Salicylate Biosynthesis in *Pseudomonas aeruginosa*

PURIFICATION AND CHARACTERIZATION OF PchB, A NOVEL BIFUNCTIONAL ENZYME DISPLAYING ISOCHORISMATE PYRUVATE-LYASE AND CHORISMATE MUTASE ACTIVITIES*

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ABSTRACT

Isochorismate pyruvate-lyase (IPL), the second enzyme of pyochelin biosynthesis and the product of the pchB gene, was purified to homogeneity from Pseudomonas aeruginosa. In the reaction catalyzed by this enzyme, isochorismate → salicylate + pyruvate, no cofactors appear to be required. At the pH optimum (pH 6.8), the enzyme displayed Michaelis-Menten kinetics, with an apparent $K_m$ of 12.5 μM for isochorismate and a $k_{cat}$ of 106 min$^{-1}$, calculated per monomer. The native enzyme behaved as a homodimer, as judged by molecular sieving chromatography, electrophoresis under non-denaturing conditions, and cross-linking experiments. PchB has about 20% amino acid sequence identity with AroQ-class chorismate mutases (CMs). Chorismate was shown to be converted to prephenate by purified PchB in vitro, with an apparent $K_m$ of 150 μM and a $k_{cat}$ of 7.8 min$^{-1}$. An oxabicyclic diacid transition state analog and well-characterized inhibitor of CMs competitively inhibited both IPL and CM activities of PchB. Moreover, a CM-deficient E. coli mutant, which is auxotrophic for phenylalanine and tyrosine, was functionally complemented by the cloned P. aeruginosa pchB gene for growth in minimal medium. A mutant form of PchB, in which isoleucine-88 was changed to threonine, had no detectable IPL activity, but retained wild-type CM activity. In conclusion, the 11.5-kDa subunit of PchB appears to contain a single active site involved in both IPL and CM activity.
INTRODUCTION

Salicylate is a biosynthetic product and a precursor of secondary metabolites and siderophores (iron chelators) in several bacterial genera, e.g. *Pseudomonas, Burkholderia, Azospirillum, Vibrio, Yersinia* and *Mycobacterium* (1-7). The first evidence for a bacterial salicylate biosynthetic pathway was obtained in *Mycobacterium smegmatis*, which incorporates shikimate into salicylate and the siderophore mycobactin derived from salicylate (8-10). Since isochorismate decomposes slowly into salicylate and 3-carboxylphenylpyruvate at pH 7 and room temperature, in the absence of enzymes, isochorismate was proposed to be the immediate precursor of salicylate (11). It was then shown that crude cell extracts of *M. smegmatis* convert chorismate and isochorismate to salicylate (Fig. 1), by the successive action of isochorismate synthase (ICS) and IPL (10, 12). Whereas ICS has been purified from *E. coli* (13, 14), from *Bacillus subtilis* (15) and recently also from a plant (16), IPL has not yet been characterized as a pure enzyme from any organism.

In the ubiquitous bacterium and opportunistic human pathogen *Pseudomonas aeruginosa*, as in other organisms, chorismate is a precursor of aromatic amino acids, ubiquinone and folate (Fig. 1). In addition, phenazine compounds such as pyocyanine (17, 18) and the siderophore pyochelin (19) are produced by pathways branching off from chorismate (Fig. 1). Salicylate is a biosynthetic precursor of pyochelin in *P. aeruginosa* (1, 20). Genetic evidence indicates that the *pchA* and *pchB* genes encode ICS and IPL, respectively, in this bacterium (2). These genes are part of the *pchDCBA* operon which, together with the *pchEFGHI* operon, code for the enzymes of the pyochelin pathway (21-24).
The deduced protein products of the *P. aeruginosa* *pchB* gene and its *P. fluorescens* homolog *pmsB* both show about 20% sequence identity with CMs of the AroQ family (25-28); the significance of this low-level similarity is unclear. CM catalyzes a Claisen rearrangement of chorismate, leading to prephenate, a common precursor in phenylalanine and tyrosine biosynthesis (Fig. 1).

*P. aeruginosa* has at least two CM activities (29-31). The first of these is due to the bifunctional P-protein (AroQ•PheA) consisting of a CM domain (AroQ) and a prephenate dehydratase domain, which produces phenylpyruvate, a phenylalanine precursor (Fig. 1). The second CM is a monofunctional, periplasmic enzyme (*AroQ*).

The aims of the present work are to characterize the properties of purified PchB from *P. aeruginosa* and to investigate its relationship to CM of the AroQ class.
EXPERIMENTAL PROCEDURES

Materials—The endo-oxabicyclic diacid transition state analog inhibitor of CM and Na-iscochorismate (used as a reference) were generous gifts from Drs. P. Bartlett (University of California, Berkeley) and E.W. Leistner (University of Cologne), respectively. Prephenic acid and phenylpyruvic acid were obtained from Sigma (Fluka Chemie AG, Switzerland). Carbenicillin was a gift from GlaxoSmithKline. Chorismic acid was isolated from the CM-deficient Klebsiella pneumoniae strain 62-1 (ATCC 25306; Phe -Tyr -Trp -; 32), purified as the free acid according to the method of Grisostomi et al. (33) and stored at -80°C. The purity of chorismic acid was 98% as determined by UV spectroscopy using $\varepsilon_{274nm} = 2,630 \text{ M}^{-1}\text{cm}^{-1}$ (34) and 95.3% by analytical C-18 reverse phase HPLC at 280 nm. Prephenic acid, phenylpyruvic acid and $p$-hydroxybenzoic acid were present at 0.6%, 1.4% and 0.06%, respectively, as judged by HPLC. Isochorismate was isolated from K. pneumoniae 62-1 harboring the $entC$ plasmid pKS3-02 (35), which was grown in medium A containing kanamycin (60 μg/ml), and transferred to 2-liter production medium BN (11) containing 0.1 mM of each tyrosine, phenylalanine and tryptophan plus 0.3 mM isopropyl-$\beta$-D-thiogalactopyranoside (IPTG) (35).

After precipitation with 2 M barium acetate, a 1:2 mixture of isochorismate and chorismate (600 mg from 2 liter of culture) was obtained, of which 50 mg portions were purified on a Nucleosil 120 C-18 column (10 x 250 mm) in aqueous 0.27% formic acid, pH 2.5. Elution at 4.1 ml/min was carried out by a methanol (% v/v) step gradient: 10 min, 0%; 10-16 min, 0%-19%; 16-41 min, 19%; 41-51 min, 19%-28.5%; 51-58 min, 28.5%; 58-63 min, 28.5%-95%; 63-73 min, 95% [(35); E.W. Leistner, personal communication].
Isochorismic acid and chorismic acid were eluted with 19% methanol after 30 and 48 min, respectively, neutralized with NaOH and freeze dried. Isochorismate \( \varepsilon_{278\text{nm}} = 8300 \text{ M}^{-1} \text{ cm}^{-1} \) \(^{(36)}\) preparations adjusted to 200 μM contained 65 mM Na-formate, 43 μM Na-prephenate and 20 μM Na-phenylpyruvate. No contamination of the isochorismate preparations with chorismate or with salicylate could be detected. At an isochorismate concentration giving half maximal velocity (12.5 μM), the addition of 20 μM Na-prephenate, 8 μM Na-phenylpyruvate, or 26 mM Na-formate (i.e. a 10-fold excess over the contamination levels present in our isochorismate preparations) had no effect on IPL activity.

**Isochorismate Determination by HPLC**—An analytical detection method at pH 7.0 was worked out for isochorismate, using a Nucleosil C-18 column (4 x 250 mm). Elution at a flow rate of 1 ml/min was carried out with a 0-65% (v/v) linear gradient of acetonitrile in 10 mM K-phosphate buffer pH 7.0 containing 5 mM of the ion pair reagent tetrabutylammonium hydrogen sulfate (Fluka), using a Hewlett-Packard 1050 system equipped with a diode array detector. \( p \)-Hydroxybenzoate (10.2 min), prephenate (10.9 min), chorismate (11.7 min), isochorismate (12.4 min), phenylpyruvate (15.6 min) and salicylate (16.4 min) were eluted at the retention times indicated and quantitated at \( \lambda_{\text{max}} \) with a diode array UV detector.

**DNA Manipulation and Nucleotide Sequence Analyses**—Routine procedures were used for the isolation of DNA and for cloning experiments \(^{(37, 38)}\). *K. pneumoniae* and *Escherichia coli* strains were transformed with plasmid DNA by electroporation \(^{(39)}\). Plasmid pME6152 containing *pchB* under the *tac* promoter, was constructed by cloning the 0.75-kb *pchB XhoI-PstI* fragment of pME3368 \(^{(2)}\) in pMMB67EH \(^{(40)}\). A 0.75-kb *BamHI/HindIII* fragment encoding *pchB*(I88T) from mutated pME6152 (see below) was subcloned into pUK21 \(^{(38)}\) and pMMB67EH, resulting in pME6169 and pME6179, respectively.
The nucleotide sequence of the pchB (I88T) gene on pME6179 was determined by using the Thermo Sequenase II dye terminator cycle sequencing kit (Amersham) and the ABI PRISM™ 373 sequencer.

_**Purification of the PchB Enzyme—P. aeruginosa ADD1976/pME3324** [a T7 promoter expression vector with pchB (2)] was grown in 250 ml of nutrient yeast broth (2) containing carbenicillin (250 μg/ml) in 1 liter flasks with shaking at 37°C. At A_{600}= 0.8, the chromosomal T7 RNA polymerase gene was induced for 1 h by the addition of IPTG at 1 mM final concentration. Rifampicin was then added at 200 μg/ml and the incubation was continued for 2 h. All subsequent operations were carried out at 4°C. Cells from 3 flasks were harvested by centrifugation at 8000 x g for 10 min and washed twice in 50 mM K-phosphate buffer (pH 7.5) containing 10% (v/v) glycerol and 1 mM dithiothreitol (DTT) (buffer A). Cells (wet weight 3 g), which could be stored at -20°C, were broken in 15 ml buffer A by sonication for 5 x 30 s. Cell debris was removed by centrifugation at 10,000 x g for 30 min; the resulting crude extract containing 8-10 mg of protein per ml was applied to a 1.6 x 20 cm DEAE-Sepharose CL-6B column (Pharmacia) equilibrated with buffer A. The enzyme was eluted with 300 ml of buffer A at a flow rate of 1 ml/min. The fractions containing PchB, detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in 10-20% gradient gels stained with Coomassie Blue, were combined (100 ml) and loaded onto a column of Phenyl-Sepharose CL-4B (1.6 x 10 cm) previously equilibrated with 200 ml of buffer A. The enzyme was eluted as a broad peak with 150 ml of buffer A. Fractions containing PchB were pooled (80 ml) and a solution of 0.4% (w/v) n-octyl-β-glucopyranoside (Calbiochem) was slowly added by stirring to a final concentration of 0.1% (w/v), to avoid protein precipitation during the concentration steps.
This fraction was concentrated to 2 ml by ultrafiltration through an Amicon YM3 membrane in a stirred cell, followed by further concentration with an Amicon Centriplus C–10 concentrator (cut-off: 10,000 Da). The concentrated fraction was chromatographed on a 1.6 x 70 cm column of Bio-Gel P100 polyacrylamide gel (Bio-Rad) in buffer A at a flow rate of 6 ml/h. PchB-containing fractions were pooled, aliquoted and stored at -80°C. The protein concentration was determined by the method of Bradford (41) using a commercial reagent (Bio-Rad) and bovine serum albumin as the standard.

**Molecular Mass Estimation and NH₂-terminal Analysis**—The subunit molecular mass of PchB was estimated by SDS-PAGE (42) in 10-20% gradient gels (Ready Gel, Bio-Rad), with standards of the Low Molecular Weight Calibration Kit (Pharmacia). The molecular mass of the native PchB was estimated by gel filtration chromatography on Sephadex G-150 (1.6 x 70 cm, 0.1 ml/min), Bio-Gel P100 (1.6 x 70 cm, 0.1 ml/min) and FPLC Superose 12 HR (10/30, 0.4 ml/min) columns in buffer A, with ribonuclease A (13.7 kDa), lysozyme (14.6 kDa), proteinase K (28.8 kDa), pepsin (34.5 kDa), protein A (42 kDa), ovalbumin (43 kDa), and bovine serum albumin (67 kDa) as markers. The elution volumes were plotted against the logarithm of molecular masses for the standards and the linear regression curve was used to estimate the apparent molecular mass of PchB.

In addition, the molecular mass of native PchB was estimated from PAGE in non-denaturing gels of 7.5, 10, 12, 15 and 20% polyacrylamide, with the Low Molecular Weight Calibration Kit (Pharmacia) as a standard, by Ferguson plot analysis (43). The slopes obtained from plots of the logarithm of relative mobility versus polyacrylamide concentration were plotted against the molecular weight of the standard proteins.

The N-terminal sequence of PchB was determined by Dr. P. James (ETH, Zürich) on an Applied Biosystems, Inc. Peptide Sequencer Model 473A, using the Edman degradation procedure.
Cross-linking of PchB—Samples of 50 μl purified PchB (0.54 μg/μl) in buffer A were treated with 3,3’-dithiobis-(sulfosuccinimidylpropionate) [DTSSP] to a final concentration of 0.5 mM. The crosslinker was prepared immediately before use as a 5 mM solution in 50 mM K-phosphate, pH 7.5, containing 0.75 M NaCl. The samples were incubated with constant agitation at room temperature for 30 min. The reaction was quenched by addition of 40 mM Tris-HCl buffer, pH 7.5. Each sample was split into two aliquots, treated with the solubilizing solution (final concentrations: 1.2% (w/v) SDS, 10% (w/v) sucrose, 10 mM Tris-HCl pH 7.5) with or without 40 mM DTT to cleave the crosslinks, incubated at 50°C for 3 min and subjected to SDS-PAGE on a 10-20% polyacrylamide gradient. Standard proteins (Low and High Molecular Weight Kits, Pharmacia) and the PchB control were not exposed to DTSSP, but otherwise treated in the same way.

Enzyme Assays—IPL activity of PchB was determined by measuring the fluorescence of salicylate formed using an excitation wavelength of 305 nm and an emission wavelength of 440 nm in a Perkin-Elmer LS30 fluorimeter. Unless stated otherwise, the incubation mixture contained, in a final volume of 500 μl, 100 mM K-phosphate buffer, pH 7.0, 600 μM isochorismate (purified by HPLC), 10% (v/v) glycerol, 10 mM MgCl₂, 1 mM DTT and enzyme. Controls without enzyme were included routinely, to correct for non-enzymatic reactions. The reaction at 37°C was initiated by the addition of isochorismate and terminated by the addition of 10 μl concentrated HCl, followed by extraction with 3 ml ethyl acetate. Blanks were obtained from non-incubated reaction mixtures. The amount of salicylate formed was determined from a standard curve obtained with 0.5 to 8 μM salicylic acid (Fluka) in ethyl acetate. One IPL unit of enzyme activity is defined as the amount of enzyme converting 1 μmol isochorismate to salicylate per min. The reaction was stopped within the first 10% of the isochorismate-to-salicylate conversion.
CM activity of PchB was assayed by the method of Cotton and Gibson (44) as modified by Ahmad and Jensen (45). The product of the enzymatic reaction, prephenate, is converted to phenylpyruvate under acidic conditions, which is subsequently measured by its absorbance at 320 nm at basic pH. The reaction mixture contained, in a final volume of 0.2 ml, 50 mM K-phosphate, pH 7.0, 1 mM DTT, 1 mM chorismate and enzyme. Control reaction mixtures lacking enzyme were always included to estimate the amount of prephenate formed non-enzymatically during the incubation period. Reactions were carried out at 37°C and terminated by the addition of 0.1 ml of 1 M HCl, followed by incubation at 37°C for 10 min to convert the prephenate formed to phenylpyruvate. Phenylpyruvate concentrations were determined at 320 nm after the addition of 0.7 ml of 2.5 M NaOH, using \( \varepsilon_{320\text{nm}} = 17,500 \) M\(^{-1}\)/cm\(^{-1} \) (44). One CM unit of enzyme activity is defined as the amount of enzyme needed for the formation of 1 \( \mu \)mol prephenate (assayed as phenylpyruvate) per min.

**Kinetic Studies**—Assays were performed in triplicate and repeated three times. Initial velocity data were fitted to the equations of Hanes (Table II), Lineweaver-Burk (Fig. 3A/B/C) and Dixon using Enzpack software (Biosoft).

**In Vivo CM Activity of PchB**—The CM-negative E. coli strain KA12 harboring pKIMP-UAUC (47, 48) with or without pME6152 (pchB\(^{+}\)) was grown at 37°C with shaking in 500-ml flasks containing 100 ml minimal medium E (48), amended with 0.5% (w/v) glucose, 1 mM thiamine and 1 mM IPTG. Control cultures were grown in minimal medium E supplemented with 1 mM tyrosine and 1 mM phenylalanine. The cultures were inoculated with about \( 10^7 \) washed cells/ml, previously grown in minimal medium E supplemented with both amino acids, ampicillin (100 \( \mu \)g/ml) and chloramphenicol (25 \( \mu \)g/ml). Cell growth was monitored by following the OD\(_{600\text{nm}}\).
Western Immunoblot Procedure—Rabbit antibodies were generated by subcutaneous injection of purified PchB (110 μg/500 μl) emulsified with 500 μl of complete Freund's adjuvant. 3 booster injections with the same amount of antigen at 4-week intervals were applied. Ten days after the final injection, blood was collected and aliquots of the serum obtained were kept at -80°C. The enzyme (typically 0.1 μg) and cell extracts were subjected to SDS-PAGE and electrotransferred to a nitrocellulose sheet (Bio-Rad) in a Trans-Blot apparatus (Bio-Rad) with a transfer buffer containing 25 mM Tris Base (pH 8.3), 192 mM glycine, and 20% (v/v) ethanol. The antiserum was diluted 1:1000 with Tris-buffered saline-Tween [20 mM Tris-HCl, pH 7.5, 137 mM NaCl, 3 mM KCl, 0.05 % (w/v) Tween-20] for use in PchB immunoblots, which were treated with goat anti-rabbit IgG conjugated to horse radish peroxidase (Sigma). PchB was visualized by the enhanced chemiluminescence system according to the manufacturer's instructions (Amersham).
RESULTS

Purification of the PchB Enzyme—IPL was purified from the overproducing strain *P. aeruginosa* ADD1976/pME3324 as described in “Experimental Procedures” and summarized in Table I. In crude extract, PchB constituted approximately 20% of the soluble protein. Purification by DEAE-ion exchange chromatography resulted in a 5.9-fold increase in specific activity. Hydrophobic interaction chromatography on Phenyl-Sepharose and molecular sieving on Bio-Gel P100 removed some contaminants, but did not improve the specific activity (Table I). PchB protein tended to form insoluble aggregates in concentrated solution. Therefore, pooled fractions after Phenyl-Sepharose chromatography were concentrated by ultrafiltration in the presence of 0.1% *n*-octyl-β-D-glucopyranoside, allowing a higher recovery. Used at this concentration, *n*-octylglucose had no effect on IPL activity. Glycerol, which was present in buffer A, did not influence IPL activity either, in concentrations of up to 30%. PchB was eluted from the Bio-Gel column in a single symmetric peak, with a purity of >98% as judged by SDS-PAGE (Fig. 2, lane 1). The PchB polypeptide (calculated molecular mass 11.5 kDa) migrated slightly ahead of the 14.4 kDa marker, α-lactalbumin (Fig. 2).

The N-terminal amino acid sequence of the PchB polypeptide (met–lys–thr–pro–glu–asp–x–thr–gly–leu) obtained by Edman degradation matched that predicted from the DNA sequence (2) after processing of the first methionine.

Molecular Mass of Native PchB Enzyme—Mobility of PchB in PAGE under non-denaturing conditions was similar to that of carbonic anhydrase (28.7 kDa) and a Ferguson
plot indicated an apparent molecular mass of 30 ± 2 kDa. Analytical size exclusion chromatography on Sephadex G-150, Superose 12 HR and Bio-Gel P100 gave values of approximately 32 kDa, 31 ± 1 kDa and 34 ± 2 kDa, respectively, for native PchB.

PchB was cross-linked with the homobifunctional, water-soluble agent DTSSP and treated with the solubilizing buffer as described in “Experimental Procedures”. After SDS-PAGE, a substantial fraction of PchB was found to migrate as a covalently linked dimer (Fig. 2, lane 2). Larger forms corresponding probably to trimers and tetramers were also seen with much lower intensities (Fig. 2, lane 2). Increasing the cross-linker concentration to 2 mM appeared to favor the dimeric form (data not shown). Treatment of cross-linked PchB with DTT converted these forms back to the monomeric subunit (data not shown). The fact that the band corresponding to the dimer was dominant after cross-linking can be interpreted in favor of a dimeric structure of native PchB. The apparent molecular mass obtained by molecular sieving would also be compatible with a trimeric PchB structure. However, CM of *Methanococcus jannaschii* (27), a dimeric protein whose 99-amino-acid sequence is related to that of PchB (see below), showed an apparent molecular mass of 29.4 kDa in gel filtration experiments. This value is also higher than that expected for a dimeric form and was assumed to result from the non-spherical, elongated form of the dimeric species (27). In conclusion, PchB is very likely to adopt a dimeric structure in solution, but a trimeric structure cannot be totally excluded at this stage.

*Catalytic Properties of PchB*—Purified PchB protein had a broad optimum of IPL activity around pH 6.8 in 100 mM K-phosphate buffer (data not shown). Preincubation of the PchB protein (1.1 µg/ml) at 37°C for 10 min with the cation chelators EDTA, EGTA, or o-phenanthroline (each at 1 mM in assay buffer) did not affect IPL activity significantly.
Addition of 10 mM MgCl₂, 1 mM ZnCl₂, 1 mM FeCl₂, or 1 mM CaCl₂ changed IPL activity by ≤ 20%. Thus, no metal cofactor requirement was found for PchB.

The enzyme displayed Michaelis-Menten kinetics for IPL, as derived from initial velocity data collected at isochorismate concentrations varying from 1 to 600 μM under standard assay conditions. No substrate inhibition was observed at isochorismate concentrations of up to 1200 μM (data not shown). From Hanes plots, $V_{\text{max}}$ and $K_m$ values for isochorismate were obtained and the catalytic rate constant ($k_{\text{cat}}$) was calculated per PchB monomer (11.5 kDa) (Table II).

As sequence comparisons pointed to a possible relationship of PchB to CMs of the AroQ class, purified PchB enzyme was assayed with chorismate as the substrate. Interestingly, the enzyme displayed CM activity and showed Michaelis-Menten kinetics with chorismate as the substrate (Table II). The $K_m$ value for chorismate (150 μM) was higher than that for isochorismate (12.5 μM), whereas the $k_{\text{cat}}$ value calculated per monomer was lower for the Claisen rearrangement of chorismate (7.8 min⁻¹) than that for the isochorismate-to-salicylate conversion (106 min⁻¹), suggesting that PchB primarily functions as an IPL rather than as a CM.

*Inhibitors of PchB*—Bartlett et al. (49, 50) have described an *endo*-oxabicyclic diacid resembling the conformation of the presumed transition state of the enzymatic rearrangement of chorismate to prephenate. This transition state analog (TSA) acts as a potent inhibitor of CM (49, 50). TSA competitively inhibited both CM and IPL activities of PchB (Fig. 3, A & B), with similar $K_i$ values of 28 μM and 22 μM, respectively (Table II). This result suggests and raises the possibility that the IPL and CM activities of PchB involve a shared active site.
Chorismate was a weak competitive inhibitor of the IPL reaction (Fig. 3C), with a $K_i$ of 400 $\mu$M, as determined by a Dixon plot (Table II). The reciprocal test, i.e. potential inhibition of the CM reaction by isochorismate, could not be performed because the presence of isochorismate interfered with the standard CM assay.

Pyruvate, which is produced in the IPL reaction, did not inhibit IPL when added at 100 $\mu$M. Tryptophan, phenylalanine, tyrosine and dihydroaeruginoate (= hydroxyphenyl-thiazolinyl-carboxylate, an intermediate in pyochelin biosynthesis; Fig. 1), at 100 $\mu$M each, had no effect on IPL activity, when measured with 12 $\mu$M isochorismate. At 1 mM, phenylalanine and tyrosine were weak inhibitors of IPL, causing ca. 30% inhibition.

PchB Has CM Activity in Vivo—We tested whether PchB could restore a CM-deficiency in vivo. As no CM-negative mutant of *P. aeruginosa* was available, this test was carried out with a CM-negative strain of *E. coli*, KA12/pKIMP-UAUC (46, 47), harboring pME6152 (*pchB* expressed from the inducible tac promoter).

In the presence of 1 mM IPTG, but not in the absence of the inducer, PchB was produced at high levels in *E. coli* as revealed by Western blotting (Fig. 4; lanes 2 + 3). Under inducing conditions, PchB represented about 5% of the total soluble protein in *E. coli* (data not shown).

Moreover, PchB allowed functional complementation of the CM deficiency, i.e., growth in unsupplemented minimal medium. The parent strain *E. coli* KA12/pKIMP-UAUC grew only when phenylalanine and tyrosine were added to the minimal medium. When this strain carried pME6152, growth occurred in minimal medium without phenylalanine and tyrosine, provided that IPTG was added (Table III). However, the doubling time $t_d$ (240 min) was about 3 times longer than that observed in minimal medium with added phenylalanine.
and tyrosine (80 min), suggesting that PchB could only partially complement the CM deficiency of the *E. coli* host (Table III).

The minimal medium used does not contain added iron (48). Under these conditions, we expect the iron-repressible *entC* gene, which encodes ICS in the *E. coli* host, to be derepressed (52). This facilitates the rapid interconversion of the intracellular pools of chorismate and isochorismate (53, 35). Conversion of isochorismate to the thermodynamically favored salicylate by the IPL activity of PchB may therefore efficiently deplete the chorismate pool, which could result in the inability of PchB to fully complement the CM-deficiency (see Fig. 1). Thus, under iron limitation, PchB would mostly function as an IPL, rather than as a CM. This scenario is consistent with the significant increase in growth rate of *E. coli* KA12/pKIMP-UAUC + pME6152 when 100 μM FeCl₃ was added to the medium (Table III).

**Isolation of an IPL-Defective Mutant Form of PchB**—In order to improve the ability of the PchB enzyme to carry out the CM reaction *in vivo*, we grew *E. coli* KA12/pKIMP-UAUC + pME6152 for about 80 generations in minimal medium containing IPTG and antibiotics, but lacking tyrosine and phenylalanine. After subculturing several times, a strain showing faster growth (*t_d* = 120 min) could be isolated. The mutation causing this growth difference could be localized to the *pchB* gene of pME6152. When the mutated *pchB* gene was excised and cloned back into the same vector (pMMB67EH), the resulting plasmid pME6179 enabled strain *E. coli* KA12/pKIMP-UAUC to grow with the same doubling time (120 min; Table III), thus excluding the possibility that the growth advantage might have been caused by a mutation in the host or in the vector.

The mutated *pchB* gene of pME6179 was sequenced and a T:A to G:C transversion was identified at nucleotide 263, leading to a replacement of isoleucine-88 with threonine.
The affected position is not among those conserved between the PchB polypeptide and CMs of various origins (27). PchB showed the greatest similarity to the CM moiety (the AroQ-domain) of AroQ•TyrA from Haemophilus influenzae (26.3% identity) and to the same enzyme from E. coli (24.7% identity). PchB also shows similarity (17% identity) to the CM moiety of AroQ•PheA (the P-protein) of E. coli (Fig. 5).

The catalytic properties of PchB I88T were compared to the ones of wild-type PchB in cell extracts of E. coli. The apparent $K_m$ values obtained under these conditions for wild-type PchB were essentially the same as those of the purified enzyme (Tables II and III). Interestingly, PchB I88T had lost all IPL activity, but its CM activity remained unchanged (Table III). The observed accelerated growth of the host strain carrying the mutant pchB gene can thus be explained by an improved channeling of chorismate towards phenylalanine and tyrosine, which are essential products for growth in minimal medium. The fact that iron addition to the growth medium did not further reduce the doubling time (Table III) is consistent with the expectation that depletion of the intracellular chorismate pool towards salicylate is not possible anymore without the IPL function, irrespective of the induction state of entC. Thus, loss of IPL activity enabled the mutant PchB protein to carry out the CM job more effectively in the CM-deficient E. coli host.
The PchB protein, the first IPL purified from any organism, is a small enzyme. Its 101-amino acid subunit forms a homodimer, as suggested by the cross-linking experiments shown in Fig. 2. IPL catalyzes the second reaction in the pyochelin biosynthetic pathway of \textit{P. aeruginosa}, following the initial PchA-dependent conversion of chorismate to isochorismate [(2), Fig. 1]. We discovered that PchB can also carry out the CM-reaction (Fig. 1), \textit{in vitro} and \textit{in vivo}. However, the catalytic efficiency ($k_{\text{cat}}/K_m$) of this reaction is more than two orders of magnitude lower than that of the IPL reaction (Table II). Moreover, while PchB’s $K_m$ for chorismate is in the range of the Michaelis constants of other typical CMs (27, 54, 55), its catalytic rate constant $k_{\text{cat}}$ is significantly smaller by two to three orders of magnitude. When PchB was overproduced at a level about 5\% of the total soluble protein in \textit{E. coli}, its CM function (or that of the selected IPL-deficient variant PchB$^{188T}$) was apparently the growth-limiting factor when the recombinant organism was cultivated in minimal medium (Table III). It is therefore unlikely that PchB plays a significant role in aromatic amino acid biosynthesis of \textit{P. aeruginosa}, even under conditions of derepression of the \textit{pchDCBA} operon, i.e. when iron is limiting (22).

PchB structurally resembles CMs of the AroQ class in several respects. First, the length of the PchB sequence corresponds to the typical length of an AroQ domain, and the degree of sequence similarity of PchB with other AroQ proteins is in the range of similarities among other CMs of the AroQ family (27, 31). Second, secondary structure analysis using a neural network (56) predicts that PchB has the same all-$\alpha$-helical structural organization as the CM moiety of AroQ•PheA from \textit{E. coli} (Fig. 5). The crystal structure of the latter
enzyme has been solved as a complex with TSA (51). It is an elongated dimer formed by two identical subunits which are composed of three α-helices each. Two symmetrical active sites at the dimer interface bury the ligand in the core of a four-helix-bundle motif. Third, the quaternary structure of PchB was found to be dimeric, too, resulting in a similar abnormal migration behavior in gel filtration experiments as described previously for the monofunctional dimeric AroQ-class CM from *M. jannaschii* (27). Fourth, sequence identities between PchB and AroQ proteins include amino acids that make prominent interactions with ligand in the active site of CM [(51), Fig. 5]. Specifically, of the 20 residues that are identical between PchB and *E. coli* AroQ•PheA (Fig. 5), ten are among the 19 residues situated within a distance of 6 Å from any heteroatom of the TSA molecule bound in the active site of the CM domain of *E. coli* AroQ•PheA (Fig. 6A).

Besides the structural similarities of the two enzymes, a series of functional resemblances points to common catalytic features of IPL and CMs of the AroQ class. First, and most importantly, PchB has CM activity. Second, TSA, which is known to mimic the transition state of the CM reaction, shows similar *K*<sub>i</sub> values for both the CM and the IPL activities of PchB (Table II). Third, chorismate and isochorismate, the two alternative substrates of PchB, are distinguished only by the position of their respective hydroxyl groups (Fig. 1), leaving most of the functional groups and overall geometry of the substrates available for common binding interactions in the active site.

From the structural and functional correspondences it is clear that there can only be a single type of active site in PchB to which both substrates must bind and which carries out the Claisen rearrangement of chorismate in addition to the conversion of isochorismate to salicylate and pyruvate. Using the three-dimensional structure of *E. coli* AroQ•PheA (51) complexed with TSA as a template (Fig. 6A), we built a model of the active site of PchB with bound isochorismate (Fig. 6B) to illustrate the large degree of structural conservation.
between the active sites of the two enzymes. It is reasonable to assume that many of the amino acids that line the binding pockets play similar roles for CM and IPL activity, and experiments addressing mechanistic questions of PchB function are in progress (D. Künzler and P. Kast, unpublished results).

Among the dissimilarities in the active sites of the *E. coli* CM and *P. aeruginosa* PchB are residues in the vicinity of the hydroxyl group of the substrates. For instance, in the *E. coli* enzyme, Glu52, which is strongly conserved in the AroQ family (27), provides a hydrogen bond to the ligand’s 4-hydroxyl group [(52), Fig. 6A]. In PchB, this residue is replaced by a valine (Val55; Fig. 6B) which appears to be less favorable for chorismate binding but could provide a more suitable hydrophobic environment for the corresponding edge of isochorismate. The 12-fold lower affinity of PchB for chorismate versus isochorismate (Table II) may well be a consequence of this structural feature. Other differences between the active sites are apparent in the region of the 2-hydroxyl group of isochorismate. Interestingly, the residue mutated in the selected variant PchB<sup>I88T</sup> is located in this area (Fig. 6B). The exchange of isoleucine at position 88 by a threonine may prevent productive binding of isochorismate and thereby explain abolishment of all IPL activity. Since chorismate lacks a substituent at position 2, the mutation does not necessarily interfere with chorismate binding. The unchanged $K_m$ for the CM reaction of PchB<sup>I88T</sup> (Table III) is in agreement with this view.

Based on our observations, we speculate that PchB was derived from an AroQ-class CM by a gene duplication event followed by selection for efficient IPL function in the course of the evolution of the pyochelin siderophore pathway. From the fact that PchB can still use the same active site either for the IPL or for the CM reaction, it appears that the active site of the ancestral CM needed only modest alteration to be turned into an efficient IPL. Nevertheless, the required changes had a pronounced deleterious effect on the CM activity.
According to this scenario, the residual CM activity of PchB would reflect no more than a remnant of its evolutionary past. If an efficient IPL can relatively easily evolve from a CM, it can be further speculated that contemporary CMs may already possess (weak) IPL activity. It should be interesting to test established or presumed CM enzymes for the presence of this possible side activity, particularly those AroQ class members that possess a valine at the position corresponding to Glu52 in *E. coli* AroQ•PheA [see alignments in refs. 27 and 31].
REFERENCES


The abbreviations used are: AroQ•PheA, bifunctional enzyme consisting of an N-terminal chorismate mutase and a C-terminal prephenate dehydratase domain; AroQ•TyrA, as AroQ•PheA but with a C-terminal prephenate dehydrogenase domain; CM, chorismate mutase; DTSSP, 3,3'-Dithiobis[sulfosuccinimidylpropionate]; DTT, dithiothreitol; EGTA, ethylene glycol-bis-(β-aminoethyl)-N,N,N',N'-tetraacetic acid; ICS, isochorismate synthase; IPL, isochorismate pyruvate-lyase; IPTG, isopropyl-β-D-thiogalactopyranoside; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TSA, transition state analog.
FIGURE LEGENDS

FIG. 1. Chorismate metabolism in *Pseudomonas aeruginosa*. Biotransformations particularly relevant to this work are: 1, isochorismate synthase reaction (EC 5.4.99.6), catalyzed by PchA (2); 2, isochorismate pyruvate-lyase reaction (EC 4.1.99.-), catalyzed by PchB (2); 3, chorismate mutase reaction (EC 5.4.99.5), catalyzed by *AroQ*•PheA (bifunctional chorismate mutase-prephenate dehydratase), #AroQ (monofunctional periplasmic chorismate mutase) (29-31) and PchB (this study).

FIG. 2. Estimation of the molecular mass of purified PchB from *P. aeruginosa* by SDS-PAGE. Purified PchB (13.5 µg) was treated with 0.5 mM DTSSP and analyzed as described under "Experimental Procedures". *Lane 1*, PchB without DTSSP; *lane 2*, cross-linked PchB; *lane 3*, high molecular mass marker proteins, the 36 kDa band is lactate dehydrogenase; *lane 4*, low molecular mass marker: phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), soybean trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.4 kDa).
FIG. 3. Competitive inhibition by TSA and chorismate of purified PchB from *P. aeruginosa.* **A,** Inhibition of IPL by TSA. Double-reciprocal plot of the initial velocity v versus isochorismate concentration at different concentrations of TSA (mM): 0 (○); 0.05 (□); 0.2 (△). **B,** Inhibition of CM by TSA. Double-reciprocal plot of the initial velocity v versus chorismate concentration at different concentrations of TSA (mM): 0 (○); 0.01 (◇); 0.05 (□). **C,** Inhibition of IPL by chorismate. Double-reciprocal plot of the initial velocity v versus isochorismate concentration at different concentrations of chorismate (mM): 0 (○); 0.5 (□); 1 (△).

IPL and CM activities of PchB were assayed at 37°C as described in "Experimental Procedures". The reactions were started by adding the enzyme to the prewarmed reaction mixture. Each measurement was performed at least three times.

FIG. 4. Western blot analysis of PchB production in *E. coli* KA12/pKIMP-UAUC harboring pME6152. Crude cell extracts (2 μg of protein) were subjected to SDS–PAGE (10-20 % gradient gels) followed by immunoblot analysis with anti-PchB polyclonal antibodies as described under "Experimental Procedures". **Lane 1,** purified PchB (0.1 μg); **lane 2,** crude cell extract of *E. coli* KA12/pKIMP-UAUC/pME6152 grown in supplemented minimal medium with IPTG-induction of *pchB* expression or, **lane 3,** without IPTG-induction.
FIG. 5. Amino acid sequence alignment of PchB from *P. aeruginosa* with the AroQ domain of the *E. coli* chorismate mutase-prephenate dehydratase (AroQ•PheA) and secondary structure prediction for PchB. The protein sequences for PchB and the AroQ domain of the *E. coli* chorismate mutase-prephenate dehydratase (AroQ•PheA) were each analyzed with the program PHDsec [(56), http://www.embl-heidelberg.de/Services/sander/predictprotein/]. The analysis employed a neural network which was not trained with AroQ proteins. The predicted secondary structure is indicated for each residue as H (helical) or L (loop). N- or C-terminal regions denoted with “x” do not align well with the core AroQ sequences and were thus not included in the analysis. Experimental secondary structure assignments for AroQ•PheA from x-ray crystallographic data (51) are indicated for all structurally resolved residues. Bold letters highlight conserved amino acids found in the active site from the chorismate mutase moiety of the bifunctional *E. coli* chorismate mutase-prephenate dehydratase (AroQ•PheA) which make prominent polar interactions with the ligand TSA in the crystal structure (51). The position of the isoleucine-to-threonine mutation identified in the *pchB* mutant gene is highlighted with a black box. Numbers in italic correspond to PchB amino acid residues (1-102).
FIG. 6. Structure of the active site of the AroQ domain of AroQ•PheA from E. coli and the corresponding model for P. aeruginosa PchB. A, Relevant section of the crystal structure of E. coli AroQ•PheA complexed with the transition state analog TSA (PDB #1ECM) (51), showing the catalytic site of the CM domain. Displayed are the 19 residues whose side chains are within 6 Å of any heteroatom of the ligand. B, Homology model of PchB with bound isochorismate in a conformation suitable to fit into the presumed active site. The model was derived from the crystal structure of E. coli AroQ•PheA. While the protein backbone was left unaltered, the nine E. coli AroQ•PheA-specific side chains were replaced by the corresponding PchB-specific counterparts using appropriate side chain rotamer conformations to reduce steric clashes. An Ile88-to-Thr88 mutation results in loss of IPL, without affecting CM activity. Carbon atoms in A) and B) are color coded in turquoise (belonging to the ligand), green (fully conserved residues between E. coli AroQ•PheA and PchB) and white (non-identical residues between AroQ•PheA and PchB).
**TABLE I**

*Purification of the PchB enzyme from the overproducing strain P. aeruginosa ADD1976/pME3324, based on IPL activity*\textsuperscript{a}

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Protein (mg)</th>
<th>Activity (unit)</th>
<th>Specific activity (unit/mg)</th>
<th>Yield (%)</th>
<th>Purification -fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude cell extract</td>
<td>107.4</td>
<td>251.3</td>
<td>2.34</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>DEAE-Sepharose CL-6B</td>
<td>14.6</td>
<td>200.6</td>
<td>13.74</td>
<td>79.8</td>
<td>5.9</td>
</tr>
<tr>
<td>Phenyl-Sepharose CL-4B</td>
<td>8.9</td>
<td>100.8</td>
<td>11.33</td>
<td>40.1</td>
<td>4.8</td>
</tr>
<tr>
<td>Bio-Gel P100</td>
<td>4.4</td>
<td>56.7</td>
<td>12.89</td>
<td>22.6</td>
<td>5.5</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Data shown are taken from a typical purification; in four independent experiments the purification factors were reproducible ± 10%.
### TABLE II

*Kinetic parameters* of PchB for IPL and CM activities at 37°C

<table>
<thead>
<tr>
<th>Activity</th>
<th>Substrate</th>
<th>$K_m$ [μM]</th>
<th>$V_{max}$ [μmol min$^{-1}$]</th>
<th>$k_{cat}$ [min$^{-1}$]</th>
<th>$K_i^b$ [μM]</th>
</tr>
</thead>
<tbody>
<tr>
<td>IPL</td>
<td>Isochorismate</td>
<td>12.5</td>
<td>$4.9 \times 10^{-3}$</td>
<td>106</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>Chorismate</td>
<td>150</td>
<td>$3.4 \times 10^{-3}$</td>
<td>7.8</td>
<td>28</td>
</tr>
</tbody>
</table>

$^a$ Substrate saturation curve data were fitted to the Hanes equation ($v^{-1} = V_{max}^{-1} [S] + K_m V_{max}^{-1}$). The resulting $V_{max}$ values were transformed to catalytic rate constants ($k_{cat} = V_{max} M_r E_0^{-1}$), calculated per PchB monomer. The values represent the mean values of 3 independent determinations carried out as described in "Experimental Procedures". In the IPL assay, 0.54 μg purified PchB was incubated for 15 s; in the CM assay, 5 μg PchB was incubated for 10 min. Standard deviations of the values shown did not exceed 10%.

$^b$ For the determination of the inhibition mode, data were fitted to the Lineweaver-Burk equation ($v^{-1} = K_m V_{max}^{-1} [S]^{-1} + V_{max}^{-1}$) (see Fig. 3). The $K_i$ values were calculated from a Dixon plot ($v^{-1}$ against $[I]$).

Three to four concentrations of each the transition state analog (TSA) (10 to 200 μM) and chorismate (100 μM to 1 mM) were tested.
### TABLE III
**Complementation of the phenylalanine and tyrosine auxotrophies of the CM-deficient strain**

*E. coli* KA12/pKIMP-UAUC by PchB from *P. aeruginosa*<sup>a</sup>

<table>
<thead>
<tr>
<th>Plasmid / genotype</th>
<th>Addition to growth medium&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Doubling time&lt;sup&gt;c&lt;/sup&gt; (&lt;td&gt;)</th>
<th>IPL&lt;sup&gt;d&lt;/sup&gt;</th>
<th>CM&lt;sup&gt;d&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>&lt;i&gt;K&lt;sub&gt;m&lt;/i&gt;&lt;/i&gt;</td>
<td>&lt;i&gt;V&lt;sub&gt;max&lt;/sub&gt;&lt;/i&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>μM</td>
<td>μmol min&lt;sup&gt;-1&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>μM</td>
<td>μmol min&lt;sup&gt;-1&lt;/sup&gt;</td>
</tr>
<tr>
<td>—</td>
<td>—</td>
<td>NG</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>IPTG</td>
<td>NG</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>IPTG + Fe</td>
<td>NG</td>
<td>82 ± 4</td>
<td>153</td>
<td>4.2</td>
</tr>
<tr>
<td>Phe + Tyr</td>
<td></td>
<td>240 ± 20</td>
<td>13.3</td>
<td>6.4</td>
</tr>
<tr>
<td>—</td>
<td>NG</td>
<td>120 ± 6</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>IPTG</td>
<td>120 ± 6</td>
<td>124</td>
<td>3.8</td>
<td></td>
</tr>
<tr>
<td>IPTG + Fe</td>
<td>120 ± 10</td>
<td>124</td>
<td>3.8</td>
<td></td>
</tr>
<tr>
<td>Phe + Tyr</td>
<td>80 ± 4</td>
<td>124</td>
<td>3.8</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Cells of *E. coli* KA12/pKIMP-UAUC (Δ<sup>pheA-tyrA-aroF</sup> thi-1 endA1 hsdR17 Δ<sup>argF-lac</sup> U169 supE44 Δ<sup>srlR-recA</sup>) [prephenate dehydrogenase of *E. herbicola*], *pheC* [prephenate dehydratase of *P. aeruginosa*]) (46, 47), without or with a *pchB* plasmid, were grown in minimal medium E with 0.5% glucose and 1 mM thiamine as described in “Experimental Procedures”.

<sup>b</sup> Concentration of supplements were: IPTG, Phe, Tyr: 1 mM each; Fe (= FeCl<sub>3</sub>, 0.1 mM).

<sup>c</sup> Doubling times (<td>) were calculated from the exponential growth phase of three independent cultures; NG, no growth in 96 h.

<sup>d</sup> IPL and CM were assayed in crude extracts under standard conditions, with 35 μg and 310 μg of protein, respectively; standard deviations of the values shown did not exceed 10%; ND, no activity detected.
FIG. 2
FIG. 3, A/B/C
FIG. 5
FIG. 6
Salicylate biosynthesis in Pseudomonas aeruginosa: Purification and characterization of pchB, a novel bifunctional enzyme displaying isochorismate pyruvate-lyase and chorismate mutase activities
Catherine Gaille, Peter Kast and Dieter Haas

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