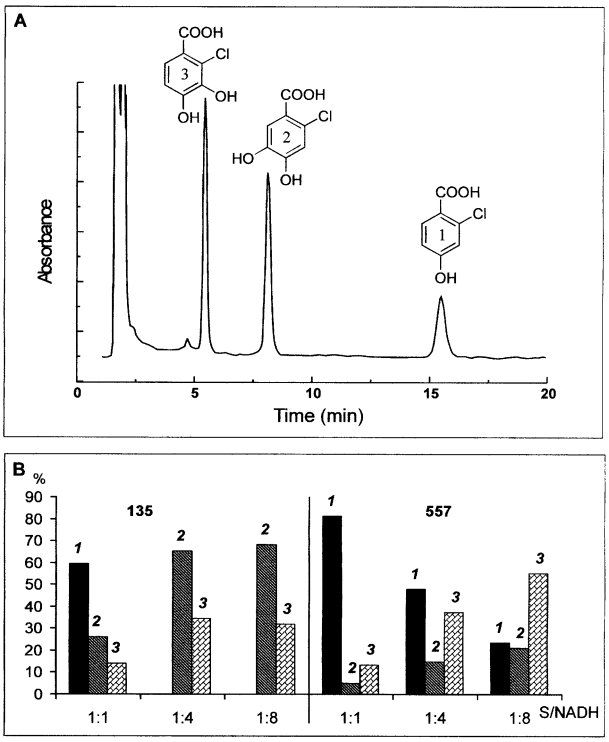


Figure 4. HPLC product analysis of the reactions of PHBH from *R. rhodnii* 135 (135) and *R. opacus* 557 (557) with 2-chloro-4-hydroxybenzoate. The concentration of 2-chloro-4-hydroxybenzoate was 200 μM in all experiments. The substrate/NADH ratio varied from 1:1 to 1:8. Detection was at 260 nm. (**A**) HPLC chromatogram of the reaction of PHBH from *R. opacus* 557 with 2-chloro-4-hydroxybenzoate at substrate/NADH ratio 1:4. (**B**) Diagram showing the percentage content of substrate (***1***) and reaction products (***2***, ***3***) at the end of the reactions.

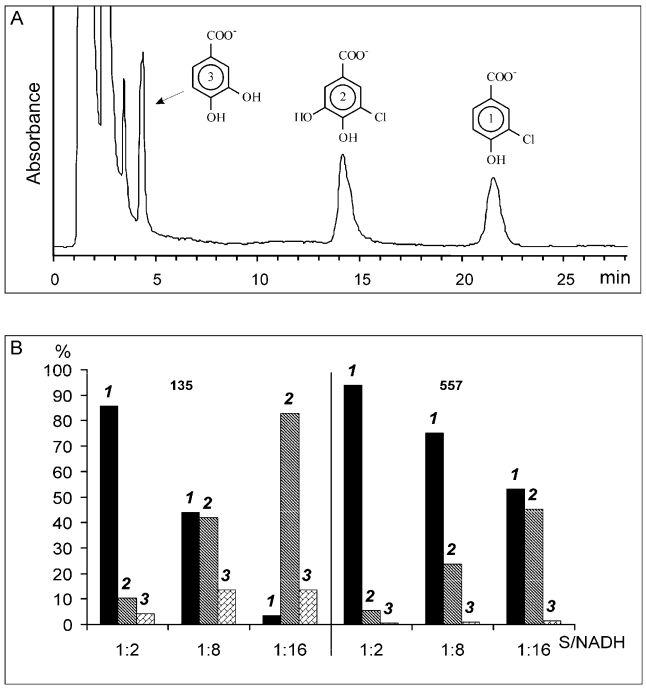


Figure 5. HPLC product analysis of the reactions of PHBH from *R. rhodnii* 135 (135) and *R. opacus* 557 (557) with 3-chloro-4-hydroxybenzoate. The concentration of 3-chloro-4-hydroxybenzoate was 150 μM in all experiments. The substrate/NADH ratio varied from 1:2 to 1:16. Detection was at 260 nm. (**A**) HPLC chromatogram of the reaction of PHBH from *R. rhodnii* 135 with 3-chloro-4-hydroxybenzoate at substrate/NADH ratio 1:8. (**B**) Diagram showing the percentage content of substrate (***1***) and reaction products (***2***, ***3***) at the end of the reactions.

3,4-dihydroxybenzoate product. Furthermore, at high NADH/substrate ratio, no oxidative dehalogenation of 3-chloro-4,5-dihydroxybenzoate to 3,4,5-trihydroxybenzoate was observed.

Regioselectivity of Hydroxylation of Monofluorinated Substrates

In analogy to the reactions with monochlorinated substrates, the PHBH-mediated conversion of monofluorinated 4-hydroxybenzoates may result in two different *ortho*-hydroxylation products.[31] ^{19}F NMR product analysis showed that conversion of 2-fluoro-4-hydroxybenzoate by *Rhodococcus* PHBH mainly resulted in the production of 2-fluoro-3,4-dihydroxybenzoate (Fig. 6). With PHBH from *R. rhodnii* 135, no other fluorinated aromatic products were formed but significant amounts of fluoride anion were released (Fig. 6B). HPLC product analysis established that the formation of fluoride anion was not due to the potential enzymatic oxidative dehalogenation of 2-fluoro-3,4-dihydroxybenzoate to 2,3,4-trihydroxybenzoate. When the conversion of 2-fluoro-4-hydroxybenzoate by PHBH from *R. rhodnii* 135 was performed in the presence of 1 mM ascorbate almost no fluoride anion was released and only a small trace of 2-fluoro-3,4,5-trihydroxybenzoate was observed (not shown). This supports an earlier finding that in the absence of ascorbate, considerable non-enzymatic oxidation of fluorinated intermediate products occurs.[19]

PHBH from *R. opacus* 557 was less specific for C3 hydroxylation of 2-fluoro-4-hydroxybenzoate. With this enzyme, significant amounts of 2-fluoro-4,5-hydroxybenzoate were formed (Fig. 6). Conversion at high NADH/substrate ratio resulted in the decrease of both dihydroxylated products and increase of fluoride anion (Fig. 6). When this reaction was performed in the presence of ascorbate, considerable amounts of 2-fluoro-3,4,5-trihydroxybenzoate and less fluoride anion were produced. This is in line with the above notion that ascorbate inhibits the formation of non-enzymatic oxidation products.

^{19}F NMR analysis indicated that both *Rhodococcus* PHBH enzymes formed two products from 3-fluoro-4-hydroxybenzoate (Fig. 7). Accumulation of fluoride

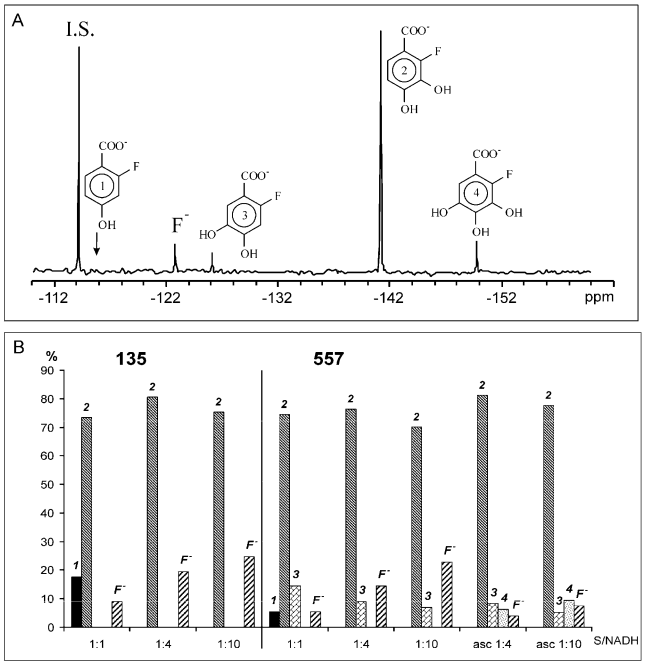


Figure 6. ^{19}F NMR product analysis of the reactions of PHBH from *R. rhodnii* 135 (135) and *R. opacus* 557 (557) with 2-fluoro-4-hydroxybenzoate. The concentration of 2-fluoro-4-hydroxybenzoate was 250 μM in all experiments. The substrate/NADH ratio varied from 1:1 to 1:10. (**A**) ^{19}F NMR spectrum of the reaction of PHBH from *R. opacus* 557 with 2-fluoro-4-hydroxybenzoate at substrate/NADH ratio 1:10 in the presence of ascorbate. Note that the substrate is not present anymore but that its position is marked. (**B**) Diagram showing the percentage content of substrate (***1***) and reaction products (***2***, ***3***, ***4*** and F^-) at the end of the reactions.

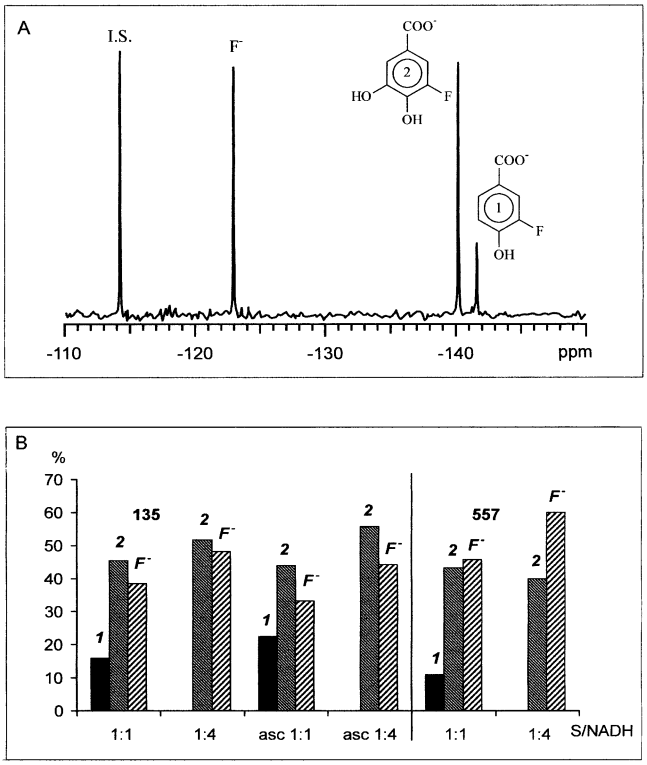


Figure 7. ^{19}F NMR product analysis of the reactions of PHBH from *R. rhodnii* 135 (135) and *R. opacus* 557 (557) with 3-fluoro-4-hydroxybenzoate. The concentration of 3-fluoro-4-hydroxybenzoate was 250 μM in all experiments. The substrate/NADH ratio varied from 1:1 to 1:4. (**A**) ^{19}F NMR spectrum of the reaction of PHBH from *R. rhodnii* 135 with 3-fluoro-4-hydroxybenzoate at substrate/NADH ratio 1:1. (**B**) Diagram showing the percentage content of substrate (***1***) and reaction products (***2*** and F^-) at the end of the reactions.

anion was taken as evidence for oxidative dehalogenation at C3, leading to 3,4-dihydroxybenzoate. This was confirmed by HPLC analysis, which showed that in the presence of equimolar amounts of NADH and aromatic substrate, conversion of 3-fluoro-4-hydroxybenzoate by PHBH from *R. opacus* 557 resulted in the formation of nearly equal amounts of 3,4-dihydroxybenzoate and 5-fluoro-3,4-dihydroxybenzoate (Fig. 8). Because formation of fluoride anion may also originate from the further hydroxylation of 5-fluoro-3,4-dihydroxybenzoate, enzymatic conversion of 3-fluoro-4-hydroxybenzoate also was studied at higher NADH/substrate ratios. When NADH was added in 4-fold excess, with PHBH from *R. rhodnii* 135 hardly any change in 5-fluoro-3,4-dihydroxybenzoate/fluoride anion product ratio occurred (Fig. 7B). The presence of ascorbate only slightly influenced the amount of free fluoride formed, indicating that defluorination was not due to non-enzymatic oxidation of quinone intermediates. With PHBH from *R. opacus* 557 and a 4-fold excess of NADH, the amount of fluoride anion increased at the expense of 5-fluoro-3,4-dihydroxybenzoate (Fig. 7B). HPLC analysis showed the formation of a small amount of 3,4,5-trihydroxybenzoate (Fig. 8) which increased to about 10% of total product when the substrate/NADH ratio was increased to 1:10 (not shown).

Conclusions

In this paper we have described the activity and regioselectivity of two newly characterized PHBH enzymes from *Rhodococcus* species. Because of their preference for NADH as electron donor, these flavoenzymes may develop as useful biocatalysts for the synthesis of commercially unavailable dihydroxybenzoates. Besides serving as synthons and antioxidants, these compounds could be developed into useful drugs for treating infections.[32]

Monofluorinated 4-hydroxybenzoates were good substrates for *Rhodococcus* PHBH with tight coupling of NADH consumption to product formation. PHBH from *R. rhodnii* 135 turned out to be especially useful for the regioselective synthesis of 2-fluoro-3,4-dihydroxyben-

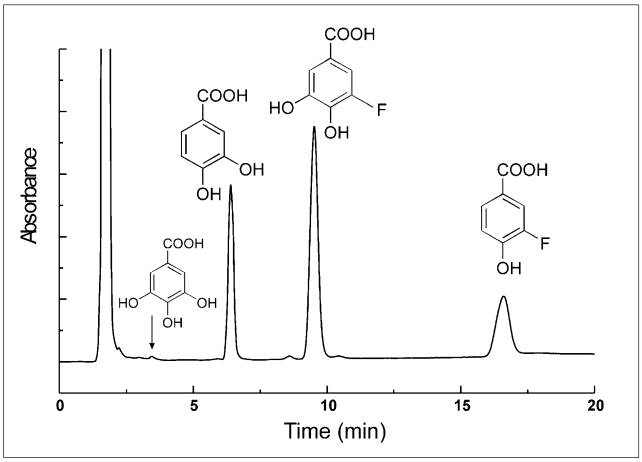


Figure 8. HPLC product analysis of the reaction of PHBH from *R. opacus* 557 with 3-fluoro-4-hydroxybenzoate. The concentrations of 3-fluoro-4-hydroxybenzoate and NADH were 250 μM.

zoate as no substrate inhibition with 2-fluoro-4-hydroxybenzoate occurred. *Rhodococcus* PHBH produced significant higher amounts of 2-fluoro- and 3-fluoro-4,5-dihydroxybenzoate than *Pseudomonas* PHBH,[18,30] suggesting that the performance of C5 hydroxylation of monofluorinated 4-hydroxybenzoates may be increased by protein redesign.

Opposite regioselectivity was observed in the reactions of *Rhodococcus* PHBH with 2-chloro-4-hydroxybenzoate. This suggests that the *Rhodococcus* enzymes can bind the 2-chloro-4-hydroxybenzoate substrate in flipped orientations. Another possibility is that the chlorinated substrate binds in only one orientation and that enzyme dynamics allow some rotational mobility of the substrate aromatic ring, allowing the distal oxygen of the flavin hydroperoxide to approach the C3 and C5 atoms with different efficiency.[19] Clearly, more insight into the active site topologies of the *Rhodococcus* enzymes is needed to address this topic in further detail.

To our best knowledge, PHBH-mediated *ortho*-hydroxylation of 3-chloro-4-hydroxybenzoate has not been reported before.[33] The strong uncoupling of hydroxylation observed with this compound points to a deactivating effect of the 3-chloro group and is in agreement with the electrophilic aromatic substitution mechanism, proposed for this class of flavoenzymes.[34] Our results suggest that the yield of conversion of chlorinated PHBH substrates may be increased by efficient cofactor regeneration. Such a system should also contain catalase and ascorbate to prevent the formation of unwanted by-products.

Experimental Section

Chemicals

NADH and dithiothreitol (DTT) were from Boehringer. Tris, ethylenediaminetetraacetate (EDTA) and flavine adenine dinucleotide (FAD) were from Sigma. Aromatic compounds were purchased as reported earlier.[33] Fluorinated substrates were synthesized and purified as described before.[33]

Enzymes

PHBH from *Rhodococcus rhodnii* 135, PHBH from *Rhodococcus opacus* 557 and protocatechuate 3,4-dioxygenase from *Rhodococcus rhodnii* 135 were purified as recently described.[29] Catalase was purchased from Sigma.

Kinetics

Enzyme activity with different substrates was measured spectrophotometrically at 340 nm, following NADH consumption. All experiments were performed in air-saturated 50 mM potassium phosphate pH 7.2. For determination of kinetic parameters, activity determinations were carried out in the presence of saturating concentrations of NADH (300–350 μM). Because of very low Michaelis constants, apparent K_m values for 2-fluoro-4-hydroxybenzoate and 2-chloro-4-hydroxybenzoate were determined from kinetic substrate depletion experiments. For this purpose, the reaction mixture contained 10–30 μM haloaromatic substrate, 300–350 μM NADH, 50–100 mU/mL PHBH, and about 1 U/mL protocatechuate 3,4-dioxygenase from *Rhodococcus rhodnii* 135. The reaction was initiated by the addition of PHBH and changes in absorption at 340 nm were recorded until all substrate was consumed (t_{end}). Kinetic traces obtained were numerically differentiated, time of half-maximum rate ($t_{1/2}$) was determined, and the apparent K_m value was calculated from the differences in absorbance at $t_{1/2}$ and t_{end} taking into account the efficiency of substrate hydroxylation.

Efficiency of Substrate Hydroxylation

The efficiency of substrate hydroxylation was determined by the following methods:

i) Oxygen consumption and hydrogen peroxide accumulation was measured polarographically with a Clark electrode.[13] For this purpose excess of aromatic substrate (200–500 μM) was reacted with limited amounts of NADH (20–200 μM). The reaction was initiated by the addition of PHBH (1–2 U/mL) and at the end of the reaction catalase (50 U/mL) was added to determine the amount of hydrogen peroxide formed.

ii) Spectrophotometric determination of NADH consumption at 340 nm. The reaction mixture contained aromatic substrate (10–50 μM), excess of NADH (100–350 μM) and protocatechuate 3,4-dioxygenase (5 U/mL) to remove the aromatic reaction product. The reaction was initiated by the addition of PHBH (0.1–0.5 U/mL).

iii) Qualitative information from HPLC and ^{19}F-NMR data (see below).

NMR

The enzymatic conversions of 2-fluoro-4-hydroxybenzoate and 3-fluoro-4-hydroxybenzoate were analyzed by ^{19}F NMR using a Bruker DPX 400 NMR spectrometer, essentially as described previously.[31] Reaction mixtures contained 250 – 500 μM fluorinated substrate, varying amounts of NADH and 0.2 U PHBH in 2.0 mL air-saturated 50 mM potassium phosphate pH 7.2, containing 0.5 mM DTT, 1 mM EDTA and 10 μM FAD, either in the absence or presence of 1 mM ascorbate. The reaction was followed spectrophotometrically at 340 nm until completion (about 2 hours). The sample was frozen in liquid nitrogen and stored at $-20\,^{\circ}C$. Before ^{19}F NMR analysis, the sample was thawed and precipitated protein was removed by centrifugation. ^{19}F NMR spectra were recorded at $7\,^{\circ}C$ with 4-fluorobenzoate serving as internal standard. Usually 3000 – 15000 scans were collected. Chemical shifts of ^{19}F NMR resonances at pH 7.2 of 2-fluoro-4-hydroxybenzoate products were equal to published values:[31] 2-fluoro-4-hydroxybenzoate – 116.4 ppm; 2-fluoro-3,4-dihydroxybenzoate – 141.2 ppm; 2-fluoro-4,5-dihydroxybenzoate – 126.2 ppm; 2-fluoro-3,4,5-trihydroxybenzoate – 149.8 ppm. Newly determined ^{19}F NMR chemical shifts for 3-fluoro-4-hydroxybenzoate and 5-fluoro-3,4-dihydroxybenzoate were – 141.7 ppm and – 140.7 ppm, respectively.

The conversion of 2,4-dihydroxybenzoate by PHBH from *Rhodococcus* sp. was analyzed with ^{1}H NMR. ^{1}H NMR measurements were performed on a Bruker AMX 500 spectrometer as essentially described previously.[35]

HPLC and MS

The enzymatic conversions of 2-chloro-4-hydroxybenzoate, 3-chloro-4-hydroxybenzoate and 2,4-dihydroxybenzoate were analyzed by HPLC using an Applied Biosystems 400 pump equipped with a Waters 996 photodiode-array detector. Reaction products were separated with a 4.0×60 mm C18 reverse-phase column (Spherisorb, ODS 2, Pharmacia). Reaction mixtures contained 100 – 200 μM substrate, varying amounts of NADH and about 1 – 2 U PHBH in 1 mL air-saturated 50 mM phosphate pH 7.2, containing 0.5 mM DTT, 1 mM EDTA, 1 mM ascorbate and 10 μM FAD. The reaction was followed spectrophotometrically at 340 nm. At the end of the reaction, 35 μL of 2 M sulfuric acid were added and precipitated protein was removed by centrifugation. HPLC analysis of the resulting supernatant was carried out by gradient elution with 0.1% acetic acid and 0 – 30% methanol as mobile phase. Relative yields of aromatic products were determined using the millimolar absorption coefficients of 2-chloro-4-hydroxybenzoate ($\varepsilon_{254} = 13.0\ mM^{-1}\ cm^{-1}$), 3-chloro-4-hydroxybenzoate ($\varepsilon_{254} = 14.0\ mM^{-1}\ cm^{-1}$), 3,4-dihydroxybenzoate ($\varepsilon_{254} = 10.0\ mM^{-1}\ cm^{-1}$), 2-chloro-3,4-dihydroxybenzoate ($\varepsilon_{254} = 8.5\ mM^{-1}\ cm^{-1}$), 5-chloro-3,4-dihydroxybenzoate ($\varepsilon_{254} = 11.0\ mM^{-1}\ cm^{-1}$) and 2-chloro-4,5-dihydroxybenzoate ($\varepsilon_{254} = 11.0\ mM^{-1}\ cm^{-1}$).

HPLC analysis of the enzymatic conversions of 2-fluoro-4-hydroxybenzoate and 3-fluoro-4-hydroxybenzoate was performed on a Waters M600-PDA system using a 4.6×150 mm Alltima C18 reverse-phase column running in 20% methanol containing 0.8% acetic acid at a flow rate of 1.0 mL/min. With this system, the following retention times (absorption maxima) of substrates, products and reference compounds were observed: 4-hydroxybenzoate, 11.7 min (254 nm); 2-fluoro-4-hydroxybenzoate, 14.2 min (250 nm); 3-fluoro-4-hydroxybenzoate, 16.4 min (253 nm); 2,4-dihydroxybenzoate, 16.0 min (255, 293 nm); 3,4-dihydroxybenzoate, 6.2 min (260, 293 nm); 2,3-dihydroxybenzoate, 15.1 min (246, 312 nm); 2,5-dihydroxybenzoate, 10.3 min (233, 326 nm); 2,6-dihydroxybenzoate, 21.2 min (251, 307 nm); 2-fluoro-3,4-dihydroxybenzoate 6.9 min (257 nm); 5-fluoro-3,4-dihydroxybenzoate 9.5 min (260, 293 nm); 2,3,4-trihydroxybenzoate, 7.3 min (264, 301 nm); 3,4,5-trihydroxybenzoate, 3.4 min (272 nm); 2,4,6-trihydroxybenzoate, 7.8 min (255, 293 nm); 2-fluoro-3,4,5-trihydroxybenzoate, 3.6 min (267, 297 nm). Enzymatic reaction conditions were the same as in ^{19}F NMR experiments.

Mass spectrometry was performed on a Mat95 (Thermo-Finnigan, San Jose, USA) mass spectrometer by direct insertion of freeze-dried HPLC fractions dissolved in acetone. The mass spectrometer was operated in the 70 eV EI mode with scanning from 24 to 400 amu at 2 s decade^{-1}.

Acknowledgements

We thank Dr. Maarten Posthumus (Wageningen University) for performing MS experiments. This work was supported by grant 047.007.021 for Dutch-Russian research cooperation from the Dutch Scientific Organisation NWO, by EC-Copernicus grant ICA-2-CT-2000-10006, and by a grant (to A. P. J.) from the Dutch Graduate School of Environmental Chemistry and Toxicology (M&T).

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