Phenylalanine hydroxylase was purified approximately 3000-fold to apparent homogeneity with a 13% yield and crystallized from L-phenylalanine-induced cells of *Chromobacterium violaceum*. The enzyme was shown to be composed of a single polypeptide chain with an estimated molecular weight of approximately 32,000. Some of the physical properties of the enzyme include: a Stokes radius of 26.0 Å, a sedimentation coefficient of 2.71 S, a diffusion coefficient of 8.20 × 10^{-7} cm^2/s, a frictional ratio of 1.23, and an isoelectric point of pH 4.5. No detectable iron was found in the purified enzyme. Apparent $K_m$ values for L-phenylalanine and 2-amino-4-hydroxy-6,7-dimethyltetrahydropteridine were 140 and 54 μM, respectively.

### EXPERIMENTAL PROCEDURES

The "Experimental Procedures" and part of the "Results" are described in the miniprint supplement.1

1 This research was supported in part by a grant-in-aid for scientific research from the Ministry of Education, Science, and Culture of Japan. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The "Experimental Procedures," part of the "Results," Figs. 1 to 5, Tables III and IV, and "References" are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, Md. 20014. Request Document No. 78M-1435, cite author(s), and include a check or money order for $1.95 per set of photocopies.

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**RESULTS**

**Purity of Enzyme**—A typical purification procedure of phenylalanine hydroxylase from *C. violaceum* was summarized in Table I. The yield of the enzyme increased almost 2-fold after the step of DEAE-cellulose chromatography. By this procedure, the enzyme with a specific activity of 13.2 units/mg of protein was obtained in an overall purification of about 3000-fold with a 13% yield. The purified enzyme gave a single protein band upon disc gel electrophoresis as shown in Fig. 1A. A single band was also found in gel electrophoresis in 0.1% sodium dodecyl sulfate as shown in Fig. 1B.

The purified enzyme was crystallized by vapor diffusion technique using ammonium sulfate (29). The crystals took the form of rectangular plates as shown in Fig. 2.

**Physical Parameters**—The molecular weight of the enzyme was determined by a number of procedures. The value of 32,400 was obtained from Ultrogel AcA 44 gel filtration, as shown in Fig. 3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis gave a molecular weight of 33,000 (Fig. 4), which was in good agreement with the value determined by gel filtration on Ultrogel AcA 44, indicating that phenylalanine hydroxylase from *C. violaceum* consisted of a single polypeptide chain. The results of sedimentation equilibrium studies were plotted as the logarithm of the equilibrium protein concentration (ln $A_{280\text{ nm}}$) versus the square of radial distance from the center of the rotor ($r^2$) as shown in Fig. 5. Taking a partial specific volume of 0.739 cm^3/g, calculated as described below, together with the slope from Fig. 5, a molecular weight of 31,200 was obtained for the enzyme. The sedimentation velocity of the enzyme was measured by analytical centrifugation. A single, symmetrical schlieren peak was observed to sediment at $s_{20,\text{w}} = 2.71$ S. Gel filtration on Ultrogel AcA 44 (Fig. 3) gave a Stokes radius of 26.0 Å and the diffusion coefficient, $D_{20,\text{w}}$, was calculated to be $8.20 \times 10^{-7}$ cm^2/s from the value of the Stokes radius. A molecular weight of 31,700 was calculated from these values and a partial specific volume of 0.739 cm^3/g was determined by the Svedberg equation. A frictional ratio of the enzyme was calculated to be 1.23, indicating that the enzyme was nearly spherical in shape. The physical parameters of phenylalanine hydroxylase from *C. violaceum* are summarized in Table II.

Polycrylamide gel electrophoresis showed a single protein band and the isoelectric point which was estimated to be 4.5.

The absorption spectrum of the enzyme represented a typical absorption pattern of protein solution, with an absorption maximum at 279 nm and a small shoulder at 290 nm. The enzyme exhibited no absorption in the visible range.
Phenylalanine Hydroxylase

Purification of phenylalanine hydroxylase from C. violaceum

| Purification step          | Protein activity | Specific activity | Yield % | Purification Fold |
|---------------------------|------------------|-------------------|---------|------------------|
| Sonic extract             | 49,600 units     | 91.6 units/mg     | 100     | 1                |
| Protamine treatment       | 50,000 units     | 202 units/mg      | 100     | 1                |
| DEAE-Sephadex A-50        | 5,100 units      | 60.0 units/mg     | 27      | 3                |
| Acid treatment            | 2,080 units      | 56.5 units/mg     | 24      | 6                |
| DEAE-cellulose            | 266 units        | 98.6 units/mg     | 61      | 1                |
| 1st Ultrogel AcA 44       | 60 units         | 90.0 units/mg     | 41      | 339              |
| 2nd Ultrogel AcA 44       | 45 units         | 85.4 units/mg     | 39      | 430              |
| Hydroluxydate             | 5.0 units        | 42.5 units/mg     | 19      | 1,920            |
| Blue dextran-phenylbutylamine-Septa-rose | 2.2 | 29.1 units/mg | 13 | 2,990 |

Some physical parameters of phenylalanine hydroxylase from C. violaceum

| Parameters                          | Values               | Methods                      |
|-------------------------------------|----------------------|------------------------------|
| Molecular weight                    | 32,400 units         | Gel filtration              |
| Average molecular weight            | 32,000 units         | Analytical ultracentrifugation |
| Sedimentation coefficient, s_{20w}  | 2.71 S               | Gel filtration              |
| Diffusion coefficient, 8.20 × 10^{-7} cm^2/s | Gel filtration    |
| Stokes radius, a                    | 26.0 Å               | Gel filtration              |
| Partial specific volume, 8          | 0.739 cm^3/g         | Amino acid composition      |
| Frictional ratio, f/f₀              | 1.23                 | Gel filtration and amino acid composition |

Chemical Properties—The amino acid composition of phenylalanine hydroxylase from C. violaceum is given in Table III. Since 3 mol of half-cystine/mol of enzyme were determined as cysteic acid after performic acid oxidation and three sulfhydryl groups/mol of enzyme were determined by the use of 5,5'-dithiobis(2-nitrobenzoic acid) in 2% sodium dodecyl sulfate, there appeared to be no disulfide linkage in the enzyme. When the native enzyme was titrated with 5,5'-dithiobis(2-nitrobenzoic acid), only 1 mol of sulfhydrol residue/mol of enzyme reacted with the reagent. Therefore, it appeared that 2 mol of cysteine were buried inside the enzyme molecule. A partial specific volume of 0.739 cm^3/g was estimated from the amino acid composition (23).

The iron content of the enzyme was determined by the atomic absorption spectrophotometer. Prior to determination, the purified enzyme (0.37 mg/ml) was dialyzed overnight at 4°C against 50 mM 2-(N-morpholinol)ethanesulfonic acid/NaOH buffer, pH 6.5, without an appreciable loss of the activity. No detectable iron was found in this preparation.

Catalytic Properties—The optimum pH for the enzyme activity was found to be in the range of 7.3 to 7.5. The apparent K_m values for L-phenylalanine and DMPH_t were estimated from the linear double reciprocal plots to be approximately 140 and 54 μM, respectively. The apparent V_max value was also estimated to be approximately 14 μmol of tyrosine/min/mg of protein from the double reciprocal plots. Under the conditions described under “Experimental Procedures,” the rate of hydroxylation of L-tryptophan was found to be approximately 0.4% of that of L-phenylalanine. No detectable activity of tyrosine hydroxylation was observed.

DMPH_t, 6MPH_t, and tetrahydrofolate served as electron donors for the enzyme. DMPH_t was found to be the most active among these three synthetic substrates. The enzyme activity with GMPH_t and tetrahydrofolate was approximately 60 and 10% of that with DMPH_t, respectively.

Stoichiometry of the enzyme reaction is presented in Table IV. L-Phenylalanine was converted to a stoichiometric amount of L-tyrosine with the consumption of equimolar amounts of oxygen and DMPH_t.

DISCUSSION

In the present study, phenylalanine hydroxylase was obtained for the first time as a homogeneous and crystalline preparation. Since Letendre et al. (2) reported that C. violaceum contained a phenylalanine hydroxylase and a cell-free preparation was easily obtained from this organism, it was anticipated that the bacterium contained a relatively large amount of phenylalanine hydroxylase. However, a 3000-fold purification was required for obtaining a homogeneous enzyme preparation.

The molecular weight of the enzyme was determined by a number of procedures as summarized in Table II. The values ranging from 31,200 to 32,400 were consistent with the value of 33,000 determined by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate, indicating that the enzyme consisted of a single polypeptide chain. Letendre et al. (24) reported that phenylalanine hydroxylase from Pseudomonas species was a single protein chain of molecular weight between 25,000 and 27,000. In contrast to these bacterial enzymes, phenylalanine hydroxylases from mammalian livers are believed to exist in polymeric forms (4, 25, 26). Rat liver enzyme consisted of a dimer or tetramer of subunit of M_r = 55,000 (25), human liver enzyme consisted of a dimer of subunit of M_r = 54,000 (4), and monkey liver enzyme consisted of two proteins of very similar molecular weight between 45,000 and 57,500 (26).

Hydroxylation of tryptophan by rat liver phenylalanine hydroxylase was demonstrated by Renson et al. (27) using partially purified enzyme and confirmed by Kaufman and Fisher (1) using highly purified (90% pure) enzyme. Bacterial phenylalanine hydroxylase from C. violaceum also catalyzed hydroxylation of L-tryptophan, although it was much slower than that of L-phenylalanine.

Kaufman and his co-workers (28) demonstrated that phenylalanine hydroxylase of rat liver was an iron enzyme and the metal might be involved in the enzymic reaction. Bacterial phenylalanine hydroxylase from Pseudomonas species was shown to be activated by mercuric, cadmium, cupric, and cuprous ions as well as ferrous ions (29). Contrary to our expectations, no iron was detected in phenylalanine hydroxylase from C. violaceum by atomic absorption spectroscopy. Further investigations on the involvement of iron or other metals in the enzymic reaction are now in progress.

Acknowledgments—We thank Mr. K. Miyakawa for performing the ultracentrifugation studies and Mrs. Y. Ohishi for performing the amino acid analysis.

REFERENCES

References are found in the miniprint section, p. 1833.

The abbreviations used are: DMPH_t, 2-amino-4-hydroxy-6,7-dimethyltetrahydropteridine; 6MPH_t, 2-amino-4-hydroxy-6-methyltetrahydropteridine.
Phenylalanine Hydroxylase

**Phenylalanine Hydroxylase from Chromobacterium violaceum**

**PURIFICATION AND CHARACTERIZATION**

By Hiroshi Nakata, Katsuhiko Yamasaki and Hitoshi Furusawa

**EXPERIMENTAL PROCEDURES**

**Materials** - Chromobacterium violaceum (ATCC 12456) was obtained from the Culture Collection, Osaka University, Japan. An NAD+-dependent phenylalanine hydroxylase from C. violaceum was purified to homogeneity as described by Yamasaki et al. (1).

**Preparation of Enzyme** - Phenylalanine hydroxylase was purified from C. violaceum grown in a 15-liter fermenter, as described by Yamasaki et al. (1).

**Enzyme Assay** - The enzyme activity was determined by measuring the formation of tyrosine according to the method described by Yamasaki et al. (1). The reaction was started by the addition of NADH to a final concentration of 100 μM. After incubation at 37°C for 30 min, the reaction was stopped by the addition of 100 μL of 10% trichloroacetic acid.

**Results** - The enzyme was purified 4200-fold with a yield of 10%.

**Discussion** - Phenylalanine hydroxylase was purified to homogeneity, and the enzyme activity was determined by the method described by Yamasaki et al. (1).

**References** - 1. Yamasaki, K., Nakata, H., and Furusawa, H. (1979) J. Biol. Chem. 254, 11001-11006.

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**Supplementary Material**

**Phenylalanine Hydroxylase**

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Phenylalanine Hydroxylase

Fig. 1. Electrophoresis of purified phenylalanine hydroxylase from Chromobacterium violaceum. A, disc gel electrophoresis on 7% polyacrylamide gel; B, disc gel electrophoresis on 10% polyacrylamide gel in the presence of sodium dodecyl sulfate. Electrophoretograms were performed as described under "Experimental Procedures." Approximately 40 μg of purified protein were applied to each gel. Arrows indicated the dye front.

Fig. 2. Crystalline phenylalanine hydroxylase from Chromobacterium violaceum.

Fig. 3. Determination of molecular weight of phenylalanine hydroxylase from Chromobacterium violaceum by gel filtration on a Ultrogel A A-4.7. The gel was equilibrated and run with 0.1 M sodium acetate buffer, pH 6.0, containing 100 mM NaCl. The markers used were cytochrome c (Mr = 12,300), chymotrypsinogen (Mr = 25,000), ovalbumin (Mr = 45,000), and bovine serum albumin (Mr = 67,000).

Fig. 4. Determination of molecular weight of phenylalanine hydroxylase from Chromobacterium violaceum by nondenaturing polyacrylamide gel electrophoresis. The markers used were cytochrome c (Mr = 12,300), chymotrypsinogen (Mr = 25,000), ovalbumin (Mr = 45,000), catalase (Mr = 69,000), and bovine serum albumin (Mr = 67,000).

Fig. 5. Sedimentation equilibrium analysis of phenylalanine hydroxylase from Chromobacterium violaceum. The initial protein concentration was 0.27 mg/mL. The analysis was done in a buffer containing 1 M dithiothreitol.

Table III

Amino acid composition of phenylalanine hydroxylase from Chromobacterium violaceum

| Amino acid | Mol % | Residues/molecule |
|------------|-------|-------------------|
| Lysine     | 3.6   | 16.4              |
| Histidine  | 1.9   | 5.4               |
| Arginine   | 9.5   | 15.8              |
| Aspartic Acid | 13.6 | 30.9              |
| Threonine  | 4.5   | 12.9              |
| Serine     | 3.0   | 4.7               |
| Glutamic Acid | 7.8  | 21.8              |
| Proline    | 6.8   | 13.4              |
| Glycine    | 6.6   | 13.0              |
| Alanine    | 10.1  | 29.0              |
| Cysteine   | 3.1   | 11.1              |
| Tyrosine   | 4.0   | 11.2              |
| Phenylalanine | 5.6  | 11.9              |
| Tryptophan | 1.3   | 3.7               |

*Estimated by extrapolation to zero time of hydrolysis.

* Determined by the use of 5,5'-dithiobis(2-nitrobenzoic acid) in 24% sodium dodecyl sulfate (11) and as cysteic acid after performic acid oxidation (10).

** Determined spectrophotometrically (11).
### Stoichiometry of phenylalanine hydroxylase reaction

In Experiment 1, reaction mixture (250 µl) contained enzyme (15 µg), 3-methylglutaconylase (0.5 unit), phenylalanine (100 µg), and Tris-CI buffer, pH 7.3 (25 µl). Reaction was started by addition of DMGA (0.15 µmol) at 24°C and incubated with occasional mild shaking with air. The enzymic reaction was followed by the increase in absorbance at 280 nm during the incubation. The amount of phenylalanine consumed was estimated from the increase in absorbance at 280 nm by the addition of 100 µl of KI solution to the solution of the product. After the reaction, the samples were subjected to thin-layer chromatography for the determination of phenylalanine and tyrosine by using amino acid analyzer (Hitachi Model KLA-5).

In Experiment 2, reaction mixture (0.78 µl) contained enzyme (2 µg), 3-methylglutaconylase (0.5 unit), phenylalanine (50 µg), and Tris-CI buffer, pH 7.3 (87 µl) in a spectrophotometer cell. Reaction was initiated by addition of DMGA (0.15 µmol) at 24°C and the change in absorbance at 350 nm due to the oxidation of DMGA to its diphenyl form was recorded. The amount of DMGA consumed was estimated from the increase in absorbance at 350 nm by the addition of 200 µl of KI solution. The reaction mixture was filtered through a Millipore filter, and the filtrate was subjected to thin-layer chromatography for the determination of tyrosine by using amino acid analyzer (Hitachi Model KLA-5).

### Table

| Experiment | Phenylalanine | DMGA | Oxygen | Tyrosine | Consumption | Formation |
|------------|---------------|------|--------|----------|-------------|-----------|
|            |               |      |        |          | consumed    | consumed  | formed   |            |
| 1          | 100           | 375  |        |          |             |           | 1.61     |            |
| 2          | 78.7          | 92.7 |        |          | 0.92        | 112.2     | 0.82     |            |
| 3          | 83.4          | 174.3|        |          | 0.93        | 363.6     | 0.90     |            |

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