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1 **Purification and characterization of a three-component salicylate 1-hydroxylase from**
2 ***Sphingomonas* CHY-1**

3

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24

1 **Summary**

2

3 In the bacterial degradation of polycyclic aromatic hydrocarbons (PAHs), salicylate
4 hydroxylases catalyze essential reactions at the junction between the so-called upper and
5 lower catabolic pathways. Unlike the salicylate 1-hydroxylase from pseudomonads, which is
6 a well-characterized flavoprotein, the enzyme found in sphingomonads appears to be a three-
7 component Fe-S protein complex, which has not so far been characterized. Here, the
8 salicylate 1-hydroxylase from *Sphingomonas* CHY-1 has been purified and characterized with
9 respect to its biochemical and catalytic properties. The oxygenase component called PhnII,
10 exhibited an $\alpha_3\beta_3$ heterohexameric structure and contained one Rieske-type [2Fe-2S] cluster
11 and one mononuclear iron per α subunit. In the presence of purified reductase (PhnA4) and
12 ferredoxin (PhnA3) components, PhnII catalyzed the hydroxylation of salicylate to catechol
13 with a maximal specific activity of 0.89 U/mg and showed an apparent K_m for salicylate of
14 $1.1 \pm 0.2 \mu\text{M}$. The hydroxylase exhibited similar activity levels with methylsalicylates and
15 low activity with salicylate analogues bearing additional hydroxyl or electron-withdrawing
16 substituents. PhnII converted anthranilate to 2-aminophenol and exhibited a relatively low
17 affinity for this substrate ($K_m = 28 \pm 6 \mu\text{M}$). 1-Hydroxy-2-naphthoate, which is an
18 intermediate in phenanthrene degradation, was not hydroxylated by PhnII, but induced a high
19 rate of uncoupled oxidation of NADH. It also exerted a strong competitive inhibition of
20 salicylate hydroxylation, with a K_i of $0.68 \mu\text{M}$. The properties of this three-component
21 hydroxylase are compared with those of analogous bacterial hydroxylases and discussed in
22 the light of our current knowledge of PAH degradation by sphingomonads.

23

1 INTRODUCTION

2 Bacterial isolates belonging to the sphingomonad group have been shown to degrade a wide
3 range of organic pollutants, including recalcitrant compounds such as chlorinated xenobiotics
4 (16), dioxins (26) and polycyclic aromatic hydrocarbons (PAHs) (9, 18, 20, 32). The
5 remarkable capabilities of this group of bacteria suggested that they might be useful for
6 bioremediation purposes, and prompted studies on the identification of the catabolic enzymes
7 involved. Independent genetic analyses on different PAH-degrading strains revealed that they
8 contain a unique set of catabolic genes, often carried on a megaplasmid, which is probably
9 responsible for their versatile metabolism (15, 20, 23). Extending over a 40-kb DNA region,
10 the main catabolic gene cluster includes *xyl* genes involved in the catabolism of
11 monoaromatics interspersed with genes predicted to function in the degradation of polycyclic
12 substrates (designated as *bph*, *nah*, *ahd* or *phn*). This unique gene arrangement, which is
13 remarkably conserved between strains of various origins, contrasts with that found in other
14 degraders such as pseudomonads. In *Sphingobium yanoikuyae* B1, the biodegradation of
15 naphthalene is thought to proceed through a pathway similar to that found in *Pseudomonas*
16 species, with a first set of enzymes converting naphthalene to salicylate and a second set
17 responsible for the conversion of salicylate to Krebs cycle intermediates (15). In this strain as
18 in other sphingomonads, many of the gene products involved in this pathway have been
19 identified based on sequence similarity with known *Pseudomonas* enzymes, but no
20 counterpart was found for NahG which catalyzes the monohydroxylation of salicylate to
21 catechol (30). In addition, the function of many genes in the catabolism of other PAHs
22 remains to be elucidated.

23 Peculiar to sphingomonads is the occurrence of multiple copies of genes predicted to encode
24 the terminal component of Rieske-type oxygenases (21, 23). Genes coding for two cognate
25 electron carriers are generally found adjacent to such oxygenase-encoding genes in other

1 bacteria degrading aromatic hydrocarbons, but it is not the case in sphingomonads. Rieske-
2 type oxygenases are known to catalyze the dihydroxylation of various aromatic compounds,
3 and often initiate the oxidative biodegradation of pollutants. They constitute a large family of
4 two or three component metalloenzymes the catalytic component of which is generally an
5 heteromeric $\alpha_3\beta_3$ hexamer containing one Rieske-type [2Fe-2S] cluster and one non-heme
6 iron atom per α subunit. The enzyme responsible for the initial attack on PAHs has recently
7 been identified in *Sphingomonas* CHY-1, a strain able to grow on chrysene as sole carbon
8 source (9, 25). The oxygenase, called PhnI, is functionally associated with a NAD(P)H-
9 oxidoreductase (PhnA4) and a ferredoxin (PhnA3) in a three-component enzyme complex
10 able to oxidize a wide range of 2- to 5-ring PAHs (13). Analysis of a knockout mutant
11 demonstrated that PhnI was absolutely required for growth on PAHs, indicating that no other
12 oxygenase could replace PhnI in the initial dihydroxylation of PAHs (9). Another oxygenase
13 named PhnII, which catalyzes the C1-hydroxylation of salicylate to catechol has been
14 identified in strain CHY-1. When overproduced in recombinant form in *E. coli*, PhnII
15 required the co-expression of the ancillary proteins PhnA4 and PhnA3 for full activity,
16 indicating that it is a three-component enzyme which shares electron carriers with PhnI (9).
17 PhnII resembles salicylate 5-hydroxylase from *Ralstonia* sp. strain U2, which oxidizes
18 salicylate to gentisate (31) but differs from salicylate 1-hydroxylases found in pseudomonads,
19 which are monomeric flavoproteins (29). Interestingly, PhnII is also related to anthranilate
20 dioxygenase from *Burkholderia cepacia* DBO1, a three-component enzyme that exclusively
21 catalyses dioxygenation reactions (6). The occurrence of three-component salicylate 1-
22 hydroxylases was first reported by Pinyakong et al., in *Sphingobium* sp. strain P2, a
23 phenanthrene degrading strain (22). This strain synthesized three isoenzymes homologous to
24 PhnII, which showed different specificities toward substituted salicylates. Sequence
25 comparisons using BLAST indicated that PhnII showed substantial similarities with one

1 hydroxylase from strain P2 (83% and 71% identities between the α and β subunits,
2 respectively) but only moderate similarities with the other two P2 enzymes (>51% and >45%
3 identities). Such comparisons of protein sequences did not reveal any clue that would explain
4 the differences in selectivity between these enzymes. In *Sphingobium yanoikuyae* B1, indirect
5 evidence indicated that an analogous hydroxylase catalyzed both salicylate hydroxylation and
6 conversion of 1-hydroxy-2-naphthoate to 1,2-dihydroxynaphthalene (7). The latter reaction is
7 a key step in the biodegradation of phenanthrene by Gram negative bacteria.

8 So far, none of the three-component hydroxylases considered above has been purified, and
9 therefore biochemical and catalytic data on this important class of enzymes are lacking. Here,
10 we have purified the PhnII enzyme to near homogeneity and have investigated the kinetics of
11 the catalytic hydroxylation of salicylate and several analogues. We also show that 1-hydroxy-
12 2-naphthoate is not a substrate but triggers NADH oxidation at a high rate in a totally
13 uncoupled reaction. This reaction might inhibit PAH degradation by sphingomonads when
14 phenanthrene is an abundant substrate.

15

16 MATERIALS AND METHODS

17 Bacterial strains, plasmids and growth conditions

18 Strains of *Escherichia coli* and *Pseudomonas putida*, and plasmids used for gene
19 overexpression, as well as general culture conditions, have been previously described (9).
20 Plasmid pVEHD11 carrying the *phnA1bA2b* coding sequence fused to a 5'-extension coding
21 for a Histag, was constructed in two steps. A NdeI-BamHI fragment carrying the two genes of
22 interest was isolated from plasmid pDAB11 (9) and subcloned into pET15b (Novagen) to
23 give pEHD11. The insert was then extracted from pEHD11 as a XbaI fragment and cloned
24 into pVLT31 (8). A plasmid carrying *phnA1bA2b* in the right orientation with respect to the
25 P_{tac} promoter was selected and designated pVEHD11. Overproduction of ht-PhnII was

1 performed either in *E. coli* BL21(DE3) carrying pEHD11 or in *P. putida* KT2442 carrying
2 pVEHD11. Overproduction of ht-PhnA3, ht-PhnA4 and ht-Red_{B356} that were used as electron
3 carriers for ht-PhnII in oxygenase assays has been previously described (13).

4 Large-scale cultures of strain KT2442(pVEHD11) were grown on Luria-Bertani rich medium
5 in a 12-L fermentor as previously described (13). Expression of the recombinant oxygenase
6 was induced at 25°C with 0.2 mM IPTG, and incubation was prolonged for 20 h before
7 harvesting the culture by centrifugation. The bacterial pellet was washed with 50 mM Tris-
8 HCl buffer (pH 7.5) and kept frozen until use.

9

10 **Purification of the oxygenase component PhnII**

11 All purification procedures were carried out under argon, using buffers equilibrated for at
12 least 24 h in a glove box maintained under anoxic conditions ($O_2 < 2$ ppm, Jacomex, France).
13 The temperature was kept at 0-4 °C except when otherwise indicated. A crude extract was
14 prepared by thawing the bacterial pellet (100 g) in two volumes of lysis buffer (50 mM Tris-
15 HCl, pH 7.5) by volume, followed by lysozyme treatment (0.5 mg/ml) for 15 min at 30°C.
16 The suspension was then subjected to ultrasonication for a total time of 10 min including 5
17 min of pause at 75% of maximal intensity using a Vibra Cell apparatus run in pulse mode at 5
18 s/pulse (Fisher Bioblock Scientific, Illkirch, France). The lysate was centrifuged at 12,000 g
19 for 30 min, and the resulting cell extract was diluted two-fold with TG buffer (25 mM Tris-
20 HCl, pH 7.5, containing 5% glycerol and 2 mM β -mercaptoethanol) and applied to two 40-ml
21 columns of DEAE-cellulose (DE52, Whatman) equilibrated with TG buffer. After washing
22 the columns with four bed volumes of the same buffer, the oxygenase was eluted as a brown
23 band with buffered 0.3 M NaCl. The eluate (about 50 ml) was immediately applied to a small
24 column (7 ml) of immobilized metal affinity chromatography (IMAC) resin loaded with Co^{2+}
25 (TALON, BD Biosciences Ozyme, France). The column was washed successively with 8 bed

1 volumes of TG buffer containing 0.5 M NaCl and 5 bed volumes of the same buffer
2 supplemented with 20 mM imidazole and 1 mM salicylate. A brown protein fraction was then
3 eluted with TG buffer containing 0.15 M imidazole and 1 mM salicylate. A portion of the
4 preparation (1.3 ml containing about 40 mg of protein) was applied to a 1.5 × 60 cm column
5 of Superdex SD200 equilibrated in TG buffer containing 0.1 M NaCl and eluted at a rate of
6 0.43 ml/min. The purified protein fraction was concentrated onto a small column of DEAE-
7 cellulose, eluted in about 1 ml of TG buffer containing 0.3 M NaCl, and frozen as pellets in
8 liquid nitrogen. This preparation, designated ht-PhnII, was judged to be at least 95% pure by
9 SDS-PAGE (Fig 1).

10

11 **Purification of other proteins**

12 The ferredoxin (ht-PhnA3) from strain CHY-1 and the reductase component of the biphenyl
13 dioxygenase from *C. testosteroni* B-356 (ht-Red_{B356}) were purified as previously described
14 (13). The catechol 2,3-dioxygenase from *Pseudomonas putida* mt2 (Xyle) was purified
15 according to a published procedure (11).

16

17 **Enzyme assays**

18 Salicylate hydroxylase activity was determined using three different assays. In the coupled
19 assay, the formation of catechol from salicylate was coupled to ring cleavage by catechol 2,3-
20 dioxygenase (Xyle). Each reaction mixture contained 0.1 μM ht-PhnII, 1.5 or 3 μM ht-
21 PhnA3, 0.3 μM ht-Red_{B356}, 0.03 μM Xyle, 0.2 mM NADH and 0.5 mM salicylate in 50 mM
22 potassium phosphate buffer, pH 7.0. Reactions were carried out at 30°C in quartz cuvettes,
23 and initiated by the injection of a 100-fold concentrated solution of the protein mixture with a
24 gas-tight syringe. The protein mixture was prepared 30 min before starting the assays and
25 kept on ice under argon in phosphate buffer containing 1 mM dithiothreitol, 0.05 mM ferrous

1 ammonium sulfate and 10% glycerol. The absorption at 376 nm was recorded at 0.1-s
2 intervals over 1 min with a HP8452 spectrophotometer (Agilent Technologies, Les Ulis,
3 France). The enzyme activity was calculated from the initial linear portion of the time course,
4 using an absorption coefficient of $34,000 \text{ M}^{-1} \cdot \text{cm}^{-1}$ for 2-hydroxymuconate semi-aldehyde
5 (11). Assuming that each molecule of the latter product resulted from the oxidation of one
6 molecule of catechol, one unit of activity was defined as the amount of enzyme that converted
7 one micromole of salicylate to catechol per minute.

8 The enzyme activity was also measured by recording NADH oxidation at 340 nm, and
9 calculated using an absorbance coefficient of $6,220 \text{ M}^{-1} \cdot \text{cm}^{-1}$. The protein mixture used had
10 the same composition as that described above, except that XylE was omitted. Alternatively,
11 the oxygenase activity was determined by monitoring O_2 uptake using a Clark-type O_2
12 electrode under conditions essentially similar to those previously described (13). Assays
13 contained NADH at an initial concentration of 0.4 mM. After approximately 60 nmol O_2 had
14 been consumed, 300 U of liver catalase (Sigma-Aldrich) was added to estimate the amount of
15 H_2O_2 formed during the assay. In some cases, 0.6 ml of reaction mixture was immediately
16 withdrawn, and acidified with 0.1 % acetic acid. After centrifugation and filtration, samples
17 were analyzed by HPLC to quantify the catechols formed as described below.

18

19 **Steady-state kinetics**

20 Sets of salicylate hydroxylase assays were carried out at substrate concentrations that were
21 varied over a 0.5-100 μM range. Activity was measured in the XylE-coupled assay as
22 described above. All assays were performed in duplicates, and at least 12 concentrations were
23 tested. For titration of the hydroxylase with anthranilate, enzyme activity was measured by
24 monitoring NADH oxidation at 340 nm. Assays were performed for substrate concentrations
25 ranging between 5 and 500 μM . Oxidation rates were calculated using an absorption

1 coefficient incremented of $0.874 \text{ M}^{-1} \cdot \text{cm}^{-1}$ to take into account the absorbance of anthranilate
2 at 340 nm. Plots of the initial reaction rate versus substrate concentration were fitted to the
3 Michaelis-Menten equation using the curve fit option of Kaleidagraph (Synergy Software).
4 Only curve fits showing correlation coefficients better than 0.98 were considered.

5

6 **Chemical identification and quantification of the enzymatic products**

7 The oxidation products generated by PhnII were analyzed by GC-MS. Samples were
8 derivatized with bis(trimethylsilyl)trifluoroacetamide : trimethylchlorosilane (99:1) from
9 Supelco (Sigma-Aldrich), prior to analysis using a HP6890 gas chromatograph coupled to a
10 HP5973 mass spectrometer (Agilent Technologies). Operating conditions were as previously
11 described (12), and mass spectrum acquisitions were carried out in the scan mode.

12 Concentrations of catechol and methylcatechols formed by salicylate 1-hydroxylase were
13 determined by HPLC using a Kontron system equipped with a F430 UV detector. Samples
14 (0.1 ml) were injected onto a 4×150 -mm C8 reverse-phase column (Zorbax, Agilent
15 Technologies) run at 0.8 ml/min. The column was eluted with a linear gradient of 0 to 50%
16 acetonitrile in water over 15 min. Detection was carried out at 276 nm. Calibration curves
17 were generated by injecting known amounts of 3-methyl-, 4-methyl- or unsubstituted
18 catechol.

19

20 **Determination of the iron content of proteins**

21 To extract iron from proteins, samples (150 μl) were treated with 2.5 N HCl for 30 min at
22 95°C , then diluted with 135 μl of ultrapure water. Iron was assayed in triplicate as a complex
23 with bathophenanthroline by absorbance measurement at 536 nm (2). A calibration curve was
24 generated by diluting a standard solution of ferric nitrate containing 1g/L Fe (Merck).

25

1 **Protein analyses**

2 Routine protein determinations were performed using the Bradford assay (4), or the
3 bicinchoninic acid reagent kit (Pierce) using bovine serum albumin as a standard. A modified
4 microbiuret assay was used for measuring the protein content of purified preparations of ht-
5 PhnA3 and ht-PhnII (19). SDS-PAGE on mini-slab gels was performed as previously
6 described (14). The molecular mass of purified ht-PhnII was determined by size-exclusion
7 chromatography on a column (1.5 x 60 cm) of Superdex SD200 (Amersham Biosciences).
8 The column was run at a flow rate of 0.2 ml/min and calibrated with the following protein
9 markers: Ferritin (443 kDa), catalase (240 kDa), aldolase (150 kDa), bovine serum albumin
10 (67 kDa), ovalbumin (43 kDa) and myoglobin (17 kDa), all from Sigma-Aldrich.

11

12 **EPR analysis**

13 Purified ht-PhnII was adjusted to 30 μ M in 25 mM Tris-HCl, pH 7.5 containing 5% glycerol
14 and reduced with 1 mM dithionite. The EPR spectrum was recorded at 4 K as previously
15 described (13).

16

1 RESULTS

2 Purification and biochemical properties of salicylate 1-hydroxylase from strain CHY-1

3 The structural genes encoding the hydroxylase were cloned into pET15b in order to produce
4 the enzyme with a His-tag fused at the N-terminus of the large subunit. When introduced into
5 an appropriate *E. coli* overexpression strain, two polypeptides of the expected sizes were
6 detected in the cell extract by SDS-PAGE, but the enzyme was essentially inactive. Transfer
7 of the genes into plasmid pVLT31 under the P_{tac} promoter resulted in an efficient expression
8 system producing active enzyme in both *E. coli* and *P. putida*. The latter organism was
9 preferred as a host to overproduce the recombinant enzyme because it consistently afforded
10 better purification yields compared to *E. coli*. The hydroxylase was purified under anoxic
11 conditions using a three-step procedure as described under Materials and Methods, yielding a
12 protein preparation that was judged more than 95 % pure based on SDS-PAGE (Fig. 1). It
13 consisted of two polypeptides with apparent M_r of 47,000 and 21,000. We observed that the
14 enzyme activity fell dramatically upon IMAC chromatography, most likely because the
15 enzyme lost a good part of its active site Fe(II) ion. This loss of activity could be prevented by
16 supplementing buffers with 1 mM salicylate, while ethanol or isopropanol had no protecting
17 effect. After the last purification step, maximal activity was obtained by incubating the
18 protein preparation for 1 h at 4°C with 0.1 mM ferrous ammonium sulfate. The purified
19 enzyme exhibited a specific activity of 0.89 U/mg in a coupled assay in which the catechol
20 formed by the hydroxylase was oxidized to 2-hydroxymuconic acid semialdehyde by catechol
21 dioxygenase (see Methods). The molecular mass of the hydroxylase as deduced from gel
22 filtration experiments was 190 kDa, consistent with an $\alpha_3\beta_3$ hexameric structure. The enzyme
23 exhibited a broad absorbance maximum at 456 nm with a shoulder around 530 nm, which is
24 typical for proteins containing Rieske-type [2Fe-2S] clusters. The occurrence of such clusters
25 in the protein was confirmed by EPR analysis of a dithionite-reduced sample, which gave a

1 rhombic signal centered around $g = 1.91$ (data not shown). The iron content of the enzyme
2 was calculated to be 8.52 ± 0.21 Fe/mol based on chemical assay, which is close to the
3 theoretical value for an $\alpha_3\beta_3$ hexamer containing one [2Fe-2S] cluster and one non-heme iron
4 per α subunit. Overall, the salicylate hydroxylase from strain CHY-1 exhibited biochemical
5 properties similar to those of classical Rieske-type dioxygenases that catalyze the
6 dihydroxylation of various aromatic hydrocarbons (5).

7

8 **Catalytic properties and substrate specificity**

9 The activity of the purified ht-PhnII oxygenase required ancillary proteins PhnA4 and PhnA3
10 to transfer electrons derived from NADH oxidation to the enzyme active site. The dependence
11 of the enzyme complex activity on the concentration of each electron carrier was studied in
12 assays where their molar ratio relative to the oxygenase component was independently varied.
13 While half-saturation was reached at a reductase-to-oxygenase ratio close to 0.5, a much
14 higher relative concentration of ferredoxin ht-PhnA3 was needed to reach maximum activity.
15 At the highest protein ratio tested (about 200-fold molar excess of ferredoxin), the enzyme
16 activity was not fully saturated. Nevertheless, in the assays described herein this ratio was set
17 at 30 or below, in order to prevent excessive substrate-independent NADH oxidation by the
18 enzyme complex. Also, the reductase component of the biphenyl dioxygenase from
19 *Comamonas testosteroni*, ht-RED_{B356}, was substituted for PhnA4 in most assays because the
20 reductase from strain CHY-1 proved to be an unstable protein (13). No loss of hydroxylase
21 activity was observed as a result of this replacement.

22 The substrate specificity of the hydroxylase toward various salicylate analogues was first
23 determined by NADH oxidation assays (Table 1). At the end of each assay, the product
24 formed was extracted with ethyl acetate, and analyzed by GC-MS. The enzyme showed high
25 levels of activity with all the methylsalicylates tested, with a preference for 4-

1 methylsalicylate. Analogues bearing a substituent with a high electron withdrawing effect,
2 like 5-chloro- and 5-nitrosalicylate were poor substrates. 2,4- and 2,6-Dihydroxybenzoates
3 were converted to products identified as trihydroxybenzenes by GC-MS, but gentisate (2,5-
4 dihydroxybenzoate) gave no detectable product. These three compounds yielded relatively
5 small amounts of product (if any), which did not correlate with the high NADH oxidation
6 rates observed, indicating that the catalytic reaction was uncoupled. Anthranilate also gave
7 rise to substantial NADH oxidation while producing little 2-aminophenol as the only
8 detectable product. When 1-hydroxy-2-naphthoate and 2-hydroxy-1-naphthoate were
9 provided as substrates, NADH oxidation was the highest but no product was formed,
10 suggesting that the reaction was fully uncoupled. To further investigate the catalytic
11 properties of strain CHY-1 salicylate 1-hydroxylase, enzyme reactions were monitored by
12 amperometric measurements of O₂ uptake.

13

14 **Uncoupled reactions catalyzed by salicylate 1-hydroxylase**

15 Substrate-dependent oxygen uptake by CHY-1 hydroxylase as well as hydrogen peroxide
16 formation during the assays were measured for several salicylate analogues (Table 2). When
17 possible, catechol products were quantified in order to determine the coupling efficiency
18 between oxygen consumption and catechol formation. A fairly good coupling was observed
19 upon enzymatic oxidation of salicylate and methylsalicylates, although a small proportion of
20 the O₂ consumed was reduced to H₂O₂. As the enzyme also formed hydrogen peroxide in the
21 absence of substrate (data not shown), we assume that this low activity might result from an
22 autooxidation of the ht-PhnA3 ferredoxin, as previously observed with a similar enzyme
23 system (13). When anthranilate and nitrosalicylate were used as substrates, a greater
24 proportion of the oxygen consumed was converted to peroxide. The two hydroxynaphthoate
25 molecules gave rise to high rates of oxygen uptake with no product formation. HPLC

1 measurements at the end of the reaction revealed that the substrate concentration had not
2 significantly decreased. These results indicated that both hydroxynaphthoates triggered a
3 highly uncoupled enzymatic reaction, as confirmed by the high proportion of oxygen
4 recovered as hydrogen peroxide. Some of the reactions catalyzed by the hydroxylase from
5 strain CHY-1 are illustrated in figure 2.

6

7 **Steady-state kinetics**

8 Steady-state rates of salicylate hydroxylation by the purified enzyme were measured at
9 substrate concentrations in the range 0.5 to 100 μM . The XylE-coupled assay was used to
10 monitor the reaction because it directly measured the rate of product formation, whereas other
11 assays might have introduced a bias due to the imperfect coupling of the reaction. The
12 enzyme titration gave data that could be fitted by the Michaelis function, from which an
13 apparent K_m of $1.10 \pm 0.2 \mu\text{M}$ was calculated. This value remained constant when the ht-
14 PhnA3/ht-PhnII molar ratio was varied. On the other hand, the apparent turnover number of
15 the hydroxylase was found to depend on this ratio, and to vary from one enzyme preparation
16 to another, probably as a function of the iron content of the enzyme. Catalytic velocity ranged
17 between $0.73 \pm 0.04 \text{ s}^{-1}$ at a ratio of 15 for one preparation, and 2.9 s^{-1} at a ratio of 30 for
18 another preparation. Inhibition of the hydroxylase activity was observed at high salicylate
19 concentration, reaching 30% at 0.5 mM and 51% at 1 mM. The kinetic parameters of the
20 enzyme towards anthranilate were also examined at substrate concentrations that were varied
21 between 5 and 400 μM . Steady-state rates were determined using NADH oxidation assays.
22 PhnII exhibited an almost 30-fold higher K_m ($28 \pm 6 \mu\text{M}$) for this substrate compared to
23 salicylate, whereas its V_{max} taken as the NADH oxidation rate, was very similar for both
24 substrates. However, since NADH oxidation was poorly coupled to anthranilate

1 hydroxylation (Table 2), the actual rate of substrate hydroxylation was lower in the case of
2 anthranilate.

3

4 **Inhibition of salicylate hydroxylation by 1-hydroxy 2-naphthoate**

5 The bacterial degradation of phenanthrene leads to the intermediate metabolite 1-hydroxy 2-
6 naphthoate, which in gram-negative species like *Pseudomonas* sp., is thought to be converted
7 to 1,2-dihydroxynaphthalene by the same enzyme that hydroxylates salicylate (1). Since 1-
8 hydroxy 2-naphthoate was not a substrate of the hydroxylase studied herein, and caused
9 uncoupling of the reaction, we examined whether this compound would inhibit salicylate
10 hydroxylation *in vitro*. To this end, we performed salicylate titration curves at various
11 concentrations of 1-hydroxy 2-naphthoate (Fig. 3A). Results showed that the hydroxylation
12 reaction was strongly inhibited through a mechanism that appeared to be competitive. A plot
13 of K_m / V_{max} values versus inhibitor concentration gave a straight line, which allowed to
14 calculate an inhibition constant of 0.68 μM (Fig. 3B).

15

16 **DISCUSSION**

17 In this work, we describe the first characterization of a three-component salicylate 1-
18 hydroxylase, an enzyme that appears to be present only in PAH-degrading strains of the
19 sphingomonad group. The oxygenase component of the enzyme exhibited biochemical
20 properties and a co-factor content very similar to that of typical ring-hydroxylating
21 dioxygenases catalyzing the dihydroxylation of various aromatic substrates (5). In addition,
22 the PhnII protein shows significant sequence similarity with anthranilate dioxygenase from
23 *Burkholderia cepacia* DBO1 (52% and 38% identity for the α and β subunits, respectively;
24 (6)), which converts anthranilate to catechol. Nevertheless, PhnII exclusively catalyzes
25 monohydroxylations as exemplified by the conversion of anthranilate to 2-aminophenol.

1 Phylogenetic analyses have previously shown that the oxygenase components of anthranilate
2 dioxygenase from strain DBO1 and of salicylate 1-hydroxylases from sphingomonads
3 (including PhnII) form a distinct group of enzymes, together with halobenzoate dioxygenases
4 and salicylate 5-hydroxylase (6). Curiously, this group includes enzymes that function only as
5 dioxygenases on the one hand, or as monohydroxylases on the other, and which all use
6 substituted benzoates as substrates. This observation raises questions about the structural
7 features that determine whether an enzyme functions as a mono- or a dioxygenase. Such a
8 question might be addressed in experiments where a monohydroxylase is modified to acquire
9 the ability to catalyze dihydroxylations through site-directed mutagenesis of residues
10 predicted to be at the enzyme active site.

11 In *in vitro* assays, the catalytic activity of the hydroxylase was highly dependent on the molar
12 ratio of the ferredoxin (PhnA3) and oxygenase (PhnII) components, half-saturation of the
13 activity occurring for an approx. 200-fold excess of PhnA3. A similar observation was made
14 with other three-component oxygenases, including the naphthalene dioxygenase (PhnI) from
15 strain CHY-1 (13). However, in the latter case, half-saturation was achieved with a 14-fold
16 excess of PhnA3, suggesting that the ferredoxin has a different affinity for the PhnI and PhnII
17 oxygenases. This difference might have physiological significance inasmuch as the two
18 oxygenases might compete for the same ferredoxin *in vivo*. Indeed, a single ferredoxin-
19 encoding gene homologous to *phnA3* was identified in the catabolic cluster responsible for
20 aromatic hydrocarbon degradation in three sphingomonads (15, 20, 23). *In vivo*, the
21 ferredoxin requirement is certainly not as high as *in vitro*, because the high protein
22 concentration of the cytosolic compartment probably favors ferredoxin oxygenase
23 interactions.

24 The three-component salicylate hydroxylase from strain CHY-1 has a low apparent Michaelis
25 constant for its substrate, similar to that reported for counterparts of the flavoprotein-type

1 found in *Pseudomonas* species (1.72-1.9 μM ; (1, 29)). The specific activity of the two types
2 of enzymes is also of the same order (0.89 versus 0.27 U/mg for the *P.putida* enzyme; (1)),
3 although direct comparison is difficult because the *in vitro* activity of the CHY-1 enzyme is
4 strongly dependent on the ferredoxin/oxygenase molar ratio. Ht-PhnII appeared to be almost
5 as active on methylsalicylates as on salicylate. This result is consistent with our finding that
6 the aromatic ring-hydroxylating dioxygenase responsible for the initial attack on PAHs in
7 CHY-1 can oxidize several methyl- and dimethylnaphthalenes (unpublished results), which
8 are then predicted to be further oxidized to methylsalicylates. Ht-PhnII also hydroxylated
9 salicylates with chlorine, nitro and hydroxyl substituents although at slower rates. A broad
10 substrate specificity was also observed for the two flavoprotein hydroxylases from
11 *Pseudomonas stutzeri* AN10 (3) and the three-component hydroxylases from *Sphingobium* P2
12 (22). However, enzyme activity appeared to be dependent on the nature of the substituent
13 group with an order of preference as follows : $\text{CH}_3 > \text{Cl} > \text{NO}_3 > \text{OH}$. This can be clearly seen
14 by comparing the hydroxylase activity of ht-PhnII on salicylate analogues bearing either one
15 of these groups in the C5 position (Table 1). In this respect, the 5-hydroxysalicylate or
16 gentisate was not oxidized by ht-PhnII, whereas the isomers bearing an hydroxyl in the C4 or
17 C6 position were hydroxylated. This finding indicates that a polar substituent in the C5
18 position of the ring prevents productive interaction of the substrate at the enzyme active site,
19 possibly because the substrate binding pocket has a hydrophobic character that hinders access
20 of gentisate. Anthranilate was a poor substrate for ht-PhnII as indicated by the uncoupling of
21 the hydroxylation reaction and the relatively high K_m of the enzyme for this substrate. In
22 comparison, the K_m of anthranilate dioxygenase for anthranilate has been estimated to be
23 about 1 μM (10).

24 The hydroxylation of anthranilate, as did that of other poor substrates, gave rise to a
25 significant uncoupling of the reaction resulting in the formation of hydrogen peroxide. Such a

1 side reaction has been observed for enzymes with similar structures that function as
2 dioxygenases (13, 17) and seems to be a consequence of the catalytic mechanism of this type
3 of enzyme (27, 28). Indeed, it has been observed that in naphthalene dioxygenase, substrate
4 binding to the active site increases the affinity of the enzyme for oxygen (28). Such a
5 mechanism might explain the high rate of substrate-induced reduction of oxygen to peroxide
6 catalyzed by PhnII in the presence of pseudo-substrates such as hydroxynaphthoate.
7 Interestingly, salicylate hydroxylases of the flavoenzyme type, which have a different
8 catalytic mechanism, were also found to catalyze uncoupled reactions when incubated with
9 certain salicylate analogues such as benzoate (24).

10 Unlike the flavoprotein hydroxylase from *P. putida* (1), the enzyme from CHY-1 was unable
11 to hydroxylate 1-hydroxy-2-naphthoate, a metabolite that is produced in the catabolic
12 pathway of phenanthrene. Moreover, we bring evidence that this metabolite causes inhibition
13 of salicylate hydroxylation, which might be deleterious to bacterial cells growing on PAHs.
14 Indeed, salicylate is an intermediate in the degradation of several PAHs including
15 naphthalene, anthracene, phenanthrene and fluorene, and its hydroxylation might be a
16 bottleneck if subjected to metabolic inhibition. Such an inhibition could be circumvented *in*
17 *vivo* if an enzyme oxidizes 1-hydroxy 2-naphthoate and maintains its concentration to a level
18 low enough to prevent inhibition of PhnII, or if another salicylate hydroxylase functionally
19 substitutes for PhnII. In the phenanthrene-degrading strain *Sphingobium* P2, three isoenzymes
20 have been shown to catalyze salicylate hydroxylation (22) and counterparts were identified in
21 two other sphingomonads from the analysis of catabolic genes (15, 23). In *S. yanoikuyae* B1,
22 the hydroxylase encoded by *bphA1cA2c*, which shares high sequence similarities with PhnII
23 (α and β subunits show 79% and 66% identities, respectively) has been proposed to catalyze
24 hydroxylation of both salicylate and 1-hydroxy 2-naphthoate, as deduced from the analysis of
25 a *bphA1c* knockout mutant strain (7). This mutant lost the ability to grow on salicylate and

1 naphthalene, and accumulated 1-hydroxy 2-naphthoate when incubated with phenanthrene.
2 However, the ability of the enzyme to catalyze the conversion of 1-hydroxy 2-naphthoate to
3 1,2-dihydroxynaphthalene has not been clearly demonstrated. Besides, the latter study
4 indicates that the *bphA1c* product is required for hydroxylation of salicylate, implying that
5 this enzyme could not be replaced by anyone of the two other isoenzymes present in the
6 bacterium. Hence, the physiological function and raison d'être of multiple copies of three-
7 component hydroxylases in the catabolism of PAHs by sphingomonads remain to be
8 elucidated. Also, further work is needed to identify unambiguously in sphingomonads the
9 enzyme responsible for the hydroxylation of 1-hydroxy 2-naphthoate, which is an essential
10 step in phenanthrene catabolism.

11

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7

TABLE 1 : Substrate specificity of ht-PhnII in the NADH oxidation assay and GC-MS analysis of the products formed

Substrate ^a	Enzyme activity ^b		Products detected by GC-MS		Identification of product
	U.mg ⁻¹	%	RT (min)	Molecular mass	
Salicylate	0.578	100	8.87	254	catechol
3-Methyl-salicylate	0.44	76	9.86	268	3-methyl catechol
4-Methyl-salicylate	0.625	108	9.74	268	4-methyl catechol
5-Methyl-salicylate	0.396	68.5	9.74	268	4-methyl catechol
5-Chloro-salicylate	0.267	46	10.79	288	4-chloro catechol
5-Nitro-salicylate	0.184	32	13.39	299	4-nitro catechol
Anthranilate	0.39	67	10.21	253	2-aminophenol
3,5-dichloro-salicylate	ND ^d	ND ^d	12.14	322	3,5-dichlorocatechol
2,4-dihydroxybenzoate	1.38	184	11.98	342	Trihydroxybenzene
2,6-dihydroxybenzoate	0.326	44	11.34	342	Trihydroxybenzene
Gentisate	1.21	162	-	-	no product
1-hydroxy-2-naphthoate	1.35	179	-	-	no product
2-hydroxy-1-naphthoate	10.7	1430	-	-	no product

^a Initial substrate concentration was 0.5 mM except for 1-hydroxy 2-naphthoate, 2-hydroxy 1-naphthoate (0.25 mM) and 5-nitrosalicylate (0.125 mM).

^b Activities, expressed as $\mu\text{mol NADH}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$, are means of duplicate assays. Standard deviations are less than 5%. Values in italics are from a different set of assays and refer to an enzyme activity of $0.75 \text{ U}\cdot\text{mg}^{-1}$ with salicylate as substrate

^c Products were extracted with ethyl acetate after 2 min of reaction, and analyzed as trimethylsilyl derivatives.

1 Table 2 : Substrate-dependent O₂ uptake, product formation and H₂O₂ release in PhnII-catalyzed reactions
2

Substrate	O ₂ uptake ^a μmol/min/mg	H ₂ O ₂ /O ₂ ratio	Catechol produced ^b μmol/min/mg
Salicylate	0.757± 0.007	0.144	0.64 ± 0.04
3-Methylsalicylate	0.711 ± 0.010	0.23	0.50 ± 0.01
5-Methylsalicylate	0.659 ± 0.005	0.19	0.61 ± 0.01
5-Chlorosalicylate	0.630 ± 0.025	0.19	nd
5-Nitrosalicylate	0.416 ± 0.005	0.38	nd
Anthranilate	0.630 ± 0.02	0.42	nd
1-hydroxy-2-naphthoate	3.07 ± 0.01	0.70	No product
2-hydroxy-1-naphthoate	8.56 ± 0.01	0.65	No product

3
4 ^a Maximum velocities calculated from the initial O₂ uptake rate taken during the first minute of the time course.
5 The PhnA3/PhnII molar ratio was 30.

6
7 ^b As determined from HPLC quantification after about 3 min. nd means not determined because no appropriate
8 standard was available to quantify the relevant products.

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Figure legends

Figure 1 : SDS-PAGE protein analysis during purification of recombinant ht-PhnII from strain KT2442 carrying pVEHD11. Lane 1, protein markers; lane 2, soluble cell-free extract; lane 3, DEAE column eluate; lane 4, IMAC column eluate; lane 5, dialyzed purified protein.

Arrows point to the α and β subunits of ht-PhnII.

Figure 2 : Reactions catalyzed by the three-component hydroxylase from strain CHY-1.

The scheme shows reaction products formed from (a) methylsalicylate, (b) anthranilate and (c) the pseudosubstrate 1-hydroxy 2-naphthoate.

Figure 3 : Inhibition of salicylate hydroxylation by 1-hydroxy 2-naphthoate.

A) Steady state rates of salicylate hydroxylation were measured in the presence of 1-hydroxy 2-naphthoate at the following concentrations (μM): 0 (diamonds), 0.8 (filled circles), 2 (triangles), 5 (inversed triangles), 8 (open circles). Activities were measured in the coupled assay with an enzyme complex containing ht-PhnII and ht-PhnA3 in a molar ratio of 1:15. Michaelis curve fits were adjusted to the data points as described in Methods. B) Plot of K_m/V_{\max} versus inhibitor concentration.

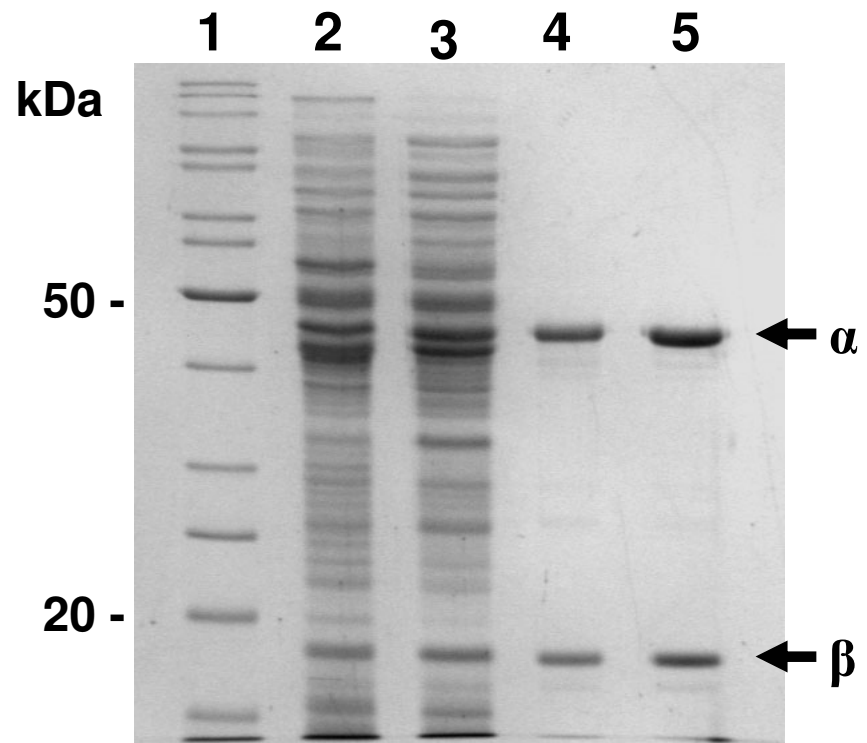


Fig.1, Jouanneau et al., 2007

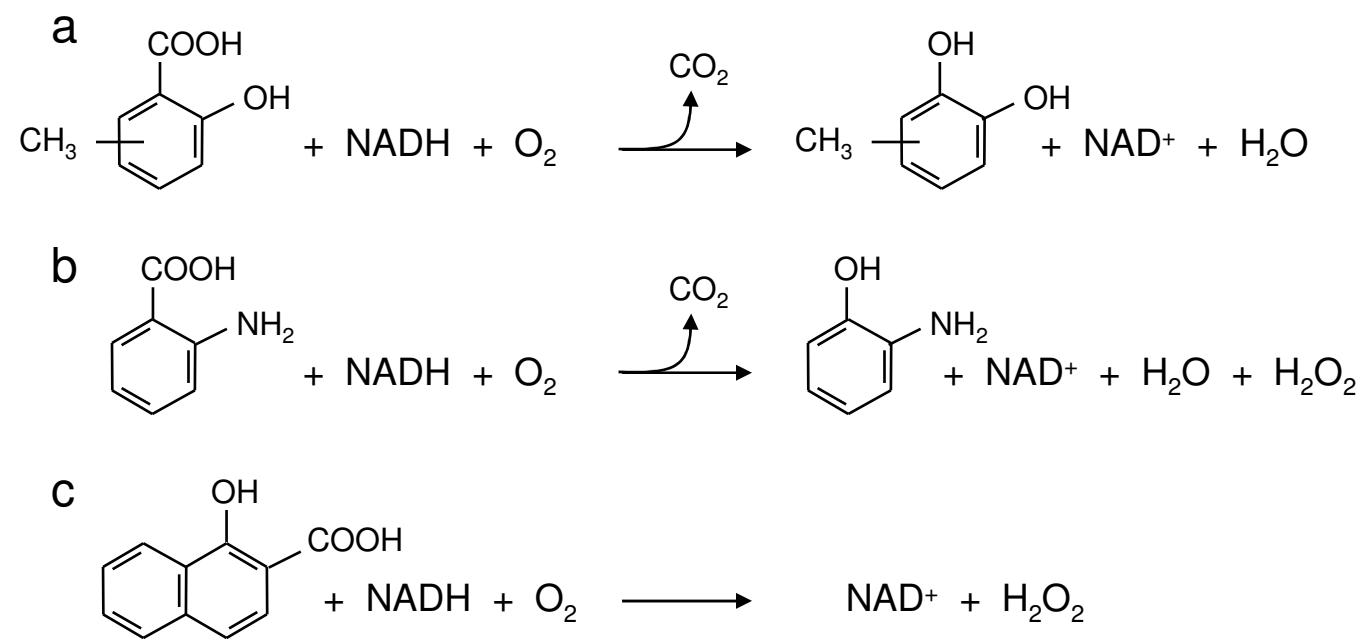


Fig. 2, Jouanneau et al.

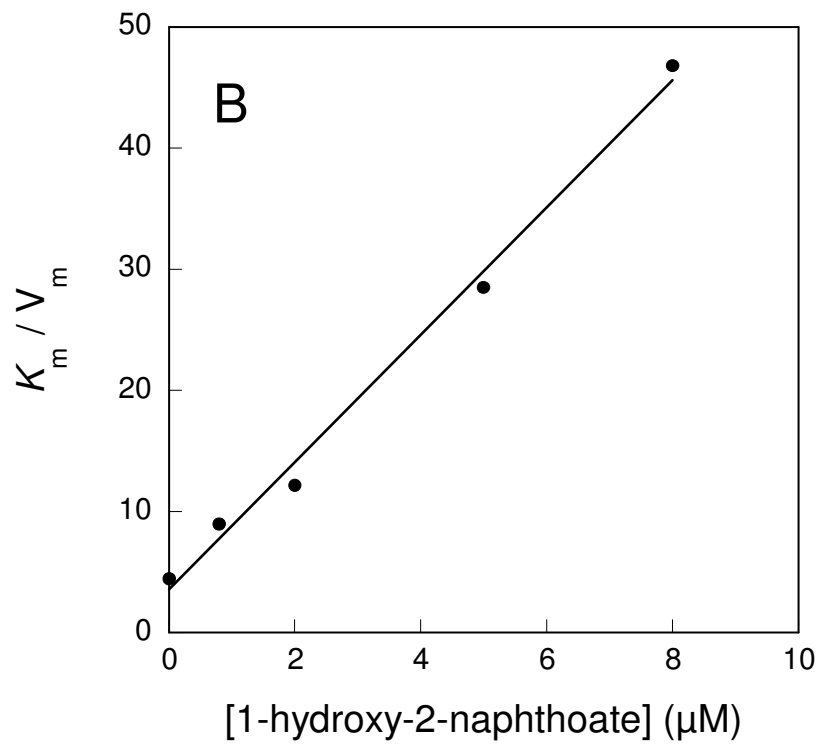
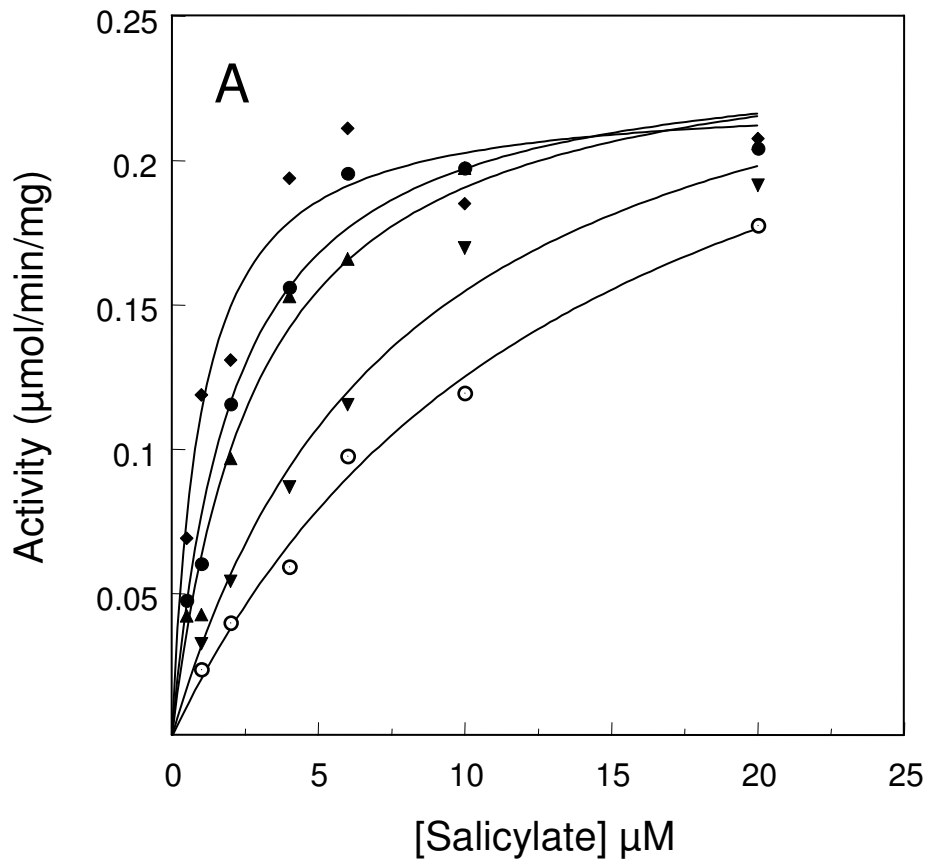


Fig. 3, Jouanneau et al., 2007