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'대장균주로부터 분리한 GSH-I 효소의 결정화

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CRYSTALLIZATION OF γ-GLUTAMYLCYSTEINE SYNTHETASE FROM Escherichia coli

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Abstract

Reduced glutathione (GSH) plays a vital role in the metabolism of all cells. Glutathione, a tripeptide composed of glutamic acid, cysteine, and glycine, is synthesized by two sequential reactions. The first is catalyzed by **γ-glutamylcysteine synthetase** (GSH-I) and the second by glutathione synthetase (GSH-II). The glutathione biosynthetic pathway of *E. coli* is mainly controlled by nonallosteric feedback inhibition of GSH-I by GSH. Determination of the three-dimensional structure of GSH-I by X-ray crystallography is necessary in order to understand the structure-function relationship at the molecular level. As the first step toward its structure determination, crystallization of *E. coli* γ-glutamylcysteine synthetase (GSH-I) has been achived using the hanging drop vapor diffusion method and capillary method. Crystals of GSH-I have been grown from

ammonium sulfate solution. The crystals grew at room temperature within 10 days to dimensions of 0.2 mm x 0.2 mm x 0.2 mm by hanging drop vapor diffusion method and diffracted to about 4 Å resolution using synchrotron X-rays. Another crystal, grown by the capillary method to dimensions of 0.25 mm x 0.25 mm x 0.3 mm within 40 days, diffracted to about 4 Å resolution using X-rays from a rotating anode.

요 약

환원된 글루타치온은 모든 세포의 대사에 중요한 역할을 하고 있다. 글루타치온은 두개의 연속적인 반응으로 합성된 세개의 펩타이드로 구성되어있다. 첫번째 반응에서의 촉매효소가 GSH-I 이며, 두번째 반응에서의 촉매효소가 GSH-II이다. 대장 균주의 글루타치온 합성 기작은 주로 GSH-I의 되돌림제어에 의해 조절되고 있다. 이러한 생화학적 작용과 구조와의 관계를 알기 위한 첫 단계로 증기 확산 방울 방법과 모세관 확산 방법으로 실온에서 결정을 얻게 되었다. GSH-I 효소의 결정은 침전제 ammonium sulfate을 사용하여 얻었으며, 증기 확산 방울 방법으로는 10일의 기간으로 실온에서 0.2 mm x 0.2 mm x 0.2 mm 크기로 자랐으며. 이 결정은 싱크로트론 X-ray를 사용하여 약 4.0 Å 까지 회절 시켰다. 또, 모세관을 이용한 방법으로 얻은 결정은 40일의 기간으로 실온에서 0.25 mm x 0.25 mm x 0.3 mm 의 크기로 자랐으며, 이 결정은 회전 음극선의 X-ray를 사용하여 약 4.0 Å 까지 회절시켰다.

Introduction

Glutathione, a tripeptide having the structure of γ-glutamyl cycteinylglycine, is an important nonprotein thiol in living systems. It has been reported that glutathione plays many important physiological roles in mammals such as maintenance of protein sulfhydryl groups and formation of the deoxyribonucleotide precusor of DNA, protection of cells against reactive oxygen compounds and free radicals, and detoxification from foreign compounds. In addition, it acts as a metabolite reducing agent, as a coenzyme in some enzymic reactions[1]. It is synthesized intracellularly by the consecutive actions of γ-glutamylcysteine synthetase (GSH-I) and glutathione synthetase (GSH-II). Three-dimensional structure of GSH-II determined at high resolution has been reported[2]. GSH-I has been purified from several sources. GSH-I from various sources such as *E. coli*, *Proteus mirabilis*, and rat liver, have been found to be different in their molecular masses and subunit structure[3-5]. In the regulation of the glutathione biosynthetic pathway of *E. coli*, the inhibitory effect of reduced glutathione on GSH-I activity seems to be of physiological significance[4,5]. Inhibition by glutathione appears to involve its binding to the glutamate site of the enzyme as well as to other enzyme sites[7]. GSH-I from *E. coli* as well as *Proteus mirabilis* contain no sulfhydryl group as an active site thiol[4,5,8]. Despite its important biological function, no three-dimensional structures of GSH-I have been reported yet. In order to study the structure-function relationship of GSH-I, determination of its three-dimensional structure by X-ray crystallography is necessary. As the first step toward its structure determination, crystallization of GSH-I from *E. coli* has been performed. We report here the results of these crystallization experiments.

Experimental Procedure

Purification

The gene encoding GSH-I from *E. coli* was cloned into *E. coli* and the protein was overproduced[6,9]. GSH-I was purified by the method of Watanabe[5]. Homogeneity of purified enzyme was determined by the discontinuous electrophoresis in 10 % (w/v) polyacrylamide gel in the presence of 0.1 % (w/v) sodium dodecyl sulfate[10]. The gel was stained by the silver staining procedure[11]. The enzyme concentration was estimated by measuring the absorbance at 280 nm, assuming that the concentration of 1.0 mg/ml corresponds to the unit absorbance at 280 nm for the path length of 1.0 cm.

Crystallization

Purified enzyme was concentrated to 22 mg/ml by ultrafiltration using YM 30 membrane (Amicon) and then dialyzed against 50 mM Hepes buffer (pH 7.50) for crystallization. Crystallization was achieved by both hanging drop vapor diffusion and capillary methods at room temperature (22 ± 2 ℃) using 24-well tissue culture plates (Flow Laboratories). Initial crystallization conditions were screened by the incomplete factorial method[12] and sparse matrix sampling[13]. Hanging drops were prepared by mixing equal volumes (3 μl) of the enzyme and the reservoir solutions. The cover glass with a hanging drop was placed over the 1-ml reservoir solution in each well of the tissue culture plate and an air-tight seal was made with grease. For the capillary method, the enzyme solution (200 μl) was injected into the capillary of diameter 2 mm using a syringe. Then the reservoir solution (200 μl) was overlayed carefully using a syringe to minimize mixing and to avoid formation of air bubbles. Both ends of the capillary were sealed tightly with wax.

X-ray Studies

A crystal was mounted in a thin-walled glass capillary for X-ray analysis. Both ends of the capillary were filled with the mother liquor and then sealed with wax. X-ray experiments were carried out at the beamline X12-C of National Synchrotron Light Source in Brookhaven National Laboratory[14]. The wavelength of the monochromatic X-rays used was 1.100 Å (1 Å = 0.1 nm). X-ray experiments were also carried out using graphite-monochromatized CuKα X-rays from a rotating anode generator (Rigaku RU-200BH), running at 40 kV and 70 mA with a 0.3 mm focus cup and a 0.6 mm collimator. FAST diffractometer systems (Enraf-Nonius) were used to record X-ray diffraction patterns.

Results and Discussion

The initial survey for crystallization conditions by the sparse matrix sampling gave useful information about crystallization conditions. Stepwise refinement of the conditions were carried out to produce larger, better crystals. Microcrystals were grown from solutions containing precipitants such as phosphate and PEG 8,000 over a wide range of pH (4 to 10) and small crystals were observed in the presence of ammonium sulfate at around pH 6.5. The crystallization condition using ammonium sulfate as precipitant seemed to be most promising and was further refined to obtain large crystals. This experiment established conditions for growing two crystal forms. The form I crystal (Fig. 1) has been obtained at room

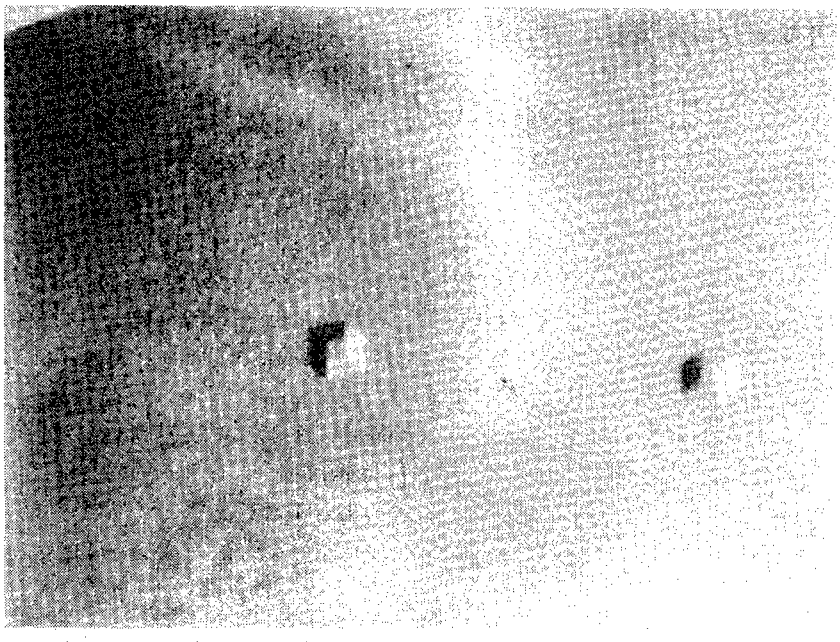

Figure 1. Form I crystals of γ-glutamylcysteine synthetase from *E. coli*. Approximate dimensions are 0.2 mm x 0.2 mm x 0.2 mm.

temperature from a reservoir solution of 100 mM ADA buffer (pH 6.5) and 1.8 M ammonium sulfate. This crystal grew in two weeks and the largest crystals are of dimensions 0.2 mm x 0.2 mm x 0.2 mm. The presence of β-octyl glucoside, EDTA, and CDTA was also found to have a positive influence on crystallization. But these crystals were unstable upon storage at room temperature and melting or cracking occurred in about two weeks after full growth. The form I-crystal diffracted to about 4 Å Bragg spacing with X-rays from synchrotron X-rays. The form I crystal was also grown by the capillary method. The crystal grew in 40 days to dimensions of 0.25 mm x 0.25 mm x 0.3 mm. This crystal was stable for about one year and it diffracted to about 4 Å Bragg spacing with X-rays from a rotating anode. Another form (II), thin plate crystal, has been obtained at room temperature from a reservoir solution of 100 mM Tris-HCl buffer (pH 8.5) and 0.4 M ammonium phosphate. Nucleation began after a week and crystals grew to dimensions of approximately 0.2 mm x 0.15 mm x 0.05 mm in two weeks. However, this plate crystal form is less suitable for X-ray analysis than form I. Further crystallization efforts are necessary to obtain larger, better-diffracting crystals.

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References

1) A. Meister & M. E. Anderson, Ann. Rev. Biochem., **57**, 711-760 (1983).
2) H. Yamaguchi, H. Kato, Y. Hata, T. Nishioka, A, Kimura, J. Oda, & Y Katsube, J. Mol. Biol. **229**, 1083-1100 (1993).
3) R. Sekura & A. Meister, J. Biol. Chem., **252**, 2599-2605 (1977).
4) H. Gumagai, R. Nakayama, & T. Tachikura, Agric. Biol. Chem., **46**, 1301-1309 (1982).
5) K. Watanabe, K. Murata, & A. Kimura, Agric. Biol. Chem., **50**, 1925-1930 (1986).
6) K. Murata & A. Kimura, Appl. Environ. Microbiol., **44**, 1444-1448 (1982).
7) P. Richman & A. Meister, J. Biol. Chem., **250**, 1422-1426 (1975).
8) Y. Iba, K. Murata, & A. Kimura, Agric. Biol. Chem., **52**, 2633-2634 (1988).
9) K. Watanabe, Y. Yamano, K. Murata, & A. Kimura, Nucl. Acids. Res., **14**, 4393-4400 (1986).
10) U. K. Laemmli, Nature, **227**, 680-685 (1970).
11) T. Rabilloud, G. Carpentier, & P. Tarroux, Electrophoresis, **9**, 288-291 (1988).
12) C. W. Carter, Jr. & C. W. Carter, J. Biol. Chem., **254**, 12219-12223 (1979).
13) J. Jancarik & S.-H. Kim, J. Appl. Crystallogr., **24**, 409-411 (1991).
14) R. M. Sweet, In *Synchrotron Radiation in Structural Biology* (Sweet, R. M. & Woodhead, A. D., eds), 63-66 (1989).