Preliminary X-ray crystallographic analysis of tryptophanase from
Escherichia coli

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Tryptophanase (L-tryptophan indole-lyase, EC 4.1.99.1), one of the pyridoxal enzymes, catalyzes the reversible degradation of tryptophan to indole, pyruvate and ammonia [1,2]. In addition, the enzyme can perform α, β-elimination [1], β-replacement [3] and α-hydrogen exchange reactions with various L-amino acids as substrates. The \( E. coli \) enzyme, of mass 208968, is composed of 4 identical subunits [4,5], each possessing a pyridoxal 5'-phosphate (PLP) binding site [1]. Monovalent cations such as K\(^+\) and NH\(_4\)\(^+\) are required for catalytic activity [3]. Although a number of amino acid residues (Lys [6], Arg [7], Thr [3-10], Met [11], His [12], Cys [13-15] and Tyr [16]) have been shown to be involved in the catalytic mechanism by chemical modification and site-directed mutagenesis experiments, detailed structural characteristics for this enzyme have not yet been elucidated. Recently, we reported the overproduction of tryptophanase from recombinant \( E. coli \) cells and crystallization of this enzyme using polyethylene glycol or potassium phosphate as precipitants [17]. Although these crystals were large enough for X-ray analysis, the quality of the crystals was not sufficient for detailed analysis. In this paper, we report the preparation of new crystals suitable for X-ray analysis and the preliminary crystallographic data.

1. INTRODUCTION

Tryptophanase (L-tryptophan indole-lyase, EC 4.1.99.1), one of the pyridoxal enzymes, catalyzes the reversible degradation of tryptophan to indole, pyruvate and ammonia [1,2]. In addition, the enzyme can perform α, β-elimination [1], β-replacement [3] and α-hydrogen exchange reactions with various L-amino acids as substrates. The \( E. coli \) enzyme, of mass 208968, is composed of 4 identical subunits [4,5], each possessing a pyridoxal 5'-phosphate (PLP) binding site [1]. Monovalent cations such as K\(^+\) and NH\(_4\)\(^+\) are required for catalytic activity [3]. Although a number of amino acid residues (Lys [6], Arg [7], Thr [3-10], Met [11], His [12], Cys [13-15] and Tyr [16]) have been shown to be involved in the catalytic mechanism by chemical modification and site-directed mutagenesis experiments, detailed structural characteristics for this enzyme have not yet been elucidated. Recently, we reported the overproduction of tryptophanase from recombinant \( E. coli \) cells and crystallization of this enzyme using polyethylene glycol or potassium phosphate as precipitants [17]. Although these crystals were large enough for X-ray analysis, the quality of the crystals was not sufficient for detailed analysis. In this paper, we report the preparation of new crystals suitable for X-ray analysis and the preliminary crystallographic data.

Abbreviations: PLP, pyridoxal 5'-phosphate

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2. MATERIALS AND METHODS

2.1. Enzyme purification

\( E. coli \) tryptophanase was purified from \( E. coli \) K-12 strain MD55 ( strains K-12 strain MD55 harboring plasmid pMD6B by methods reported previously [17]. The amount of enzyme produced from these cells corresponded to about 60% of the total soluble protein. In order to prepare new crystals with ammonium sulfate as a precipitant, the enzyme was further purified by using a column of Sepharose-bound PLP-alanine complex, SP-C-Ala [9]. SP-C-Ala was prepared by the method of Ikeda et al. [18]. The enzyme was applied to the column (1.0 x 5 cm) which was previously equilibrated with 10 mM potassium phosphate buffer, pH 7.0, at room temperature. After the column was washed thoroughly with 1 M potassium phosphate buffer, pH 7.0, to remove non-specific bound protein, tryptophanase was eluted with 50 mM potassium acetate buffer, pH 5.0, containing 50 \( \mu \)M PLP. The specific activity of the enzyme obtained thus was improved to 64 \( \mu \)g U/mg protein. The enzyme solution was concentrated by using an Amicon Centricron 30 microconcentrator. Before crystallization experiments, the enzyme was incubated with 5 mM dithiothreitol for 30 min at 37°C in 50 mM potassium phosphate buffer containing 0.5 mM PLP at pH 7.0.

The enzyme concentration was determined at 278 nm using the absorption coefficient for holoenzyme \( \left( 41^\text{cm} = 8.9 \right) [9] \). Enzyme activity was assayed spectrophotometrically with 0.33 mM S-o-nitrophenyl-L-tyrosine at pH 7.8 and 30°C [19].

2.2. Crystallization

Crystallization was performed with the hanging and sitting drop vapor diffusion methods. The hanging method was initially used for screening a wide variety of crystallization conditions. Drops of protein solution (3 \( \mu l \) at 25 mg/ml) were mixed with an equal volume of reservoir solution on a siliconized glass coverslip. Each coverslip was sealed with silicon grease on top of a well (24-well Linbro plastic tissue culture plates) containing 1.0 ml of the reservoir solution, and kept at 23°C. The sitting drop method was used for growing crystals under the optimized conditions. Fifty \( \mu l \) of protein solution was mixed with an equal volume of the crystallization solution in a depression slide. The reservoir was filled with 10 ml of the crystallization buffer, and the plastic box was then sealed with tape.
2.3. X-ray experiments

X-ray photographs were taken with an Enraf-Nonius precession camera at room temperature on Kodak DEF-5 X-ray film. X-ray diffraction intensities were measured by the rotation method [20] on an X-ray imaging plate system (Rigaku R-AXISIIIC) equipped with Franks double mirror optics [21] and a rotating-anode X-ray generator (Rigaku RU-200) operated at 40 kV, 100 mA.

2.4. Measurements of crystal and mother liquor densities

The crystal and mother liquor densities were measured by floatation in a xylene/bromobenzene linear gradient solution [22]. The linear gradient was calibrated with sodium nitrate droplets.

3. RESULTS AND DISCUSSION

Initially, we made tryptophanase crystals suitable for X-ray structure analysis from polyethylene glycol 4000 or potassium phosphate at pH 7.8 by using the hanging drop vapor diffusion method reported previously [17]. Four types of crystals were obtained: holoenzyme, apoenzyme, a complex of holoenzyme and L-alanine all crystallized from polyethylene glycol solution, and a complex of holoenzyme and L-alanine from potassium phosphate buffer. The crystals of the holoenzyme were hexagonal with unit-cell dimensions of \( a = b = 153.3 \, \text{Å}, c = 141.8 \, \text{Å} \). Although the holoenzyme crystals (hexagonal bipyramid in shape) were large enough for X-ray analysis (0.7 \( \times \) 0.7 \( \times \) 0.8 mm), they diffracted only to about 7 Å resolution. On the other hand, the crystals of holoenzyme and L-alanine complex from potassium phosphate buffer diffracted to a resolution of at least 4 Å, and thus were of a higher quality than the holoenzyme crystals. However, the reproducibility of this crystal was very low. The other two types of crystals (apoenzyme and complex from polyethylene glycol solution) were also difficult to obtain reproducibly.

In order to prepare well-ordered crystals for high-resolution X-ray analysis, we further purified the enzyme by using an affinity column of SP-C-Ala, and searched for good crystallization conditions by use of ammonium sulfate as a precipitant. At neutral pH regions, only needle-like crystals appeared. However, at pH 5.25, which is close to the isoelectric point of tryptophanase [23], crystals of holotryptophanase suitable for X-ray analysis were obtained. The crystals grew to a maximum size of 0.4 \( \times \) 0.4 \( \times \) 0.5 mm in about 1 week from a solution of 13% (w/v) ammonium sulfate and 25 mM potassium acetate at pH 5.25 containing 0.25

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Fig. 1. Crystals of tryptophanase grown from ammonium sulfate solution. The typical crystal size is approximately 0.4 \( \times \) 0.4 \( \times \) 0.5 mm.
mM PLP, 2.5 mM dithiothreitol, and 0.04% (w/v) sodium azide. As seen in Fig. 1, the crystals had a rhombic bipyramidal morphology. These crystals were obtained reproducibly. The high purification of the enzyme was thus very effective for making good crystals.

The crystals diffracted to a resolution limit of approximately 3 Å in still photographs. Precession photographs of the h01 and hk0 zones showed that the crystals are tetragonal, belonging to space group P4_2_2_2, or its enantiomorph with unit-cell dimensions of a = b = 113.4 Å and c = 232.2 Å. The number of subunit molecules of tryptophanase per unit cell in the crystals was estimated to be 16 (two subunits per asymmetric unit) from the unit-cell volume, the crystal and mother liquor densities (1.24 and 1.10 g/cm^3, respectively), and the subunit molecular weight (52 000). The resultant values of V_m and V_0ly [24] were 3.3 Å^3/Da and 0.65, respectively. These values lie within the range usually found for protein crystals [24].

The crystals thus characterized were, however, X-ray-labile. We therefore used an X-ray imaging plate system, Rigaku R-AXISIIC, for the purpose of a fast intensity measurement, and the full intensity data for the native crystal were collected to 3.0 Å resolution within 20 h. The preparation of heavy-atom derivatives is now in progress. Mercury derivatives will be obtained easily because tryptophanase has one or two highly reactive cysteine residues in the subunit [13].

Recently, we reported [9] that Trp-248 in the active site of tryptophanase interacts with PLP closely and plays an important role in the transition state of the catalytic reaction by studying a mutant enzyme in which Trp-248 had been replaced with phenylalanine. The mutant enzyme has also been crystallized under the same conditions as those of the wild-type enzyme, and the mutant crystals had an identical morphology to the wild-type. High resolution X-ray analyses of the wild-type and mutant enzymes would be very helpful to understand the role of Trp-248 in the catalytic mechanism.

The three-dimensional structure of tryptophan synthase from Salmonella typhimurium, which is also one of the pyridoxal enzymes, has been determined by X-ray diffraction analysis at high resolution [25]. Tryptophanase and tryptophan synthase share a common function, namely the synthesis of L-tryptophan, despite the fact that little sequence homology exists between the two enzymes. The structural comparison between tryptophanase and tryptophan synthase is therefore of importance in understanding the functional and evolutionary relationships of these enzymes.

REFERENCES