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A Novel Phenylserine Dehydratase from *Pseudomonas pickettii* PS22: Purification, Characterization, and Sequence of Its Phosphopyridoxyl Peptide

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A novel phenylserine dehydratase [EC 4.2.1.-], which catalyzes the deamination of L-*threo*-3-phenylserine to yield phenylpyruvate and ammonia, was purified to homogeneity from a crude extract of *Pseudomonas pickettii* PS22 isolated from soil. The enzyme was a monomer having a molecular mass of about 38 kDa and contained 1 mol of pyridoxal 5′-phosphate per mol of enzyme. The enzyme exhibited absorption maxima at 279 and 416 nm. No appreciable spectral change was observed over the pH range of 6.0 to 8.0. The maximal reactivity was obtained at about pH 7.5. The enzyme was highly specific for L-*threo*-3-phenylserine (K_m, 0.21 mM). L-*erythro*-3-Phenylserine, L-threonine, L-serine, and D-serine were inert. The enzyme was inhibited by phenylhydrazine, hydroxylamine, *p*-chloromercuribenzoate, and $HgCl_2$, but not by L-isoleucine, L-threonine, or L-serine. AMP, ADP, and ATP did not affect the enzyme activity. The N-terminal amino acid sequence was not similar to those of biosynthetic and biodegradative L-threonine dehydratases and L-serine dehydratases. The isolated tryptic phosphopyridoxyl peptide, however, contained a pyridoxal 5′-phosphate-binding consensus amino acid sequence of amino acid dehydratases.

Key words: phenylserine deaminase, phenylserine dehydratase, phosphopyridoxyl peptide, *Pseudomonas pickettii*, pyridoxal 5′-phosphate.

Although 3-phenylserine, which is an antagonist of phenylalanine in bacteria, is degraded by phenylserine aldolase (*1*), serine hydroxymethyltransferase (*2*), and L-amino acid oxidase (*3*), little attention has been paid to the metabolism of 3-phenylserine in microorganisms. During the course of a study on microbial degradation of DL-*threo*-3-phenylserine, we found that D-*threo*-3-phenylserine was a better substrate for $NADP^+$-dependent D-threonine dehydrogenase [EC 1.1.1.-] from *Pseudomonas cruciviae* IFO 12047 (*4*). To develop a means for the preparation of D-*threo*-3-phenylserine from the DL form, we screened soil bacteria that use L-*threo*-3-phenylserine as a sole carbon and nitrogen source and found a novel phenylserine dehydratase (L-*threo*-3-phenylserine dehydratase, EC 4.2.1.-) in a soil bacterium identified as *Pseudomonas pickettii* PS22 (*5*). The enzyme catalyzes the deamination of L-*threo*-3-phenylserine to yield phenylpyruvate and ammonia. To study enzymological and regulatory aspects of phenylserine dehydratase, we have purified the enzyme from *P. pickettii* PS22.

We describe here the purification and characterization of phenylserine dehydratase from *P. pickettii* PS22 and a comparison of the sequence of its phosphopyridoxyl peptide with those of other amino acid dehydratases.

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Abbreviation: TES, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid.

MATERIALS AND METHODS

Materials—DL-*threo*-3-Phenylserine, DL-*threo*-3-thienylserine, DL-*threo*-3,4-dihydroxyphenylserine, DL-*threo*-3-hydroxynorvaline, yeast alcohol dehydrogenase, bovine serum albumin, ovalbumin, and myoglobin were obtained from Sigma Chemical, St. Louis, MO; chymotrypsinogen A, and cytochrome *c* were obtained from Boehringer Mannheim GmbH, Mannheim; DEAE-cellulose was supplied by Serva, Heidelberg; Sephadex G-25 and G-150 and Mono Q HR10/10 (1.0×10 cm) and Mono P HR5/20 (0.5×20 cm) columns were purchased from Pharmacia, Uppsala; Butyl-Toyopearl 650 was obtained from Tosoh, Tokyo; and a YMC-Pack C4 column was purchased from YMC, Kyoto. DL-*erythro*-3-Phenylserine was synthesized according to the method of Greenstein and Winitz (*6*). ε-*N*-Pyridoxyllysine was prepared according to the method of Dempsey and Christensen (*7*). Other chemicals used were analytical-grade reagents.

Medium and Culture Conditions—The bacterium was cultured with a medium (pH 7.2) containing 1.0% peptone, 0.2% DL-*threo*-3-phenylserine, 0.2% K_2HPO_4, 0.2% KH_2PO_4, 0.2% NaCl, 0.01% $MgSO_4 \cdot 7H_2O$, and 0.01% yeast extract. Large-scale cultivation was carried out in 2-liter flasks containing 750 ml of the medium at 30°C for 20 h on a reciprocal shaker. The cells were harvested by centrifugation, washed twice with 0.85% NaCl, and stored at −20°C until use.

Enzyme Assay—The standard reaction mixture contain-

ed 20 μmol of DL-*threo*-3-phenylserine, 20 nmol of pyridoxal 5'-phosphate, 200 μmol of *N*-tris(hydroxymethyl)-methyl-2-aminoethanesulfonic acid (TES) buffer (pH 7.5), and enzyme in a final volume of 1.0 ml. In a blank, enzyme was replaced by water. Incubation was performed at 30°C for 10 min. The reaction was stopped by addition of 1.0 ml of 1 N HCl. Phenylpyruvate formed was determined by the 2,4-dinitrophenylhydrazine method (*5*). One unit of the enzyme was defined as the amount that catalyzed the formation of 1 μmol of phenylpyruvate per min in the reaction. Specific activity was expressed as units per milligram of protein. Protein was measured by the method of Lowry *et al.* (*8*) with bovine serum albumin as the standard. Concentrations of the purified enzyme were determined from A_{279}. The absorption coefficient ($A^{1\%}_{1cm}$ at 279 nm = 5.35) was estimated by absorption and dry weight determinations.

Purification of Phenylserine Dehydratase—All procedures were performed at 0 to 5°C, and potassium phosphate buffer containing 0.01% 2-mercaptoethanol and 1 mM ethylenediamine tetraacetic acid disodium salt (EDTA) was used in the purification procedures unless otherwise stated.

Step 1: Washed cells (244 g , wet weight) were suspended in 140 ml of 0.1 M buffer (pH 7.2) and disrupted by sonication. The supernatant obtained by centrifugation was dialyzed overnight against 10 mM buffer (pH 7.4) and used as the crude extract.

Step 2: To the crude extract (645 ml) was added 1 ml of 1.0% protamine sulfate solution (pH 7.2) per 100 mg of protein with stirring. After 10 min, the precipitate was removed by centrifugation. The supernatant was dialyzed overnight against 10 mM buffer (pH 7.4).

Step 3: The enzyme solution (798 ml) was applied to a DEAE-cellulose column (4.5 × 40.5 cm) equilibrated with 10 mM buffer (pH 7.4). After the column had been washed thoroughly with the buffer, the enzyme was eluted with the buffer supplemented with 50 mM KCl. The active fractions were pooled and concentrated by ultrafiltration with a Pellicon Labocassete (Nihon Millipore, Tokyo) equipped with PT filters.

Step 4: The enzyme solution was dialyzed overnight against 10 mM buffer (pH 8.0) and then brought to 30% saturation with solid ammonium sulfate. The enzyme solution (205 ml) was applied to a Butyl-Toyopearl 650 column (3.1 × 14.5 cm) equilibrated with 10 mM buffer (pH 8.0) containing ammonium sulfate (30% saturation). After the column had been washed with the buffer, the enzyme was eluted with 10 mM buffer (pH 8.0) containing ammonium sulfate (15% saturation). The active fractions were concentrated with an Amicon 200 ultrafiltration unit (Amicon, Lexington, MA), equipped with a PM-10 membrane filter and dialyzed against 10 mM buffer (pH 8.0).

Step 5: The enzyme solution (18 ml) was applied to a Mono Q HR10/10 anion-exchange column (1.0 × 10 cm) equilibrated with 10 mM buffer (pH 8.0). The column was equipped with a Pharmacia fast protein liquid chromatography system and developed at room temperature at a flow rate of 2.0 ml/min, with a 70-min linear gradient of KCl (0 to 0.2 M) in the same buffer. The active fractions were concentrated with the Amicon 200 ultrafiltration unit.

Step 6: The enzyme solution (12.6 ml) was dialyzed against 10 mM buffer (pH 8.0) and then brought to 30% saturation with solid ammonium sulfate. The enzyme solution was applied to a Butyl-Toyopearl 650 column (3.1 × 14.5 cm) equilibrated with 10 mM buffer (pH 8.0) containing ammonium sulfate (30% saturation). After the column had been washed with the 30 and 20% ammonium sulfate-saturated buffer, elution was done with a linear gradient of ammonium sulfate (20 to 0% saturation) in the same buffer (Fig. 1). The active fractions were concentrated with the Amicon 200 ultrafiltration unit.

Step 7: The enzyme solution (20.5 ml) was dialyzed against 10 mM buffer (pH 8.0) and applied to a Mono Q HR10/10 column equilibrated with the same buffer at room temperature. After the column had been washed with the buffer containing 25 mM KCl for 20 min at a flow rate of 1.0 ml/min, the enzyme was eluted with a 50-min linear gradient of KCl (25 to 100 mM) in the same buffer (Fig. 2). The active fractions (fraction No. 48–54) were combined,

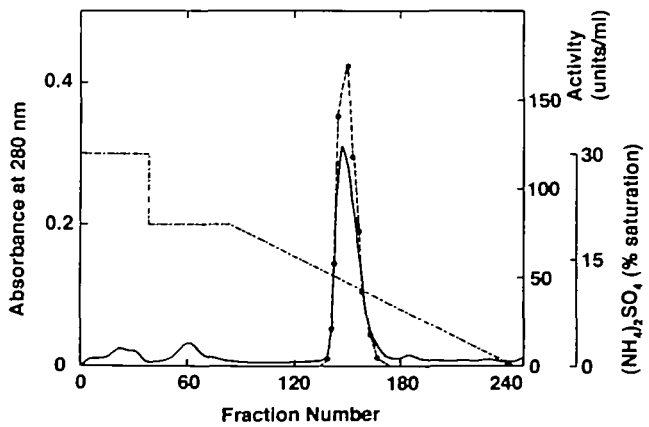

Fig. 1. **Second Butyl-Toyopearl 650 column chromatography of the enzyme.** The enzyme was chromatographed on a Butyl-Toyopearl 650 column (3.1 × 14.5 cm) equilibrated with 10 mM potassium phosphate buffer (pH 8.0) containing 0.01% 2-mercaptoethanol, 1 mM EDTA, and ammonium sulfate (30% saturation). The fractions (5 ml/tube) were collected. ○, activity; —, absorbance at 280 nm; and –·–, ammonium sulfate concentration (% saturation).

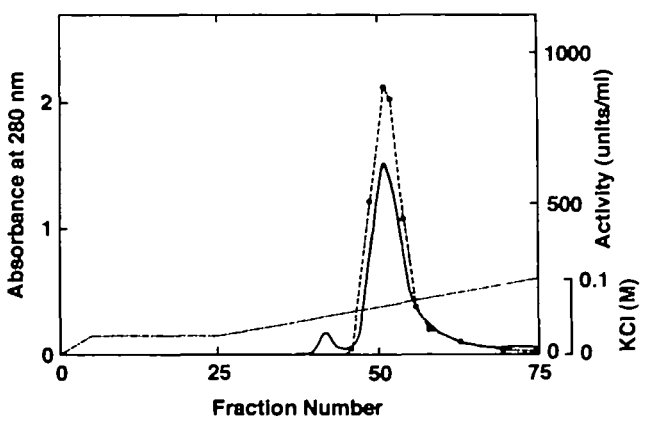

Fig. 2. **Second Mono Q column chromatography of the enzyme.** The enzyme was chromatographed on a Mono Q 10/10 column equilibrated with 10 mM potassium phosphate buffer (pH 8.0) containing 0.01% 2-mercaptoethanol and 1 mM EDTA using a Pharmacia fast protein liquid chromatography system. The fractions (1.0 ml/tube) were collected. ○, activity; —, absorbance at 280 nm; and –·–, KCl concentration (M).

dialyzed against 10 mM buffer (pH 7.4), and stored at −20°C in the presence of 30% glycerol.

Electrophoresis—Disc gel electrophoresis was performed by the method of Davis (*9*). Protein was stained with 0.04% Coomassie brilliant blue G-250 in 3.5% $HClO_4$. The enzyme was stained for activity with a solution (5.0 ml) containing 100 μmol of DL-*threo*-3-phenylserine, 0.5 mmol of TES buffer (pH 7.5), 0.2 mg of phenazine methosulfate, and 1 mg of nitroblue tetrazolium salt (*10*). SDS-disc gel electrophoresis was carried out by the method of Weber and Osborn (*11*).

Determination of Molecular Mass—The molecular mass was estimated by gel filtration on a Sephadex G-150 column, with the following standard proteins: yeast alcohol dehydrogenase (150 kDa), bovine serum albumin (67 kDa), ovalbumin (45 kDa), and myoglobin (17.8 kDa). The molecular mass was also estimated by SDS-disc gel electrophoresis, using the following standard proteins: bovine serum albumin (68 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25.7 kDa), and cytochrome *c* (11.7 kDa).

Determination of Isoelectric Point—The isoelectric point of the enzyme was measured by chromatofocusing using a Mono P HR5/20 column which was equipped with a Pharmacia fast protein liquid chromatography system.

Determination of the Pyridoxal 5′-Phosphate Content—The pyridoxal 5′-phosphate content of the enzyme was determined by the following three methods.

(i) *Phenylhydrazine method:* The enzyme (16.2 nmol) was kept at 37°C for 30 min in the presence of 0.2 N HCl to release the bound cofactor, and the amount of free pyridoxal 5′-phosphate was determined by using the phenylhydrazine method of Wada and Snell (*12*).

(ii) *Cyanohydrin method:* The enzyme sample (1.63 nmol) was hydrolyzed with 55 mM H_2SO_4 solution at 120°C for 5 h and analyzed by the cyanohydrin method of Bonavita (*13*).

(iii) *Spectrophotometric method:* The absorbance at 330 nm of the $NaBH_4$-reduced enzyme solution was measured and the phosphopyridoxyl content was calculated using its molecular extinction coefficient of 9,700 (*14*).

Isolation of Phosphopyridoxyl Peptides—The enzyme (16 nmol) was reduced with 20 mM $NaBH_4$, dialyzed against water, and lyophilized. The protein was dissolved in 0.1 ml of 0.4 M ammonium bicarbonate buffer (pH 8.0) containing 8 M urea, incubated at 37°C for 30 min, and then diluted with 0.3 ml of water. Trypsin was added to the solution in a 1 : 50 (mol/mol) ratio of protease to substrate. Digestion was performed at 37°C for 24 h. The peptides were separated on a Shimadzu HPLC system equipped with a YMC-Pack C4 column using a solvent system of 0.1% trifluoroacetic acid (A) and acetonitrile containing 0.07% trifluoroacetic acid (B). A 90-min linear gradient from 5 to 50% B was used to elute peptides at a flow rate of 1.0 ml/min. The absorbance at 210 nm and the fluorescence (excitation at 330 nm and emission at 370 nm) of the effluents were continuously monitored with a Hitachi F-1050 spectrofluorometer. The fluorescent peptides were isolated and lyophilized.

Sequence Analysis—The amino acid sequence was determined with an Applied Biosystems model 492 protein sequencer linked with a phenylthiohydantoin derivative analyzer.

Identification of ε-N-Pyridoxyllysine—ε-*N*-Pyridoxyllysine was identified by the reversed-phase isocratic HPLC method (*15*). The peptide P_3 (about 180 pmol) was incubated with 50 units of alkaline phosphatase at 37°C for 2 h and then hydrolyzed with 6 N HCl at 110°C overnight. HCl was removed by evaporation to dryness. The residue was dissolved in 2.0 ml of water. The sample (0.3 ml) was mixed with 10 μl of 9 M perchloric acid and then 100 μl of the solution was injected into a Hitachi HPLC system equipped with an ODS A-303 column (YMC, Kyoto). The column was developed with the buffer described by Edwards *et al.* (*15*) at a flow rate of 1.0 ml/min. The fluorescence (excitation at 325 nm and emission at 400 nm) of the effluent was monitored with a Hitachi F-1000 spectrofluorometer.

Spectrophotometry—Absorption spectra were taken with a Hitachi 220A spectrophotometer. Fluorescence measurements were performed with a Hitachi 650-10S spectrofluorophotometer with a 1.0-cm light path, and the intensities of emission and excitation spectra are given in arbitrary units.

RESULTS

Purification of Phenylserine Dehydratase—A summary of the purification procedure is shown in Table I. The enzyme was purified about 230-fold with a 10.3% yield from the crude extract. The purified enzyme showed a single band on disc gel and SDS-disc gel electrophoreses (Fig. 3). The mobility of the band stained for the enzyme activity on the polyacrylamide gel coincided well with the mobility of the protein band of the native enzyme stained with Coomassie brilliant blue.

Molecular Mass—The molecular mass of the enzyme was estimated to be about 40 kDa by gel filtration on a Sephadex G-150 column and 38 kDa by SDS-disc gel electrophoresis. These results suggest that the enzyme is a monomer. The isoelectric point of the enzyme was 5.1.

Stability—The enzyme could be stored at −20°C in 10 mM potassium phosphate buffer (pH 7.4) containing 0.01% 2-mercaptoethanol and 30% glycerol for several months without apparent loss of activity. When heated for 10 min in 10 mM potassium phosphate buffer (pH 7.4) containing 0.01% 2-mercaptoethanol, the enzyme was stable up to 40°C. Above 50°C, rapid inactivation occurred. The enzyme was most stable in the pH range of 6.0 to 9.5, when kept at 30°C for 10 min in the following buffers (0.1 M): sodium acetate buffer (pH 5.0-6.0), potassium phosphate buffer

TABLE I. **Summary of purification of phenylserine dehydratase from *P. pickettii* PS22.**

Steps	Total protein[a] (mg)	Specific activity (Units/mg)	Total activity (Units)	Yield (%)
Crude extract	25,100	1.27	31,900	100
Protamine sulfate	12,400	1.86	23,100	72.4
DEAE-cellulose	1,160	16.8	19,500	61.1
Butyl-Toyopearl	238	63.9	15,200	47.6
Mono Q	60.5	174	10,500	32.9
2nd Butyl-Toyopearl	38.2	258	9,860	30.9
2nd Mono Q	11.3	290	3,270	10.3

[a]The concentration of the purified enzyme after the DEAE-cellulose step was determined from the A_{279} using the extinction coefficient ($A^{1\%}_{1cm}$ at 279 nm = 5.35).

(pH 6.5-8.0), TES buffer (pH 7.2-8.0), *N*-tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid (TAPS) buffer (pH 8.0-9.0), and glycine-NaOH buffer (pH 9.0-11.5). The enzyme, however, was unstable in a Tris-HCl buffer (pH 8.0-9.0).

Effect of pH on the Enzyme Activity—The enzyme showed maximal activity at about pH 7.5 and showed high activity at pH 6.5 to 8.5 (Fig. 4).

Substrate Specificity—The enzyme was specific for the L-*threo* form of 3-phenylserine (K_m, 0.21 mM) and the other isomers were not substrates. Although DL-*threo*-3-phenylserine was used, analysis of the reaction mixture by HPLC with TSK gel Enantio L1 column showed that only the L-form was a substrate (*5*). The addition of D-*threo*-3-phenylserine (10 μmol) to the standard reaction mixture did not affect the enzyme activity, showing that the D-form of 3-phenylserine did not inhibit the reaction. In addition to L-*threo*-3-phenylserine, L-*threo*-3,4-dihydroxyphenylserine served as a substrate (17.1% of the activity obtained with L-*threo*-3-phenylserine). The enzyme did not act on DL-*threo*-3-thienylserine, DL-3-hydroxyphenylethylamine, DL-*threo*-hydroxynorvaline, DL-2-amino-3-phenyl-*n*-butanoate, DL-3-phenyllactate, DL-homoserine, L-threonine, D-threonine, L-*allo*-threonine, D-*allo*-threonine, L-serine, D-serine, D-glucosaminate, D-mannosamine, D-glucosamine, and D-galactosamine.

Inhibitors—The enzyme was inhibited 93% by 1 mM phenylhydrazine, 83% by 0.1 mM hydroxylamine, and 16% by 0.1 mM aminoxyacetate, which are typical inhibitors of the vitamin B_6 enzymes. The enzyme, however, was not inhibited by 1 mM semicarbazide, L-penicillamine, or D-penicillamine. It was inhibited completely by 0.1 mM *p*-chloromercuribenzoate or $HgCl_2$, but not by 1 mM L-cysteine, D-cysteine, or monoiodoacetate. The enzyme activity was not influenced by EDTA (1 mM), α,α'-dipyridyl (1 mM), NaF (1 mM), NaN_3 (1 mM), sodium arsenate (1 mM), ATP (0.2 mM), ADP (0.2 mM), AMP (0.2 mM), L-isoleucine (10 mM), L-threonine (10 mM), or L-serine (10 mM).

Absorption Spectrum—The enzyme exhibited absorption maxima at 279 and 416 nm at pH 7.4, with molar absorption coefficients of 20,300 and 4,570, respectively (Fig. 5, curve A). No appreciable spectral shift occurred on varying the pH (6.0 to 8.0). Reduction of the enzyme with 5 mM sodium borohydride by the dialysis method of Matsuo and Greenberg (*16*) affected both the absorption spectrum (Fig. 5, curve B) and the activity; the 416-nm peak disappeared with the appearance of an absorption peak at 330 nm, and the reduced enzyme was catalytically inactive. The borohydride-reduced enzyme showed a fluorescence maximum at 370 nm when excited at 330 nm. Addition of pyridoxal 5′-phosphate to the reduced enzyme did not increase the activity. These results showed that borohydride reduced the aldimine linkage to yield the aldamine bond.

Pyridoxal 5′-Phosphate Content—The pyridoxal 5′-phosphate content of the enzyme was 1.0 mol/38,000 g of protein by the phenylhydrazine method (*12*), 0.7 mol/38,000 g of protein by the cyanohydrin method (*13*), and

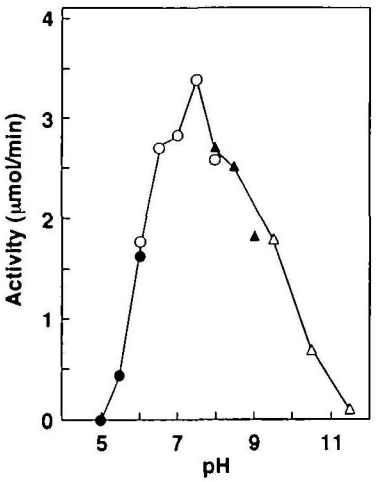

Fig. 4. **Effect of pH on the deamination of L-*threo*-3-phenylserine.** The reaction mixture contained 20 μmol of DL-*threo*-3-phenylserine, 20 nmol of pyridoxal 5′-phosphate, 200 μmol of sodium acetate (●), potassium phosphate (○), TAPS (▲), or glycine-NaOH (△) buffer.

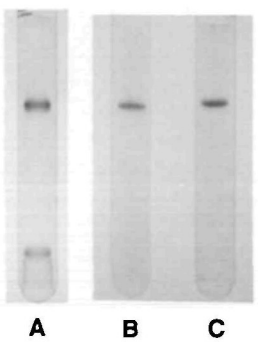

Fig. 3. **Polyacrylamide gel electrophoresis of the purified enzyme.** The purified enzyme (A, 2.0 μg; or B, 8.1 μg) was electrophoresed at a current of 2.5 mA by the method of Davis (*9*) and stained for activity (A) or protein (B) as described in "MATERIALS AND METHODS." The purified enzyme was treated with 1% SDS and 0.1% 2-mercaptoethanol by the method of Weber and Osborn (*11*). The SDS-treated enzyme (4.8 μg) was electrophoresed in the presence of 0.1% SDS at a current of 3 mA (C).

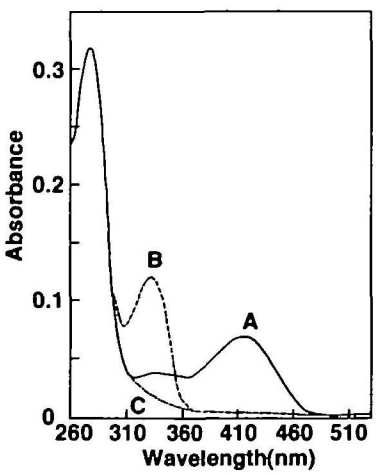

Fig. 5. **Absorption spectra of phenylserine dehydratase.** Absorption spectra were measured in 10 mM potassium phosphate buffer (pH 7.4) containing 0.01% 2-mercaptoethanol. Curve A, native enzyme; Curve B, $NaBH_4$-reduced enzyme; Curve C, apoenzyme.

0.88 mol/38,000 g of protein by the spectrophotometric method (*14*). The results obtained by these three procedures indicate that 1 mol of pyridoxal 5′-phosphate is bound to 1 mol of the enzyme protein.

Resolution and Reconstitution of the Enzyme—The addition of pyridoxal 5′-phosphate (20 μM) to the enzyme did not increase the enzyme activity, indicating that pyridoxal 5′-phosphate is tightly bound to the enzyme. To obtain the apoenzyme, the enzyme was incubated with 10 mM phenylhydrazine at 30°C for 30 min, followed by gel filtration on a Sephadex G-25 column (1.0×36 cm) equilibrated with 50 mM potassium phosphate buffer (pH 7.2) containing 1 mM EDTA and 0.01% 2-mercaptoethanol. The enzyme thus treated had no activity in the absence of added pyridoxal 5′-phosphate and no longer exhibited an absorption maximum at 416 nm (Fig. 5, curve C). Activity was almost fully restored by addition of 20 μM pyridoxal 5′-phosphate. Pyridoxal, pyridoxamine, pyridoxine, pyridoxamine 5′-phosphate, and pyridoxine 5′-phosphate neither restored the activity nor acted as inhibitors. The Michaelis constant for pyridoxal 5′-phosphate was estimated as 19 nM. The active enzyme reconstituted with pyridoxal 5′-phosphate showed the same spectrum as the native holoenzyme.

N-Terminal Amino Acid Sequence—Automated Edman degradation revealed that the N-terminal 23 amino acid sequence was Thr-Gln-Leu-Asp-Thr-Thr-Thr-Leu-Pro-Asp-Leu-Ser-Ala-Ile-Ala-Gly-Leu-Arg-Ala-Arg-Leu-Lys-Gln-.

Isolation and Sequencing of the Phosphopyridoxyl Peptides—To identify the lysyl residue which formed the Schiff-base with pyridoxal 5′-phosphate, the enzyme (16 nmol) was reduced with $NaBH_4$ followed by digestion with trypsin. The digest was separated by HPLC as described in "MATERIALS AND METHODS." Fluorescent peptides were eluted as one major peak accompanied with two minor peaks (designated P_1-P_3 in order of elution) (Fig. 6). The amino acid sequences of these three peptides were determined with a protein sequencer: P_1, Lys-Leu-Glu-Leu-Leu-Gln-Ala-Ser-Gly-Thr-Phe-X-Ala-Arg; P_2, Leu-Glu-Leu-Leu-Gln-Ala-Ser-Gly-Thr-Phe-X-Ala-Arg; and P_3, Thr-Thr-Pro-Val-Phe-Asp-Lys-Thr-Asp-Phe-Glu-Pro-Val-Pro-Gly-Thr-Ala-Val-Asn-Phe-Lys-Leu-Glu-Leu-Leu-Gln-Ala-Ser-Gly-Thr-Phe-X-Ala-Arg. These peptides contained the same sequence (13 residues). The unidentified residue (X) should correspond to the lysine residue forming the Schiff-base with pyridoxal 5′-phosphate, because the phenylthiohydantoin derivative of ε-*N*-phosphopyridoxyllysine is not identifiable with a protein sequencer. In order to identify the residue, the peptide P_3 was treated with alkaline phosphatase, hydrolyzed with 6 N HCl at 110°C overnight, and analyzed by the reversed-phase isocratic HPLC method as described in "MATERIALS AND METHODS." The hydrolyzate of the peptide showed the peak corresponded to ε-*N*-pyridoxyllysine, which was eluted between pyridoxamine and pyridoxine 5′-phosphate. Thus, the unidentified residue (X) is lysine forming the Schiff-base with pyridoxal 5′-phosphate.

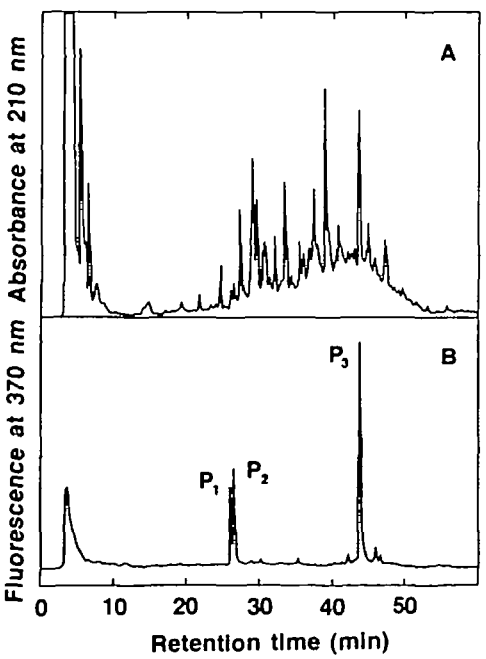

Fig. 6. **HPLC elution profile of the tryptic digest of the $NaBH_4$-reduced enzyme.** The tryptic digest of the $NaBH_4$-reduced enzyme was chromatographed on a YMC-Pack C_4 column. The peptides were detected by continuous monitoring of the absorbance at 210 nm (A) and the fluorescence (excitation at 330 nm and emission at 370 nm) (B). The peaks were designated as P_1-P_3 in order of elution.

DISCUSSION

P. pickettii PS22 could utilize L-*threo*-3-phenylserine as a sole carbon and nitrogen source (*5*). The bacterium produces phenylserine dehydratase, which catalyzes the deamination of L-*threo*-3-phenylserine to yield phenylpyruvate and ammonia. The enzyme production was induced by L-*threo*-3-phenylserine (*5*), suggesting that the enzyme functions in its degradation.

We have purified and characterized phenylserine dehydratase from *P. pickettii* PS22 to compare its properties with those of other dehydratases. The enzyme acts on L-*threo*-3-phenylserine and L-*threo*-3,4-dihydroxyphenylserine, but not on L-serine, D-serine, L-threonine, or D-threonine. Thus, this is the first example of a dehydratase specifically acting on L-*threo*-3-phenylserine.

Phenylserine dehydratase was a monomer (molecular mass, 38 kDa) similar to the *Escherichia coli* D-serine dehydratase (molecular mass, 37.3 kDa) (*17*) and the sheep liver L-threonine-serine dehydratase (molecular mass, 38 kDa) (*18*). In contrast, the biodegradative L-threonine dehydratase from *E. coli* (molecular mass, 147 kDa) (*19*) and the biosynthetic L-threonine dehydratase from *E. coli* (204 kDa) (*20*), *Salmonella typhimurium* (200 kDa) (*21*), and *Bacillus subtilis* (200 kDa) (*21*) were tetramers. Biosynthetic L-threonine dehydratase is subject to feedback inhibition by L-isoleucine (*20, 21*) and biodegradative L-threonine dehydratase is affected by AMP and ADP (*21*). These compounds, however, did not affect phenylserine dehydratase.

Although a pyridoxal 5′-phosphate-independent L-serine dehydratase, which contains an iron-sulfur cluster, has been reported (22), phenylserine dehydratase was inactivated by carbonyl reagents such as hydroxylamine and phenylhydrazine and by $NaBH_4$-reduction. EDTA and metal ions did not affect the enzyme activity. The enzyme

Fig. 7. **Comparison of the sequences of the pyridoxal 5′-phosphate binding sites of amino acid dehydratases.** Lysine residue which is bound with pyridoxal 5′-phosphate is shown by an asterisk. Conserved residues are indicated by white letters on black. PSD (*P. p.*), phenylserine dehydratase from *Pseudomonas pickettii* PS22; dThrD (*E. c.*), biodegradative L-threonine dehydratase from *Escherichia coli* (*26, 33*); sThrD (*E. c., S. t.*), biosynthetic L-threonine dehydratase from *Escherichia coli* (*25*) and *Salmonella typhimurium* (*31*); sThrD (*S. c.*), biosynthetic L-threonine dehydratase from *Saccharomyces cerevisiae* (*29*); L-SerD (*S. c.*), L-serine dehydratase from *Saccharomyces cerevisiae* (*35*); L-SerD (r. l.), L-serine dehydratase from rat liver (*23*); L-SerD (h. l.), L-serine dehydratase from human liver (*24*); and D-SerD (*E. c.*), D-serine dehydratase from *Escherichia coli* (*36, 32*).

contained 1 mol of tightly bound pyridoxal 5′-phosphate per mol of enzyme and showed the absorption peak at 416 nm. The characteristic spectral change of the enzyme upon $NaBH_4$-reduction (Fig. 5) and the identification of ε-*N*-pyridoxyllysine show that pyridoxal 5′-phosphate is bound to an ε-amino group of a lysine residue of the protein through an aldimine linkage, as reported for other vitamin B_6-enzymes. The catalytic mechanism of phenylserine dehydratase should be similar to those of other pyridoxal 5′-phosphate-dependent amino acid dehydratases (*21*).

The serine dehydratase cDNA from rat liver (*23*) and human liver (*24*) and genes for biodegradative and biosynthetic L-threonine dehydratases (*25-31*) have been cloned and sequenced. A comparison of the N-terminal 23 amino acid sequence of phenylserine dehydratase with the amino acid sequences deduced from these genes coding L-threonine and L-serine dehydratases showed no apparent similarity. The sequences of the pyridoxal 5′-phosphate-binding sites of the *E. coli* D-serine dehydratase (*32*), the *E. coli* biodegradative L-threonine dehydratase (*26, 33*), and the rat liver L-serine dehydratase (*34*) have been chemically identified. The tryptic phosphopyridoxyl peptide of phenylserine dehydratase has a similar sequence to the pyridoxal 5′-phosphate-binding site of other dehydratases (Fig. 7). Several amino acids around the lysine residue which binds pyridoxal 5′-phosphate are conserved in these dehydratases. Alexander *et al.* (*37*) divided the pyridoxal 5′-phosphate-dependent enzymes into three different families (α, β, γ) according to their primary structure. Amino acid dehydratases belong to the β-family together with the β-subunit of tryptophan synthase, threonine synthase, and cysteine synthase. In the β-family enzymes, the pyridoxal 5′-phosphate-binding lysine residue is located near the N-terminal segment of the enzymes. We recently cloned and sequenced the gene coding for phenylserine dehydratase and found that the lysine residue is positioned near the N-terminal region of phenylserine dehydratase, in the 58th position from the N-terminal (Okuda *et al.*, unpublished results).

We are currently studying the primary structure of the enzyme derived from the DNA sequence to elucidate the relationship between structure and function of the enzyme. Details of gene cloning and DNA sequencing for phenylserine dehydratase will be reported elsewhere.

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