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Purification and Characterization of *Acinetobacter calcoaceticus* 4-Hydroxybenzoate 3-Hydroxylase after Its Overexpression in *Escherichia coli*[1]

Javier Fernandez,*[,2] Anthony A. Dimarco,† L. Nicholas Ornston,† and Shigeaki Harayama*[,3]

*Department of Medical Biochemistry, University Medical Center, 1 rue Michel Servet, 1211 Geneva, Switzerland; and †Department of Biology, Yale University, New Haven, CT 06511, USA

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4-Hydroxybenzoate 3-hydroxylase [EC 1.14.13.2] from *Acinetobacter calcoaceticus* was purified to homogeneity following the 40-fold overexpression of this gene (*pobA*) in *Escherichia coli*. Overexpression was accomplished by placing the *folA* gene (encoding trimethoprim-resistant dihydrofolate reductase) directly downstream of the *pobA* gene, and demanding growth of recombinants on elevated concentration of trimethoprim. Presumably, the surviving variants have undergone a genetic alteration which allowed the overexpression of both *folA* and *pobA*. 4-Hydroxybenzoate 3-hydroxylase was purified in two chromatographic steps, characterized biochemically, and its properties were compared to those of its homolog from *Pseudomonas fluorescens*. The two enzymes differ in their reponse to Cl^- ion inhibition. A single amino acid change in the putative NADPH-binding site is proposed to account for this difference. The inhibitory and catalytic properties of substrate analogs were also examined.

Key words: *Acinetobacter calcoaceticus*, dihydrofolate reductase, 4-hydroxybenzoate 3-hydroxylase, overexpression, *pobA*.

4-Hydroxybenzoate 3-hydroxylase is a monooxygenase which converts 4-hydroxybenzoate to protocatechuate for further metabolism by one of the two branches of the β-ketoadipate pathway. The complete amino acid sequences of the isofunctional 4-hydroxybenzoate 3-hydroxylases from *Pseudomonas fluorescens* and *Pseudomonas aeruginosa* have been determined (*1-6*). The three-dimensional structure of the oxidized *Pseudomonas* enzymes complexed with the substrate has been elucidated (*7-9*) as well as the structures of the substrate-free enzyme, the reduced enzyme complexed with the substrate and the enzyme-product complex (*10, 11*). Identification of the amino acid residues of this enzyme which may be important for substrate recognition, co-factor binding and enzyme catalysis has been derived from X-ray crystallography data and computer-modelling analysis (*7, 12*). Furthermore, chemical modification and site-directed mutagenesis elucidated the roles of some residues in the catalytic reaction (*5, 13, 14*).

Recently, we have cloned the structural (*pobA*) and regulatory (*pobR*) genes for 4-hydroxybenzoate 3-hydroxylase of *Acinetobacter calcoaceticus* (*15*) and determined the nucleotide sequence (*16, 17*). The deduced amino acid sequence shows that 4-hydroxybenzoate 3-hydroxylase from *A. calcoaceticus* is a single polypeptide of 404 amino acid residues. While the *pobA* nucleotide sequence from *A. calcoaceticus* is very different from the *pobA* genes in the *Pseudomonas* species (42% identity), the amino acid sequence of the *Acinetobacter* enzyme shows 64% identity (90% overall similarity) to the enzyme from *P. fluorescens*. Nevertheless several replacements occur at or near the active site, or at positions where cofactor binding is believed to occur, suggesting that the *Acinetobacter* enzyme may exhibit different properties from the previously characterized *Pseudomonas* enzymes. This paper reports the purification and characterization of 4-hydroxybenzoate 3-hydroxylase produced from the *A. calcoaceticus pobA* gene cloned in *Escherichia coli*.

MATERIALS AND METHODS

Materials—Growth media were supplied by Difco (USA). Antibiotics, NADPH, and FAD were from Sigma Chemical (USA), and 4-hydroxybenzoate, its structural analogs, and various chemicals were purchased either from Fluka AG (Switzerland) or Aldrich (Germany). A TSK-Gel DEAE-5PW column (150×21.5 mm) and a Bio-Sil TSK-250 gel filtration column (300×7.5 mm) were from Tosoh (Japan), while Mitsui-Toatsu (Japan) supplied an HCA-hydroxyapatite column (100×7.6 mm). The Bio-Rad protein assay kit and the protein standards for gel filtration chromatography were purchased from Bio-Rad Laboratories (USA). Pharmacia (Sweden) supplied the protein

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[2] Present address: Department of Molecular Biochemistry and Biology, Faculty of Veterinary Science, Universidad Complutense, Madrid, Spain.

[3] To whom correspondence should be addressed at the present address: Marine Biotechnology Institute, Kamaishi Laboratories, 3-75-1 Heita, Kamaishi, Iwate 026, Japan. Tel: +81-193-26-6544, FAX: +81-193-26-6592

standards for SDS/polyacrylamide gel electrophoresis.

Bacterial Strains, Plasmids, and Culture Conditions—Cultures of the wild-type strain of *A. calcoaceticus* ADP1 (*18*) were grown in minimal medium M9 (*19*) supplemented with minor elements (*20*) and 5 mM 4-hydroxybenzoate as a source of carbon and energy. *E. coli* JM83 and JM101 (*21*) cultures were grown in Luria-broth under appropriate antibiotic selection. The plasmids used are shown in Fig. 1. The induction of the T7 promoter was carried out as described (*22*).

Isolation of Plasmid DNA—Plasmid DNA was purified from *E. coli* JM101 by the alkaline lysis procedure (*23*) with cesium chloride-ethidium bromide density gradient centrifugation. The same method without density gradient centrifugation was used for mini-scale preparations of plasmids.

Preparation of Cell-Free Extract—4-Hydroxybenzoate 3-hydroxylase was prepared from cultures of JM83-(pZR4057) grown in 3 liter of Luria-broth containing ampicillin (100 g/ml). Following inoculation with a preculture of JM83(pZR4057) grown in the same medium, the culture was incubated aerobically at 37°C with shaking, and harvested at the late logarithmic phase of growth (14-15 h). The yield was approximately 3-4 g wet cells per liter. The cells were washed twice with 20 mM potassium phosphate buffer (pH 7.2) containing 0.3 mM EDTA, resuspended in 1/100 initial volume of the same buffer, and ruptured by two passages through a precooled French press (SLM Instruments, USA) with a pressure differential of 76 MPa at the orifice. The broken-cell preparation was centrifuged at $20,000 \times g$ at 4°C for 20 min to remove the cell debris, and the supernatant was further centrifuged at $100,000 \times g$ at 4°C for 60 min in a Beckman 60Ti rotor.

Purification of 4-Hydroxybenzoate 3-Hydroxylase—The cell extract was applied to a TSK-BioGel DEAE-5PW anion-exchange column (150×21.5 mm) preequilibrated in 20 mM potassium phosphate buffer (pH 7.2). The enzyme was eluted by a linear gradient of 0 to 0.5 M Na_2SO_4 in 300 ml of 20 mM potassium phosphate buffer (pH 7.2) at a flow rate of 5 ml/min. The active fractions were combined and filtered through a Nalgene nitrocellulose membrane (0.45 μm pore size). The addition of exogenous FAD to these fractions increased the apparent activity of 4-hydroxybenzoate 3-hydroxylase, indicating that a fraction of 4-hydroxybenzoate 3-hydroxylase had been resolved of FAD. Prior to further purification, the active fraction was incubated with an excess amount of FAD (1 mM) for 30 min to reconstitute the FAD-bound enzyme. This preparation was applied to a Bio-Gel P-6-D column (Bio-Rad) preequilibrated with 20 mM phosphate buffer (pH 7), and eluted with the phosphate buffer. Almost all the enzyme applied was recovered in the first 3 ml, while free FAD was eluted later. The enzyme solution was subsequently diluted with water to reduce the concentration of phosphate buffer to 10 mM, loaded onto an HCA-hydroxyapatite column preequilibrated with 10 mM potassium phosphate buffer (pH 7), and eluted with the starting buffer at a flow rate of 1 ml/min. Unlike the majority of other proteins, 4-hydroxybenzoate 3-hydroxylase was not adsorbed. Yellow fractions containing enzyme activity were collected and stored at −70°C until used.

Gel Filtration Chromatography—Molecular weight of the native enzyme was estimated on a Bio-Sil TSK-250 gel filtration column calibrated with Bio-Rad gel filtration standards; samples were run in 50 mM potassium phosphate buffer pH 7 containing 0.15 M NaCl at a flow rate of 0.3 ml/min. The standard proteins were thyroglobulin (670 kDa), gamma globulin (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa), and vitamin B_{12} (1.35 kDa).

Polyacrylamide Gel Electrophoresis—Gel electrophoresis of purified 4-hydroxybenzoate 3-hydroxylase was carried out in a 12% (w/v) polyacrylamide slab gel containing 0.1% (w/v) sodium dodecyl sulfate (SDS) using 375 mM Tris-HCl (pH 8). After the run, the gel was stained with 0.1% (w/v) Coomassie Blue R250 in an aqueous solution containing 30% (v/v) methanol and 10% (v/v) acetic acid for 1 h, and destained in 30% (v/v) methanol/10% (v/v) acetic acid. The molecular mass of the enzyme was obtained from a comparison of its relative mobility to those of the standard proteins (phosphorylase *b*, 94 kDa; albumin, 67 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 30 kDa; trypsin inhibitor, 20.1 kDa; and α-lactalbumin, 14.4 kDa).

Protein Assay—Protein concentration was determined by the method of Bradford (*24*) using a Bio-Rad protein assay kit with bovine gamma globin as a standard. Protein from column effluents was monitored by measuring the absorbance at 280 nm.

Enzyme Assay—The enzyme activity was assayed spectrophotometrically using a Uvikon 940 double beam spectrophotometer (Kontron, Switzerland) by following the decrease in absorbance at 340 nm due to the oxidation of NADPH to $NADP^+$. Assay reactions were performed at 25°C in 50 mM Tris-ammonium acetate (pH 8), containing 0.14 mM 4-hydroxybenzoate, 0.14 mM NADPH, and 1 M FAD, and were initiated by addition of the enzyme. The rate of oxidation was measured during the first minute. The effect of pH on the enzymatic activity of 4-hydroxybenzoate 3-hydroxylase was determined in the standard assay conditions described above except that the following buffers were used: 50 mM Tris-ammonium acetate for pH 6.5 to 8.5, 50 mM potassium phosphate for pH 6 to 8, and 50 mM Tris-sulfate for pH 7 to 9.

Determination of N-Terminal Sequence—The N-terminal amino acid sequencing of 4-hydroxybenzoate 3-hydroxylase by Edman degradation was carried out on an automated protein sequencer (model 477, Applied Biosystems, USA).

RESULTS

Expression of pobA in A. calcoaceticus and E. coli—Measured levels of 4-hydroxybenzoate 3-hydroxylase synthesized in the natural host, *A. calcoaceticus* ADP1, and in *E. coli* (JM83) containing the cloned *pobA* gene (pZR405) were very low compared to levels observed in *P. fluorescens* (*25, 26*) and in *P. aeruginosa* (*27*) (Table I). Assuming that the specific activity of the pure enzyme from *A. calcoaceticus* is similar to that from *P. fluorescens*, the cell-free extracts of *A. calcoaceticus* and of *E. coli* (pZR405) contain 4-hydroxybenzoate 3-hydroxylase constituting only 0.1% of the total protein. In order to overexpress the *pobA* gene of *A. calcoaceticus*, we subcloned it into different vectors under the regulatory control of the *lac*- or T7 promoter. The 2.3-kb *Eco*RI-*Nsi*I fragment containing the entire *pobA* gene was cloned into pUC19 between the *Eco*RI and *Pst*I sites. This plasmid was designated pZR4053 (Fig. 1).

This construct, however, allowed the expression of *pobA* at only moderate levels (Table I). The *pobA* gene was also cloned under the T7 promoter, to construct pT762. 4-Hydroxybenzoate 3-hydroxylase was, however, not overproduced under the conditions under which T7 RNA polymerase was overproduced (Table I). The failure of the strong promoters to overexpress *pobA* may be due to the presence of a transcriptional termination signal upstream of the *pobA* gene. To remove this putative transcriptional termination signal, we constructed the plasmid pZR4054 in which the 0.8-kb *Eco*RI fragment of pBS618 containing the trimethoprim-resistant tetrahydrofolate reductase gene (*folA*) from *P. putida* was cloned into the *Eco*RI site (downstream of *pobA*) of pZR4053 in the same orientation relative to the *lac* promoter of pUC19 (Fig. 1). Genetic alterations which increase the expression of both *pobA* and *folA* may allow the host to resist higher concentrations of trimethoprim. About 10^8 cells of JM101(pZR4054) were spread onto plates containing 300 μg/ml trimethoprim. Whereas *E. coli* JM83 containing pZR4054 was resistant to trimethoprim at 100 μg/ml but sensitive to 300 μg/ml, three colonies developed with resistance to 300 μg/ml. The plasmids in these colonies were analyzed and one was selected for further analysis (pZR4056; Fig. 1). When the 4-hydroxybenzoate 3-hydroxylase activities in the cultures of JM83(pZR4054) and JM83(pZR4056) were measured, the activity in JM83(pZR4056) was 40-fold higher than that found in JM83(pZR4054). The *Eco*RI-*Hin*dIII fragment of the pZR4056 plasmid containing the *pobA* gene was then subcloned into pUC19 between the *Eco*RI and *Hin*dIII sites. The resulting plasmid pZR4057 (Fig. 1) was used to transform JM83, and the transformant, JM83(pZR4057), expressed 4-hydroxybenzoate 3-hydroxylase at a level of 1,820 mU/mg protein, 40-fold higher than the induced level of *A. calcoaceticus* (Table I). Restriction analysis of pZR4053 and pZR4057 plasmids revealed no detectable difference in size, suggesting that a point mutation or a short insertion/deletion (less than 50 bp) may be responsible for the overexpression of *pobA*.

TABLE I. **The activity of 4-hydroxybenzoate 3-hydroxylase in crude extracts.**

Strain	Medium[a]	Specific activities[b] in cell extracts
A. calcoaceticus ADP1	Succinate minimal	1
A. calcoaceticus ADP1	4-HBA minimal	50
E. coli JM83(pZR405)	L-broth + 4-HBA	45
E. coli JM83(pZR4053)	L-broth	40
E. coli JM101(pT762)	L-broth	100
E. coli JM83(pZR4054)	L-broth	65
E. coli JM83(pZR4057)	L-broth	1,820

[a]The concentrations of succinate and 4-hydroxybenzoate (4-HBA) used were 10 mM and 5 mM, respectively. [b]μmol NADPH oxidized·min^{-1}·mg protein^{-1}.

Purification of 4-Hydroxybenzoate 3-Hydroxylase—The 4-hydroxybenzoate 3-hydroxylase was purified to homogeneity in two chromatograpic steps as described in "MATERIALS AND METHODS." The procedure gave about 20-fold purification with a yield of 76% (Table II), and a specific activity of 35 to 40 units per mg protein. This activity is similar to that previously reported for *P. fluorescens* (26)

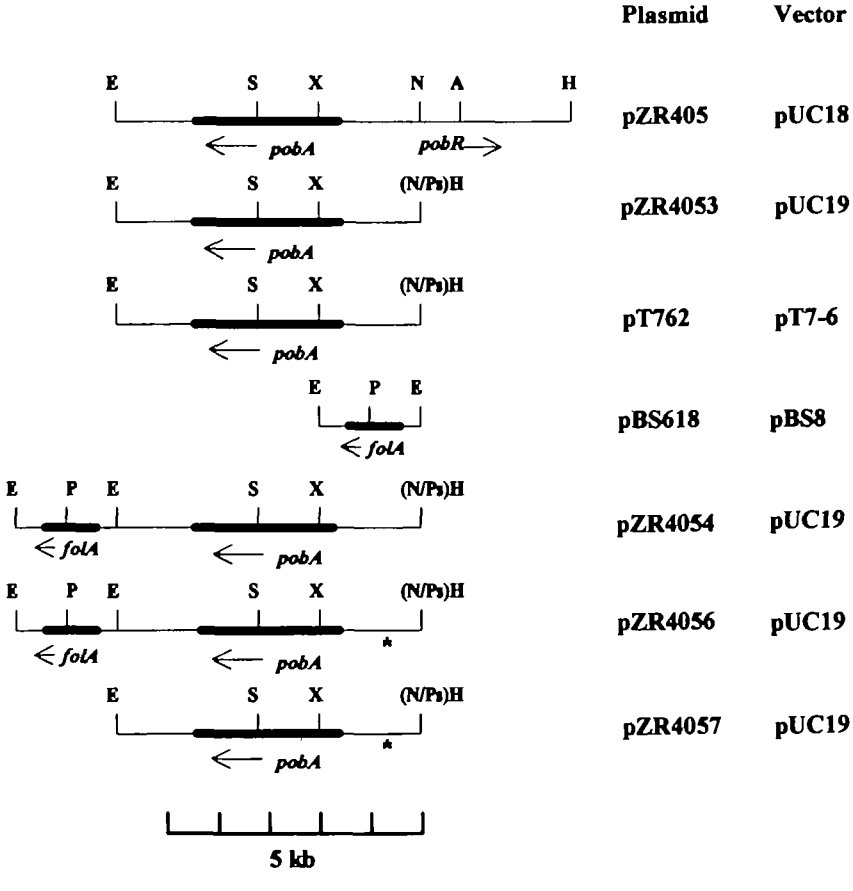

Fig. 1. **Plasmids used in this study.** Only DNA inserts are presented in this figure. Abbreviations: A, *Acc*I; E, *Eco*RI; H, *Hin*dIII; N, *Nsi*I; P, *Pvu*II; S, *Sph*I; X, *Xho*I; Ps, *Pst*I. (N/Ps)H indicates that the *Nsi*I site upstream of *pobA* was ligated to the *Pst*I site of the multiple cloning site of pUC19, which also contains the *Hin*dIII site. Between the *Nsi*I site and the initiation codon of *pobA*, a transcription termination signal may exist. This signal is modified in pZR4056 and pZR4057, and asterisks (*) indicate the modification. *folA* is the structural gene for trimethoprim-resistant tetrahydrofolate reductase from *P. putida*.

TABLE II. **Purification of 4-hydroxybenzoate 3-hydroxylase from *E. coli* JM83(pZR4057).**

Step	Volume (ml)	Protein (mg)	Total activity[a]	Specific activity[b]	Yield (%)	Purification (fold)
Cell-extract	30	1,100	2,000	1.8	100	1
DEAE-5PW	20	130	1,900	14	95	7.7
Hydroxyapatite	30	40	1,500	38	76	20

[a] μmol NADPH oxidized·min^{-1}. [b] μmol NADH oxidized·min^{-1}·mg^{-1}.

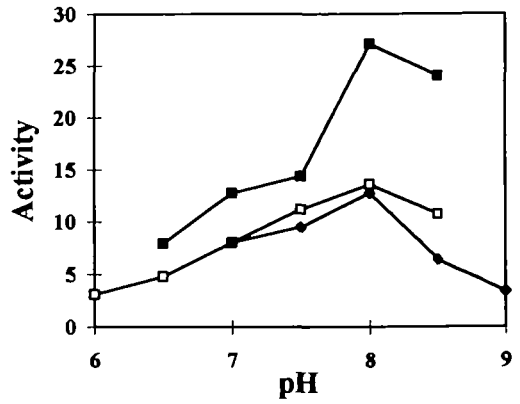

Fig. 2. **Effect of pH on the activity of 4-hydroxybenzoate 3-hydroxylase.** The buffers used were (□) potassium phosphate 50 mM; (◆) Tris-sulfate 50 mM; and (■) Tris-ammonium acetate 50 mM. The activity is expressed as mol NADPH oxidized·min^{-1}·mg protein^{-1}.

TABLE III. **Kinetic parameters of *p*-hydroxybenzoate hydroxylase.**

Substrate[a]	K_m (μM)	V_{max} apparent[b]
4-HBA	41	48.5
2,4-DHBA	250	1.5
3-Cl-4-HBA	180	3.2
Inhibitor[a]	**Type of inhibition**	**K_i (μM)**
2,4-DHBA	Competitive	310
3-Cl-4-HBA	Mixed	ND[c]
3,4-DHBA	Competitive	550
4-Aminobenzoate	Competitive	50
Benzoate	Competitive	530

[a] Abbreviations: 4-HBA, 4-hydroxybenzoate; 2,4-DHBA, 2,4-dihydroxybenzoate; 3-Cl-4-HBA, 3-chloro-4-hydroxybenzoate; 3,4-DHBA, 3,4-dihydroxybenzoate. [b] The activity (μmol NADPH oxidized·min^{-1}·mg^{-1}) was determined under the standard conditions except that the concentration of 4-hydroxybenzoate or its analogue was varied. [c] ND: not determined.

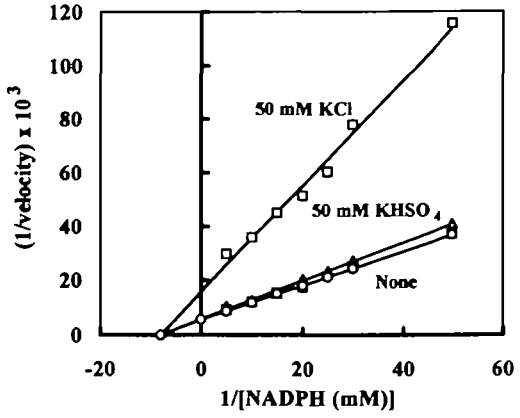

Fig. 3. **Inhibition of the 4-hydroxybenzoate 3-hydroxylase activity by KCl.** The initial velocity of the NADPH oxidation by 4-hydroxybenzoate 3-hydroxylase was measured with varying concentrations of NADPH in 50 mM Tris-ammonium acetate buffer pH 8.0 or in the same buffer containing either 50 mM KCl or 50 mM $KHSO_4$. The K_m for NADPH was unchanged under these conditions.

and *P. aeruginosa* (*27*) enzymes. The fractions obtained from the hydroxyapatite column were subjected to gel electrophoresis under denaturing conditions. In the first 3-ml fractions, only a single band of the molecular mass of 45 kDa was present, whereas in the succesive 3-ml fractions, two bands of similar molecular mass were present. This difference in the electrophoretic mobility may not be due to a difference in the molecular mass of the enzyme but the partial oxidation of Cys-122 as has been reported for the *P. fluorescens* enzyme (Cys-116) (*28*).

One difficulty we encountered during the purification was a loss of activity due to the unintentional resolution of FAD from the enzyme following chromatography on DEAE-5PW resin. This effect was also observed with hydrophobic interaction chromatography (data not shown). In both cases, activity was restored by incubating the enzyme solution in the presence of excess FAD.

The molecular weight of the enzyme estimated by gel filtration chromatography was 89,000 indicating that the enzyme exists as a dimer. The N-terminal sequence of the purified protein was MQTMKTKVAI, which confirmed the previous assignment of the initiation codon of the *pobA* gene (*16*).

Characterization of 4-Hydroxybenzoate 3-Hydroxylase—Exposure of the enzyme to temperature above 50°C for 10 min resulted in its rapid inactivation (60% inactivation), but the presence of its substrate offered some protection: in the presence of 0.1 mM 4-hydroxybenzoate, the enzyme retained 80% of its activity after heating at 50°C for 10 min. Likewise, in the presence of 4-hydroxybenzoate, FAD and EDTA, the enzyme lost only 10% of its activity after incubation at 4°C for 24 h, whereas incubation in the absence of these components resulted in a 40% loss of activity. These observations are in agreement with the results published for the enzymes from *P. fluorescens* (*29*) and from *P. aeruginosa* (*27*).

The optimum pH for 4-hydroxybenzoate 3-hydroxylase was 8.0 (Fig. 2) and is consistent with the pH optimum of 8.0 reported for the *P. aeruginosa* (*27*) and *P. fluorescens* (*26*) enzymes. The enzyme activity was about 50% lower in 50 mM potassium phosphate buffer (pH 8.0) and 50 mM Tris-sulfate buffer (pH 8.0) than in 50 mM Tris-ammonium acetate buffer (pH 8.0) (Fig. 2). We also tested the effect of salts on 4-hydroxybenzoate 3-hydroxylase activity in 50 mM Tris-ammonium acetate buffer (pH 8). Addition of 50 mM KCl inhibited 60% of the enzyme activity, but 50 mM $KHSO_4$ did not exhibit any significant inhibition (Fig.

3). The inhibition by the chloride ion was non-competitive to NADPH: the apparent K_m for NADPH was not influenced by KCl.

4-Hydroxybenzoate 3-hydroxylase from *A. calcoaceticus* oxidized NADPH in the presence of 4-hydroxybenzoate, but no activity was seen with NADH. The purified enzyme was also highly specific to 4-hydroxybenzoate. Of several analogs tested, only 2,4-dihydroxybenzoate and 3-chloro-4-hydroxybenzoate served as substrates, but the enzymatic activity with these compounds was very low. The results are summarized in Table III. The maximum activity of 4-hydroxybenzoate 3-hydroxylase was observed around 0.14 mM 4-hydroxybenzoate. At concentrations higher than 1 mM, the substrate was inhibitory.

A number of compounds structurally related to 4-hydroxybenzoate were competitively inhibitory to the oxidation of 4-hydroxybenzoate (Fig. 4). The inhibition caused by 3-chloro-4-hydroxybenzoate was mixed type. Compounds which caused over 60% of inhibition at 1 mM were 4-aminobenzoate, 2,3-dihydroxybenzoate, 2,5-dihydroxybenzoate, and 3-amino-4-hydroxybenzoate.

DISCUSSION

In this study, we purified 4-hydroxybenzoate 3-hydroxylase from *A. calcoaceticus* to homogeneity. To facilitate the purification, we overproduced this enzyme in *E. coli*. The conventional method for the overproduction of cloned genes, namely, the cloning of a gene of interest downstream of a strong promoter, did not realize our objective. We therefore devised a method to select variants which overexpressed the *pobA* gene. To perform this, we placed the *folA* gene of *P. putida* immediately downstream of the *pobA* gene, selected derivatives which overexpressed the *folA* gene, and hence exhibited higher resistance to trimethoprim. Such derivatives overexpressed the *pobA* gene. The trimethoprim resistance is not used as a marker in common cloning vectors, and the *folA* gene is small and easy to handle. The method used in this paper is therefore generally applicable to any other genes to increase their expression in *E. coli* or in other trimethoprim-sensitive bacteria.

The induced level of 4-hydroxybenzoate 3-hydroxylase in *A. calcoaceticus* corresponds to approximately 0.1% of the total protein. This amount is much less than the induced levels in *P. fluorescens* and *P. aeruginosa*. Apparently, 4-hydroxybenzoate 3-hydroxylase at a level of 0.1% of the total protein is sufficient to support the growth of *A. calcoaceticus* on 4-hydroxybenzoate.

Although the amino acid identity between 4-hydroxybenzoate 3-hydroxylase of *A. calcoaceticus* and the *Pseudomonas* enzymes is 63%, most of the specific residues implicated for important functions, namely, FAD- and substrate-binding, are conserved between these enzymes (*16*). In agreement with the sequence data, we found that the properties of 4-hydroxybenzoate 3-hydroxylase from *A. calcoaceticus* are very similar to those of the *Pseudomonas* enzymes.

It has been stated that 4-hydroxybenzoate 3-hydroxylase from *P. desmolytica* is inhibited by some organic acids, such as acetate, maleate, and propionate (*30*). In the *Acinetobacter* enzyme, however, acetate, malate, and succinate stimulated the enzymatic activity (Fig. 2 and our unpublished data). Apparently, spaces available for these solvent molecules differ between these two enzymes.

We found that the chloride ion is a non-competitive inhibitor of the 4-hydroxybenzoate 3-hydroxylase from *A. calcoaceticus*. The halide ions have been shown to be competitive inhibitors for NADPH of 4-hydroxybenzoate 3-hydroxylase from *P. desmolytica* (*30*) and *P. fluorescens* (*31*). It is therefore possible that the NADPH-binding domain in the *Acinetobacter* enzyme is slightly different from those in the *Pseudomonas* enzymes. In spite of intensive studies on the *P. fluorescens* enzyme, the NADPH-binding site of this enzyme has not yet been identified, although it is assumed that NADPH binds to the re-side of the flavin, and that the nicotinamide moiety of NADPH may bind with its ring parallel to the flavin ring. It is therefore interesting to identify amino acid residues responsible for the sensitivity to the halide ions since one of these residues in *P. fluorescens* would overlap with the NADPH-binding domain, while in the *Acinetobacter* enzyme, the chloride ion-binding site and the NADPH-binding site may be separated. From the three-dimensional structure of the *P. fluorescens* enzyme, it is possible to identify several residues which can potentially interact with NADPH. Some of these residues are substituted in the *Acinetobacter* enzyme. Three replacements occur along the proposed cavity of NADPH binding (*6, 32*): Val47Ile, Leu188Val, Leu261Ile (numbered according to the *P. fluorescens* enzyme). In the latter two cases, the residue is either at the surface (Leu261), or faces away from the cavity (Leu188). One of them, the Val47Ile substitution, is a candidate for the residue responsible for the modification of the halide-ion-binding: the Val47Ile substitution together with neighbor Tyr385 may change the orientation of FAD, hence the electrostatic environment of the NADPH-binding site.

Our results with 3-chloro-4-hydroxybenzoate suggest that this compound is oxidized by 4-hydroxybenzoate 3-hydroxylase from *A. calcoaceticus*, but its oxidation was not efficient. There are two possible reasons to account for this. First, 3-chloro-4-hydroxybenzoate exhibits a low affinity to the enzyme as demonstrated by the limited FAD-absorbance change induced by this compound (our unpublished data). Certainly, the chloro-substitution causes a structural hindrance to binding. Leu199 and/or Tyr201 (numbering according to the *P. fluorescens* enzyme), which are located in the vicinity of the C3 atom of the substrate, may be responsible for it. Secondly, the electrodrawing nature of the chloro-substitution may inhibit the electrophilic attack by 4*a*-hydroperoxide. More detailed study is required to elucidate the constraints of 4-hydroxybenzoate 3-hydroxylase for the oxidation of 3-chloro-4-hydroxybenzoate.

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