J. Biochem. **127**, 43–49 (2000)

Presence of Two *trans-o*-Hydroxybenzylidenepyruvate Hydratase-Aldolases in Naphthalenesulfonate-Assimilating *Sphingomonas paucimobilis* TA-2: Comparison of Some Properties

Takashi Ohmoto,[1] Kunihiko Moriyoshi, Kiyofumi Sakai, Nobutake Hamada, and Tatsuhiko Ohe

The Osaka Municipal Technical Research Institute, 1–6–50 Morinomiya, Joto-ku, Osaka 536–8553

Received August 19, 1999; accepted October 7, 1999

Two *trans-o*-hydroxybenzylidenepyruvate hydratase-aldolases named *t*HBP HA A and *t*HBP HA B were purified from a cell-free extract of naphthalenesulfonate-assimilating *Sphingomonas paucimobilis* (formerly *Pseudomonas* sp.) TA-2 to an electrophoretically homogeneous state by successive column chromatographies on DEAE-cellulose, DEAE-Toyopearl 650M, Sephacryl S-100, Hydroxyapatite, and Mono Q. These enzymes were similar to each other in molecular mass (*ca.* 37 kDa on SDS-PAGE, *ca.* 110 kDa on ultracentrifugation), thermal stability (<50°C) and optimum pH (pH 9.0). However, they differed from each other in N-terminal amino acid sequences, pH stability, K_m values for *trans-o*-hydroxybenzylidenepyruvate (*t*HBP), and inhibition by *p*-chloromercuribenzoic acid (PCMB). *t*HBP HA B had a homologous N-terminal amino acid sequence with *t*HBP HAs from *Pseudomonas vesicularis* DSM 6383 (strain BN6) and *Sphingomonas aromaticivorans* F119, and *t*HBP HA A had a homologous sequence with *t*HBP HAs of *Pseudomonas putida* strain OUS82, *Pseudomonas* sp. strain C18 and NAH7 plasmid. *t*HBP HA B was inhibited by PCMB, but *t*HBP HA A was not. Their K_m values for *t*HBP were 9 and 3 μM, respectively. *t*HBP HA B was stable in the range of pH 7.1 to pH 10.7, and *t*HBP HA A was stable in the range of pH 6.0 to 9.3.

Key words: aldolase, *trans-o*-hydroxybenzylidenepyruvate, naphthalene, naphthalenesulfonate, *Sphingomonas*.

S. paucimobilis (formerly *Pseudomonas* sp.) TA-2 was isolated as a 2-naphthylamine-1-sulfonate (tobias acid) (Fig. 1, 1)-assimilating bacterium, and it also assimilated 1-naphthalenesulfonate (Fig. 1, 3) and 2-hydroxy-1-naphthalenesulfonate (Fig. 1, 2) (*1*). Salicylate (Fig. 1, 11) is an intermediate in the metabolic pathway and is metabolized further through the gentisate pathway (*1, 2*). It has been shown that the metabolic pathway from naphthalenesulfonate to salicylate is similar to an upper naphthalene catabolic pathway (*1–6*). In the upper pathway, 1,2-dihydroxynaphthalene (Fig. 1, 5), 2-hydroxychromene-2-carboxylate (2HC2CA) (Fig. 1, 6), *trans-o*-hydroxybenzylidenepyruvate (*t*HBP) (Fig. 1, 7), and salicylaldehyde (Fig. 1, 10) are common intermediates (*6–10*) (Fig. 1).

A *t*HBP hydratase-aldolase (HA) (Fig. 1, enzyme C) is involved in degradation of *t*HBP to salicylaldehyde and pyruvate (*11*). Eaton and Chapman cloned the *t*HBP HA gene from the naphthalene catabolic plasmid NAH7 and found that a *t*HBP HA degraded *t*HBP to form salicylaldehyde and pyruvate (*12*). Several *t*HBP HA genes have since been cloned and sequenced (*12–20*). On the other hand, Kuhm *et al.* purified 2′-hydroxybenzalpyruvate (alias *o*-hydroxybenzylidenepyruvate) aldolase from *P. vesicularis* DSM 6383 (strain BN6), which degrades 2-naphthalenesulfonates (*10*), even though they did not elucidate whether the substrate, 2′-hydroxybenzalpyruvate, was *cis*-form or *trans*-form. However, it appears that Kuhm *et al.* prepared *t*HBP for the substrate of 2′-hydroxybenzalpyruvate aldolase, because 2HC2CA is spontaneously converted to *t*HBP in alkaline solution (*6, 12*). As a result, 2′-hydroxybenzalpyruvate aldolase seems to be *t*HBP HA. Recently, we purified the 2HC2CA isomerase (Fig. 1, enzyme B) from *S. paucimobilis* TA-2 and elucidated that 2HC2CA was isomerized to *t*HBP enzymatically (*6*).

In the present article, we describe the presence of at least three *t*HBP HAs in *S. paucimobilis* TA-2. We also describe the purification of two typical HAs and the comparison of their characteristics of the two HAs, thereby contributing to the sparse literature on isozymes of *t*HBP HAs involved in naphthalene or naphthalenesulfonate metabolism.

MATERIALS AND METHODS

Cultivation and Enzyme Production—S. paucimobilis (formerly *Pseudomonas* sp.) TA-2 was cultivated in a nutrient medium (tryptone (Difco Laboratories), 1%; yeast extract (Difco Laboratories), 0.5%; NaCl, 0.5%) and synthetic media containing glucose (0.5%), succinate (0.2%), or

[1]To whom correspondence should be addressed. Tel: +81-6-6963-8061, Fax: +81-6-6963-8079, E-mail: ohmoto@omtri.city.osaka.jp

Abbreviations: CAPS, cyclohexyaminopropanesulfonic acid; DFP, diisopropyl fluorophosphate; HA, hydratase-aldolase; 2HC2CA, 2-hydroxychromene-2-carboxylate; MIA, monoiodoacetic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; NEM, *N*-ethylmaleimide; PCMB, *p*-chloromercuribenzoic acid; PMSF, phenylmethanesulfonyl fluoride; *t*HBP, *trans-o*-hydroxybenzylidenepyruvate.

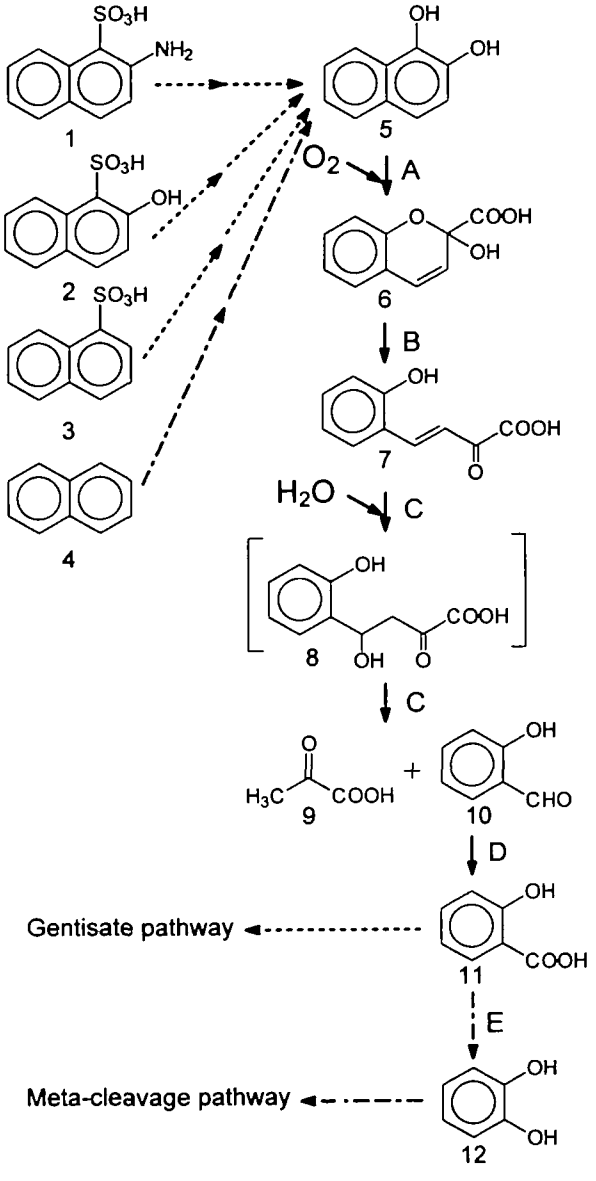

Fig. 1. **Proposed pathways for the metabolism of tobias acid and naphthalene to salicylate by *S. paucimobilis* TA-2 and NAH 7.** See Refs. *1–14* and *15*. Chemicals: 1, 2-naphthylamine-1-sulfonate (tobias acid); 2, 2-hydroxy-1-naphthalenesulfonate; 3, 1-naphthalenesulfonate; 4, naphthalene; 5, 1,2-dihydroxynaphthalene; 6, 2-hydroxychromene-2-carboxylate; 7, *trans-o*-hydroxybenzylidenepyruvate; 8, 2-oxo-4-hydroxy-4-(2′-hydroxyphenyl)butyrate; 9, pyruvate; 10, salicylaldehyde; 11, salicylate; 12, catechol. Enzymes: A, 1,2-dihydroxynaphthalene dioxygenase; B, 2-hydroxychromene-2-carboxylate isomerase; C, *trans-o*-hydroxybenzylidenepyruvate hydratase-aldolase; D, salicylaldehyde dehydrogenase; E, salicylate hydroxylase. Arrows: ·····►, pathways for *S. paucimobilis* TA-2; - - ►, pathways for NAH 7; ——►, pathways for *S. paucimobilis* TA-2 and NAH 7.

tobias acid (0.2%) as a sole carbon source and minerals as described in our previous paper (*1*). The cells were harvested from 320 ml of cultures in the late logarithmic growth phase, washed twice with 10 mM potassium phosphate buffer (pH 7.0), and resuspended (O.D. 20 at 660 nm) in the same buffer. Lysozyme was added to 10 ml of the suspension to a concentration of 0.1 mg/ml and the suspensions were incubated for at 30°C for 20 min. The cells were disrupted by sonication (Insonator 201M, Kubota) for 10 min, and cell debris was removed by centrifugation. Deoxyribonuclease I was added to the supernatants to a concentration of 0.02 mg/ml, and the solutions were dialyzed against 10 mM Tris-HCl (pH 7.4). Insoluble materials were removed by centrifugation, and the solutions were used as cell-free extracts. The protein concentration, which varied between 3.0 and 3.9 mg/ml, was adjusted to 3.0 mg/ml, and 3.0 ml of the solution was put on a Mono Q column (0.5×5 cm) equilibrated with 20 mM Tris-HCl buffer (pH 7.3). The enzymes were eluted with a linear concentration gradient of 0–0.3 M NaCl in the buffer. The activities of all fractions were measured.

Enzyme Assays—2HC2CA was prepared by use of 1,2-dihydroxynaphthalene dioxygenase from *S. paucimobilis* TA-2 as described in our previous paper (*6*). Since 2HC2CA isomerized slowly and spontaneously to *t*HBP in neutral or weak alkaline aqueous solution (*6, 12*), 250 μM 2HC2CA was incubated in 200 mM bicine buffer (pH 8.1) overnight at room temperature. Equilibrium was reached with the conversion of about 40% of 2HC2CA to *t*HBP, and this solution was used as a substrate. *t*HBP HA activity was assayed at 30°C for 5 min in a reaction mixture (80 μl) containing 80 μM *t*HBP, 120 μM 2HC2CA, 160 mM sodium-bicine buffer (pH 8.1), and 0.075–0.5 milliunit of the *t*HBP HA. The reaction was stopped by adding 15 μl of 1 M phosphoric acid. The reaction mixture was injected onto an HPLC apparatus (Shimadzu LC-5A), and the product (salicylaldehyde) was separated from the substrate and quantified as described in our previous paper (*1, 6*). One unit of each enzyme activity was defined as the amount that formed 1 μmol of salicylaldehyde per min at 30°C.

Purification of tHBP HA—All procedures were done below 4°C.

Step 1. Preparation of cell-free extracts: Bacterial cells in the late logarithmic growth phase were harvested from 14.5 liters of culture in nutrient medium, washed twice with 10 mM potassium phosphate buffer (pH 7.0), resuspended in the same buffer, then disrupted with an ultrasonic oscillator. The supernatant obtained by centrifugation at 15,000 ×*g* was used as the cell-free extract. Streptomycin sulfate was added to the cell-free extract to a concentration of 0.7%, and pH was adjusted to 7.4. The precipitate formed was removed by centrifugation (15,000 ×*g*), and the supernatant was dialyzed against 30 mM Tris-HCl buffer (pH 7.4).

Step 2. DEAE-cellulose column chromatography: The dialyzed solution was put on a DEAE-cellulose column (2.6×40 cm) equilibrated with 30 mM Tris-HCl buffer. The column was washed with the same buffer and eluted with 30 mM Tris-HCl buffer containing 0.3 M NaCl. The eluate was dialyzed against 30 mM Tris-HCl buffer (pH 7.4).

Step 3. DEAE-Toyopearl column chromatography: The dialyzed solution was put on a DEAE-Toyopearl 650M column (2.6×40 cm) equilibrated with 30 mM Tris-HCl buffer (pH 7.3). The column was washed with the buffer and eluted with a linear concentration gradient of 0–0.3 M NaCl in the buffer. The enzyme activity was eluted at 0.1 M NaCl and 0.15 M NaCl. The two eluates were concentrated separately by ultrafiltration.

Step 4. Gel filtration: Each enzyme solution was filtered

through a Sephacryl S-100 column (2.6×90 cm) equilibrated with 30 mM Tris-HCl buffer containing 0.2 M NaCl, and the active fractions were combined and dialyzed against 1 mM potassium phosphate buffer (pH 7.2).

Step 5. Hydroxyapatite column chromatography: The dialyzed solutions were put on hydroxyapatite columns (2.6 × 10 cm) equilibrated with 2 mM potassium phosphate buffer (pH 7.2). The columns were washed with 2 mM potassium phosphate buffer and eluted with a linear concentration gradient of 2–500 or 2–150 mM potassium phosphate buffer (pH 7.2). The enzyme activities were found at about 200 and 35 mM phosphate buffer, respectively.

Step 6. Mono Q column chromatography: The dialyzed solutions were put on Mono Q columns (0.5×5 cm) equilibrated with 20 mM Tris-HCl (pH 7.3). The enzymes were eluted with a linear concentration gradient of 0–0.3 M NaCl in the buffer. The enzyme activities were eluted at about 0.2 and 0.25 M NaCl, respectively. The eluates were concentrated by ultrafiltration and dialyzed against 20 mM Tris-HCl buffer (pH 7.3). The enzyme solutions were used as the final preparations of *t*HBP HA A and *t*HBP HA B, respectively.

Protein Determination—Protein was determined by measuring the absorbance at 280 nm of the sample solution or by the method of Lowry *et al.* (*21*) using BSA as a standard.

Polyacrylamide Gel Electrophoresis (PAGE), Peptide Mapping and Isoelectric Focusing—Native PAGE was done with a 7.5% acrylamide slab gel by the method of Gabriel at pH 9.4 (*22*). SDS-PAGE was done in 17% acrylamide and 0.1% SDS with a discontinuous Tris-glycine buffer system as described by Laemmli (*23*), and peptide mapping was done for the purified HAs with *Staphylococcus aureus* V8 protease as described by Cleveland *et al.* (*24*). Isoelectric focusing (IEF) was done according to the Model 111 Mini IEF Cell instruction manual (Bio-Rad, USA). Protein bands in the gel were stained with Coomassie Brilliant Blue R-250.

Analytical Ultracentrifugation—Molecular mass was determined for *t*HBP HA A and B by sedimentation equilibrium using a HITACHI 282 Analytical Ultracentrifuge. The sedimentation equilibrium experiments were conducted in 20 mM Tris-HCl (pH 7.3). Samples were loaded into cells and scanned at 280 nm at *ca.* 6×10^{-6} M monomer at 8,000 rpm. Equilibrium was presumed to be reached when no difference was detected between scans 3 h apart. The time for a run was 24 h. The partial specific volume was calculated as 0.727 for *t*HBP HA A and 0.733 for *t*HBP HA B based upon the amino acid composition of the proteins.

N-Terminal Amino Acid Sequence of tHBP HA—The purified enzymes (*ca.* 350 pmol) were electrophoresed by SDS-PAGE and blotted onto a polyvinylidene difluoride membrane. Their N-terminal amino acid sequences were analyzed by automated Edman degradation using an Applied Biosystem model 471A gas-phase sequencer. Homology searches were done using DNA and protein sequence databases obtained from GenBank, PIR, and SwissProt.

Effects of Various Reagents on the Hydratase-Aldolase Activity—*t*HBP HA was assayed under the standard assay conditions, except that inhibitors, chelators, and metal ions were added to the reaction mixture at 1 mM at the start of reaction. In separate experiments, the enzyme was preincubated with reagents before the reaction. For reagents other than sodium borohydride ($NaBH_4$), 6 µl of the enzyme (5.1 milliunits) was mixed with 1 µl of the inhibitor (20 mM) and 13 µl of Tris-HCl buffer (60 mM, pH 7.4), the mixtures were incubated at 25°C for 1 h, and 15 µl of the mixtures were used for the enzyme assay. Aldolases are generally divided into a divalent metal ion catalysis group and a Schiff base forming group. Because the aldolases forming Schiff bases are sensitive to Schiff base reducing reagents, *e.g*, $NaBH_4$, the effect of $NaBH_4$ on *t*HBP HA activity in the presence of the substrate or the products was examined (*10*, *25*). Thirty microliters of the enzyme (0.05 unit) was mixed with 18 µl of the Tris-HCl buffer (200 mM, pH 7.6), 2 µl of the $NaBH_4$ (1 M) dissolved in 0.02 N NaOH, and 50 µl of salicylaldehyde (1 mM), pyruvate (1 mM or 20 mM), or the mixture of *t*HBP (1 mM) and 2HC2CA (1.5 mM). After incubation at 25°C for 10 min, the enzyme solution was dialyzed against 110 ml of Tris-HCl buffer (20 mM, pH 7.6) overnight at 4°C. The residual activity was measured.

RESULTS

tHBP HA in Cell-Free Extracts—*S. paucimobilis* TA-2 was cultured in the nutrient medium or the mineral salt medium containing tobias acid (0.2%), succinate (0.2%), or glucose (0.5%). The *t*HBP HA activities of cell-free extracts were examined as described in "MATERIALS AND METHODS," and the production of *t*HBP HAs by the cell-free extracts was compared. When the cell-free extracts from the cells grown on the four media were chromatographed on the Mono Q column, three active fractions (A, C, and B, based on elution order) were obtained in all cases (Fig. 2). The activities of fractions A and B were approximately equal in all chromatograms except for the slightly higher activities of fraction B from the cells grown on tobias acid, but the activities of fraction C varied depending on the medium on which the cells were cultivated: the activity of fraction C from the cells grown on tobias acid was higher than the others and that from the cells grown on succinate was lower. Next, each fraction was preincubated with 0.25 mM *p*-chloromercuribenzoic acid (PCMB) for 1 h before the reaction, and the activities were measured. The activities of fraction A were not inhibited, whereas those of fraction B were completely inhibited and those of fraction C were partially inhibited. These results provide evidence for the presence of at least three *t*HBP HAs with different properties and their involvement in the degradation of tobias acid in *S. paucimobilis* TA-2.

Purification and Molecular Weight of tHBP HAs from S. paucimobilis TA-2—The *t*HBP HAs were purified to homogeneity from the cell-free extract of *S. paucimobilis* TA-2 as described under "MATERIALS AND METHODS." Table I shows summarizes of the purification of *t*HBP HA A and B for fractions A and B, respectively. The specific activities of *t*HBP HA A and B were 11 and 7.7 units per mg of protein, respectively. The final preparations appeared to be homogeneous on both native PAGE and SDS-PAGE (Fig. 3). The molecular masses of the enzymes were estimated to be 37.6 and 37.2 kDa by SDS-PAGE (Fig. 3A). To estimate molecular mass under non-denatured conditions, ultracentrifugation was done as described under "MATERIALS AND METHODS". The molecular mass was estimated to be 116 kDa for *t*HBP HA A and 104 kDa for *t*HBP HA B. These

findings suggest that the enzymes are trimeric proteins.

N-Terminal Amino Acid Sequencesof tHBPHAs—The N-terminal amino acid sequences of the HAs were determined and aligned with those of *t*HBP HAs, which are also called 2′-hydroxybenzalpyruvatealdolases, from NAH7 plasmid (*nahE*) (*14*), *Pseudomonas aeruginosa* strain PaK1 (*pahE*) (*18*), *Pseudomonas putida* strain OUS82 (*pahE*) (*15, 19*), *Pseudomonas* sp. strain C18 (*doxI*) (*13*), *Burkholderia* sp. strain RP007 (*16*), *Pseudomonas putida* plasmid NPL-1 (*nahE*) (*20*), *Sphingomonas aromaticivorans* F119 (*nahE*) (*17*), and *Pseudomonas vesicularis* DSM 6383 (strain BN6) (*10*) (Fig. 4). The alignment revealed two patterns of N-terminal sequences, and the sequences of the TA-2 HAs show homology with the sequences of different hatterns. The sequences of *t*HBP HA A and B were most highly homologous with those of NAH7 (*nahE*) HA and strain BN6 HA, respectively. Furthermore, the peptide mapping of the HAs showed different patterns (Fig. 3A). However, the N-terminal amino acid sequences of the two HAs contain homologous sequences with each other.

Physicochemical Characteristics of the Enzymes—The effects of temperature on the purified HAs from *S. paucimobilis* TA-2 were examined. After incubation for 5 min in

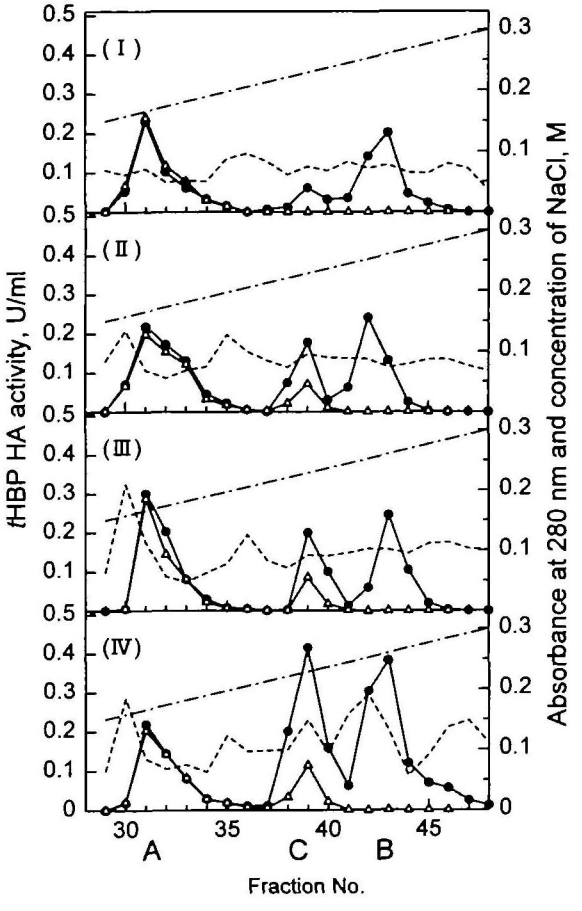

Fig. 2. **Mono Q column chromatography of cell-free extracts from *S. paucimobilis* TA-2 grown in various media.** The cells were cultivated in succinate medium (I), glucose medium (II), nutrient medium (III), and tobias acid medium (IV). Conditions: column, 0.5×5 cm; elution, a linear concentration gradient of 0–0.3 M NaCl in 20 mM Tris-HCl buffer (pH 7.3). Symbols: ●, *t*HBP HA activity; △, *t*HBP HA activity after preincubation with 0.25 mM *p*-chloromercuribenzoic acid for 1 h; ---, absorbance at 280 nm; —•—, concentration of NaCl.

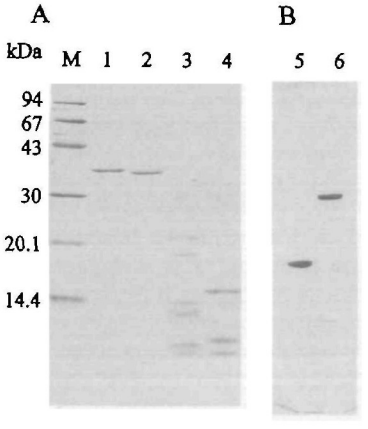

Fig. 3. **PAGE of the purified *t*HBP HA A and B and their peptide mapping.** (A) SDS-PAGE (17% gel). Lane 1, *t*HBP HA A from the Mono Q column; lane 2, *t*HBP HA B from the Mono Q column; lane 3, *t*HBP HA A (4.8 g) after digestion (37°C, 2 h) with *Staphylococcus aureus* V8 protease (1.25 g); lane 4, *t*HBP HA B (4.8 g) after digestion (37°C, 2 h) with *Staphylococcus aureus* V8 protease (1.25 g); lane M, molecular mass markers: phosphorylase *b* (94 kDa), albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa), and -lactalbumin (14.4 kDa). (B) Native-PAGE. Electrophoresis (7.5% gel) was performed at pH 9.4. Lane 5, *t*HBP HA B from the Mono Q column; lane 6, *t*HBP HA A from the Mono Q column.

TABLE I. **Purification of *t*HBP HA A and B from *S. paucimobilis* TA-2.**

Step	Volume (ml)	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Purification	Recovery (%)
Crude extract	530	2,120	16,800	0.13	1	100
(*t*HBP HA A)						
DEAE-Toyopearl 650M	265	420	1,340	0.31	2.5	20
Sephacryl S-100 HR	110	308	237	1.3	10	15
Hydroxyapatite	43.5	119	20.5	5.8	46	5.6
Mono Q	5.95	70.2	6.59	11	84	3.3
(*t*HBP HA B)						
DEAE-Toyopearl 650M	188	474	1,290	0.37	2.9	22
Sephacryl S-100 HR	24.0	92.4	171	0.54	4.3	4.4
Hydroxyapatite	53.5	82.1	30.0	2.7	22	3.9
Mono Q	6.98	79.0	10.3	7.7	61	3.7

Source	Plasmid	Gene	N-terminal amino acid sequence	Reference
Burkholderia sp. strain RP007		*phnE*	M S K Q R K Q R L G T E D V N G A W V I M P T P	16
Pseudomonas putida	NPL-1	*nahE*	M S N K I M K T S R L T A E D I N G A W T I M P T P	20
Pseudomonas sp. C18		*doxI*	M S N K I M K T S R L T A E D I N G A W T I M P T P	13
Pseudomonas putida strain OUS82		*pahE*	M L N K I S K T A R L T A E D I N G A W T I M P T P	15, 19
Pseudomonas aruginosa strain PaK1		*pahE*	M S N K I M K T S R L T A E D I N G A W T I M P T P	18
Pseudomonas putida G7	NAH7	*nahE*	M L N K V I K T T R L T A E D I N G A W T I M P T P	14
***Sphingomonas paucimobilis* TA-2 (*t*HBP HA A)**			T R K T S K A T R L T A E D I Q G A W V I M P T P	This study
***Sphingomonas paucimobilis* TA-2 (*t*HBP HA B)**			A R T L M K P D D V K G A W A I I P T P A K D D A T	This study
Pseudomonas vesicularis DSM 6383 (strain BN6)			A R T L V K P D D V K G A W A I I P T P A K D D A T	10
Sphingomonas aromaticivorans F119	pNL1	*nahE*	M A R E L L T A A D V K G A W A I V P T P A K E G A S	17

Fig. 4. **N-terminal amino acid sequence alignment of *t*HBP HAs of *S. paucimobilis* TA-2 and *t*HBP HAs of different origins.**

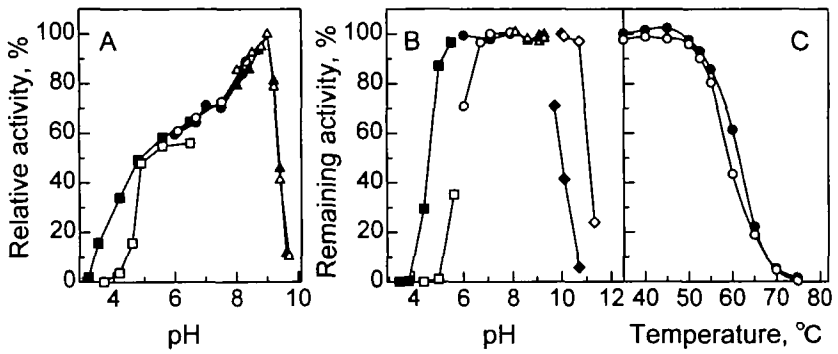

Fig. 5. **Effects of pH and temperature on *t*HBP HA activity and stability, and activation.** (A) Optimum pH: the enzyme activity was measured in 250 mM acetate buffer (*t*HBP HA A, ■; *t*HBP HA B, □), 250 mM MOPS buffer (*t*HBP HA A, ●; *t*HBP HA B, ○) and 250 mM bicine buffer (*t*HBP HA A, ▲; *t*HBP HA B, △). (B) pH stability: the remaining activity was measured under the standard assay conditions after incubation at various pHs for 20 h at 4°C. The following buffers were used: 40 mM acetate (*t*HBP HA A, ■; *t*HBP HA B, □), 40 mM MOPS buffer (*t*HBP HA A, ; *t*HBP HA B, ○), 40 mM bicine buffer (*t*HBP HA A, ▲; *t*HBP HA B, △), 40 mM CAPS buffer (*t*HBPHA A, ◆; *t*HBP HA B, ◆). (C) Thermal stability: the remaining activity was measured after incubation at various temperatures for 5 min in 20 mM Tris-HCl buffer (pH 7.3).

20 mM Tris-HCl buffer (pH 7.3), the HAs were stable at up to 50°C, and about 20% of the original activity remained after the incubation at 65°C (Fig. 5C). The effects of pH on stability were also examined. HA A was stable in the range of pH 6.0 to 9.3 (at 4°C for 20 h), whereas HA B was stable in the range of pH 7.1 to 10.7 (at 4°C for 20 h) (Fig. 5B). The effects of pH on activity are shown in Fig. 5A: the optimum pH of both enzymes was 9. The isoelectric point (pI) of *t*HBP HA A and B was determined by isoelectric focusing to be 4.8 and 4.3, respectively. Lineweaver-Burk plots gave K_m values for *t*HBP of 3 and 9 μM, respectively.

Effects of Various Compounds—The effects of SH reagents, chelators, and metal ions were examined. The experimental procedures were as described under "MATERIALS AND METHODS." When added at 1 mM at the start of the reaction, Hg^{2+} caused moderate inhibition (40%) of the *t*HBP HA B activity, while the other reagents, namely, monoiodoacetic acid (MIA), *N*-ethylmaleimide (NEM), PCMB, diisopropyl fluorophosphate (DFP), phenylmethanesulfonyl fluoride (PMSF), EDTA, NaN_3, KCN, *o*-phenanthroline, 2,2′-bipyridine, Fe^{2+}, Fe^{3+}, Mg^{2+}, Mn^{2+}, Co^{2+}, Ca^{2+}, Zn^{2+}, Cd^{2+}, Cu^{2+}, and Ni^{2+}, were ineffective. None of these reagents interfered remarkably with the *t*HBP HA A activity. To investigate the inhibitory effects of chelators and SH reagents further, the HAs were preincubated with EDTA, *o*-phenanthroline, 2,2′-bipyridine, KCN, MIA, NEM, or PCMB at 1 mM for 30 min at 25°C, then the activities were assayed. PCMB and NEM were strongly (100%) and moderately (35%) inhibitory toward *t*HBP HA B activity, respectively, and the other reagents were not. More than 85% of the initial activity of *t*HBP HA A remained.

Kuhm *et al.* indicated that 2′-hydroxybenzalpyruvate aldolase from *Pseudomonas vesicularis* DSM 6383 (strain BN6) belongs to class 1 aldolases forming Schiff-base intermediates (*10*). We examined the sensibility of *t*HBP HAs from *S. paucimobilis* TA-2 to the Schiff-base reducing agent $NaBH_4$ (Table II). The experimental procedures were as described under "MATERIALS AND METHODS". More than 89% of HA A and B was inactivated by the treatment with 20 mM $NaBH_4$ together with 0.5 mM *t*HBP or 10 mM pyruvate, whereas both HAs remained intact after the

TABLE II. **Inactivation of *t*HBP HAs after sodium borhydride reduction.** Experimental procedures are described under "MATERIALS AND METHODS."

Substrate or product	Conc. of substrate or product (mM)	Conc. of $NaBH_4$ (mM)	Remaining activity (%)	
			HA A	HA B
Salicylaldehyde	0.5	20	93	102
Salicylaldehyde	0.5	0	91	100
Pyruvate	10	20	2.6	11
Pyruvate	10	0	95	95
Pyruvate	0.5	20	83	90
Pyruvate	0.5	0	98	102
*t*HBP	0.5	20	3.2	8.8
*t*HBP	0.5	0	85	97
None (after dialysis)	0	20	96	97
None (after dialysis)	0	0	98	102
None (initial activity)	0	0	100	100

treatment with 20 mM $NaBH_4$ together with salicylaldehyde, or with each individual compound.

DISCUSSION

The cell-free extracts from the cells of *S. paucimobilis* TA-2 grown in succinate, glucose, tobias acid, and nutrient medium were applied on a Mono Q column, and three active fractions (fraction A, C, and B) were obtained (Fig. 2). The results of chromatography suggested that the HA(s) corresponding to fraction C were induced by tobias acid, whereas the HAs corresponding to fractions A and B were constitutive. Because there is a noteworthy difference in inhibition of the *t*HBP HA activity of fraction A and B by PCMB, we purified the constitutive *t*HBP HAs, namely, *t*HBP HA A and B, and characterized them. *t*HBP HA A and B were isozymes. This conclusion was confirmed by the differences in the N-terminal amino acid sequence and the pattern of the peptide fragments produced on the peptide mapping.

Some characteristics were common to *t*HBP HA A and B. The molecular masses were about 37 kDa on SDS-PAGE (Fig. 3) and about 110 kDa on analytical ultracentrifugation. This suggested that the enzymes consist of three identical subunits. Both were inactivated by $NaBH_4$ in the presence of *t*HBP or pyruvate (Table II), and neither was inhibited by chelators or activated by metal ions. Therefore, they belonged to class I aldolases, which form Schiff-base intermediates, not to class II aldolases, which use a divalent metal ion at the active site (*10, 25, 26*). These results agreed with the results for 2′-hydroxybenzalpyruvate aldolase from strain BN6 obtained by Kuhm *et al.* (*10*). In addition, the *t*HBP HAs had a similar thermal stability (<50°C) and optimum pH (pH 9).

On the other hand, there were several distinctions between *t*HBP HA A and B. *t*HBP HA B was inactivated by PCMB and NEM, but *t*HBP HA A was not. *t*HBP HA B was also inhibited by Hg^{2+}. These results suggested the presence of a SH group at or near the active site of *t*HBP HA B. The K_m value for *t*HBP of *t*HBP HA A was 3 μM, whereas that of *t*HBP HA B was 9 μM. The N-terminal amino acid sequences and the pattern of the peptide fragments on the peptide mapping were also different. The N-terminal sequences of *t*HBP HAs of several origins were found to form two groups on the basis of homology, one including the *t*HBP HA from NAH7 and the *t*HBP HA A, and the other including the *t*HBP HA from strain BN6 and the *t*HBP HA B. Kuhm *et al.* reported that 2′-hydroxybenzalpyruvate aldolase from strain BN6 was inactivated by PCMB and its K_m value was 17 μM, and the value was higher than the K_m values (4 μM) of *t*HBP HA from the cell-free extract of NAH7 (*10*). From the limited data presently available, it appears that *t*HBP HAs of the former group have low K_m values (<*ca.* 4 μM) and are not inactivated by PCMB, and *t*HBP HAs of the latter group have rather high K_m values (>*ca.* 9 μM) and are inactivated by PCMB.

Eaton assumed that *t*HBP HA of strain BN6, which belongs to the latter group, had undergone the removal of six amino acids from the N-terminal end (*14*). However, we obtained two kinds of N-terminal amino acid sequences from the identical bacteria at the same time. The N-terminal amino acid sequence of *t*HBP HA B appears to have undergone the removal of only one amino acid (methionine), because the deduced N-terminal amino acid sequence of *t*HBP HA B from *Sphingomonas aromaticivorans* F119 (*17*), which belongs to the latter group, corresponds to its open reading frame (Fig. 4). Furthermore, the N-terminal amino acid sequence of *t*HBP HA A also appears to have undergone the removal of methionine. *t*HBP HA appears, therefore, to have no special removal process except for methionine.

REFERENCES

1. Ohe, T., Ohmoto, T., Kobayashi, Y., Sato, A., and Watanabe, Y. (1990) Metabolism of naphthalenesulfonic acids by *Pseudomonas* sp. TA-2. *Agric. Biol. Chem.* **54**, 669–675
2. Ohmoto, T., Sakai, K., Hamada, N., and Ohe, T. (1991) Salicylic acid metabolism through a gentisate pathway by *Pseudomonas* sp. TA-2. *Agric. Biol. Chem.* **55**, 1733–1737
3. Brilon, C., Beckmann, W., and Knackmuss, H.-J. (1981) Catabolism of naphthalenesulfonic acids by *Pseudomonas* sp. A3 and *Pseudomonas* sp. C22. *Appl. Environ. Microbiol.* **42**, 44–55
4. Nörtemann, B., Baumgarten, J., Rast, H.G., and Knackmuss, H.-J. (1986) Bacterial communities degrading amino- and hydroxynaphthalene-2-sulfonates. *Appl. Environ. Microbiol.* **52**, 1195–1202
5. Kuhm, A.E., Knackmuss, H.-J., and Stolz, A. (1993) 2-Hydroxychromene-2-carboxylate isomerase from bacteria that degrade naphthalenesulfonate. *Biodegradation* **4**, 155–162
6. Ohmoto, T., Kinoshita, T., Moriyoshi, K., Sakai, K., Hamada, N., and Ohe, T. (1998) Purification and some properties of 2-hydroxychromene-2-carboxylate isomerase from naphthalenesulfonate-assimilating *Pseudomonas* sp. TA-2. *J. Biochem.* **124**, 591–597
7. Gibson, D.T. and Subramanian, V. (1984) Microbial degradation of aromatic hydrocarbons in *Microbial Degradation of Organic Compounds* (Gibson, D.T., ed.) pp. 181–252, Marcel Dekker, New York
8. Yen, K.-M. and Gunsalus, I.C. (1982) Plasmid gene organization: Naphthalene/salicylate oxidation. *Proc. Natl. Acad. Sci. USA* **79**, 874–878
9. Kuhm, A.E., Stolz, A., Ngai, K.-L., and Knackmuss, H.-J. (1991) Purification and characterization of a 1,2-dihydroxynaphthalene dioxygenase from a bacterium that degrades naphthalenesulfonic acids. *J. Bacteriol.* **173**, 3795–3802
10. Kuhm, A.E., Knackmuss, H.-J., and Stolz, A. (1993) Purification and properties of 2′-hydroxybenzalpyruvate aldolase from a bacterium that degrades naphthalenesulfonates. *J. Biol. Chem.* **268**, 9484–9489
11. Barnsley, E.A. (1976) Naphthalene metabolism by *Pseudomonas*: The oxidation of 1,2-dihydroxynaphthalene to 2-hydroxychromene-2-carboxylic acid and the formation of 2′-hydroxybenzalpyruvate. *Biochem. Biophys. Res. Commun.* **72**, 1116–1121
12. Eaton, R.W. and Chapman, P.J. (1992) Bacterial metabolism of naphthalene-construction and use of recombinant bacteria to study ring cleavage of 1,2-dihydroxynaphthalene and subsequent reactions. *J. Bacteriol.* **174**, 7542–7554
13. Denome, S.A., Stanley, D.C., Olson, E.S., and Young, K.D. (1993) Metabolism of dibenzothiophene and naphthalene in *Pseudomonas* strains: complete DNA sequence of an upper naphthalene. *J. Bacteriol.* **175**, 6890–6901
14. Eaton, R.W. (1994) Organization and evolution of naphthalene catabolic pathways: Sequence of the DNA encoding 2-hydroxychromene-2-carboxylate isomerase and *trans*-*o*-hydroxybenzylidenepyruvate hydratase-aldolase from the NAH7 plasmid. *J. Bacteriol.* **176**, 7757–7762
15. Kiyohara, H., Torigoe, S., Kaida, N., Asaki, T., Iida, T., Hayashi, H., and Takizawa, N. (1994) Cloning and characterization of a chromosomal gene cluster, *pah*, that encodes the upper pathway for phenanthrene and naphthalene utilization

by *Pseudomonas putida* OUS82. *J. Bacteriol.* **176**, 2439–2443
16. Laurie, A.D., Lloyd-Jones, G. (1999) The *phn* genes of *Burkholderia* sp. strain RP007 constitute a divergent gene cluster for polycyclic aromatic hydrocarbon catabolism. *J. Bacteriol.* **181**, 531–540
17. Romine, M.F., Stillwell, L.C., Wong, K.K., Thurston, S.J., Sisk, E.C., Sensen, C., Gaasterland, T., Fredrickson, J.K., and Saffer, J.D. (1999) Complete sequence of a 184-kilobase catabolic plasmid from *Sphingomonas aromaticivorans* F199. *J. Bacteriol.* **181**, 1585–1602
18. Takizawa, N., Iida, T., Yamauchi, K., Satoh, S., Wang, Y., Fukuda, M., and Kiyohara, H. (1996) GenBank accession no. D84146
19. Takizawa, N., Iida, T., Sawada, T., Yamauchi, K., Kubo, M., Wang, Y., Fukuda, M., and Kiyohara, H. (1997) GenBank accession no. AB004059
20. Bezborodnikov, S.G., Boronin, A.M., and Tiedje, J.M. (1997) GenBank accession no. AF010471
21. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) Protein measurement with the folin phenol reagent. *J. Biol. Chem.* **193**, 265–275
22. Gabriel, O. (1971) Analytical disc gel electrophoresis in *Methods in Enzymology* (Jacoby, W.B., ed.) Vol. 22, pp. 565–578, Academic Press, New York
23. Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685
24. Cleveland, D.W., Fischer, S.G., Kirschner, M.W., and Laemmli, U.K. (1977) Peptide mapping by limited proteolysis in sodium dodecyl sulfate and analysis by gel electrophoresis. *J. Biol. Chem.* **252**, 1102–1106
25. Lebherz, H.G. and Rutters, W.J. (1973) A Class I (Schiff Base) fructose diphosphate aldolase of prokaryotic origin: Purification and properties of *Micrococcus aerogenes* aldolase. *J. Biol. Chem.* **248**, 1650–1659
26. Lawrence, M.C., Barbosa, J.A., Smith, B.J., Hall, N.E., Pilling, P.A., Ooi, H.C., and Marcuccio, S.M. (1997) Structure and mechanism of a sub-family of enzymes related to *N*-acetylneuraminate lyase. *J. Mol. Biol.* **266**, 381–399