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*Salmonella typhimurium*의 Deoxy-Thymidine Diphosphate-D-Glucose-4,6-Dehydratase의 결정화와 X-선 회절에 관한 연구

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Crystallization and Preliminary X-ray Diffraction Studies of *Salmonella typhimurium* Deoxy-Thymidine Diphosphate-D-Glucose-4,6-Dehydratase

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요 약

Salmonella typhimurium LT2에서 deoxy-thymidine diphosphate-D-glucose-4,6-dehydratase의 유전자를 재조합한 *Escherichia coli* BL21 clone으로 부터 dTDP-D-glucose dehydratase를 분리 정제한 후, 이 효소의 단결정을 상온에서 sitting drop 기체확산법으로 성장시켰다. 결정은 효소에 기질이 포함되어 있는 것과 포함되어 있지 않은 것 모두가 얻어지며, 이때 침전제는 1.6-2.0 M Na,K 인산 완충용액(pH 8.0)을 사용하였다. 이 단결정은 최소 2.5 Å의 분해능으로 회절하였으며, 공간군은 hexagonal한 $P6_1$이고, 격자의 크기는 a=b=168.54 Å, c=81.08 Å이었다. Asymmetric unit에는 이량체 한 분자를 포함하고 있으며 단백질 질량당 결정의 부피는 V_M=2.4 Å^3/Da, 용매의 함유율은 부피를 기준으로 64 % 였다.

Abstract

Single crystals of deoxy-thymidine diphosphate-D-glucose-4,6-dehydratase(abbreviated as dTDP-D-glucose dehydratase) from *Escherichia coli* Strain BL21 clone which harbors the gene of dTDP-D-glucose dehydratase in *Salmonella typhimurium* LT2 have been grown with and without substrates by sitting drop vapor diffusion at room temperature. The precipitating agent was 1.6 to 2.0 M Na,K phosphate buffer(pH 8.0). The crystals diffract to at least 2.5 Å and belong to the hexagonal space group $P6_1$ with cell dimensions a=b=168.54 Å, c=81.08 Å. The asymmetric unit contains one dimer with a crystal volume per protein mass(V_M) of 2.4 Å^3/Da and solvent content ($V_{sol.}$) of 64 % by volume.

INTRODUCTION

Enzyme technology offers a great potential for the synthesis of chiral compounds as drugs and pharmaceutical intermediates.[1] Whole microorganisms as well as purified enzymes are used for the production of valuable products, even in industrial scale synthesis. Both types of biocatalysts show specific advantages and disadvantages as discussed elsewhere.[2]

The dTDP-D-glucose-4,6-dehydratase (EC 4.2.1.46) catalyzes a conversion of dTDP-D-glucose to dTDP-4-keto-6-deoxyglucose.[3] This enzyme-catalyzed product, dTDP-4-keto-6-deoxyglucose is an intermediate deoxygenated hexose sugar component that is usually essential for biological activity in many antibiotics, including macrolides such as desosamine, mycosamine, anthracyclines such as daunorubicin (daunomycin), doxorubicin (adriamycin), and the aclacinomycins[4] (Figure 1). 6-deoxyhexoses are found ubiquitously in animals, plants, and microorganisms. They are, for instance, constituents of glycoproteins and bacterial cell walls, and of many antibiotics and other natural products.

Their biosynthesis, and in fact that of all deoxyhexoses studied so far, occurs at the sugar nucleotide level and is initiated by oxidoreductases which catalyze the irreversible conversion of the hexose nucleotide into a 4-keto-6-deoxyhexose derivative. While different nucleotides, i.e., uridine, guanosine, thimidine, or cytosine derivatives, serve as substrates in different organisms, the enzymes from different sources, all seem to have a common mechanism. In all cases studied, the enzyme contains an

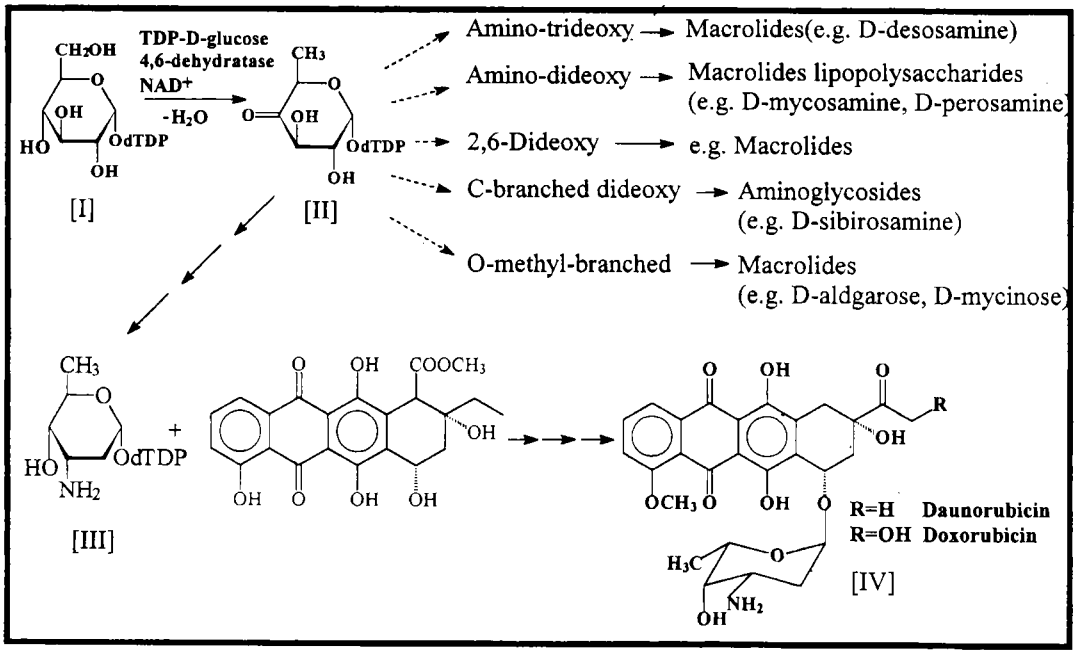

Fig. 1 : A plausible reaction pathway for the biosynthesis of daunorubicin from dTDP-D-glucose. [Ⅰ] dTDP-D-glucose, [Ⅱ] dTDP-6-Deoxy-4-keto-D- glucose, [Ⅲ] dTDP-daunosamine, [Ⅳ] R=H, Daunorubicin; R=OH, Doxorubicin

essential NAD^+ moiety and the reaction involves transfer of the hydrogen from C-4 of the substrate to C-6 of the resulting 4-keto-6-deoxyhexose derivative.[8-12]

This enzyme, dTDP-D-glucose-4,6-dehydratase, has been purified from a variety of sources, including *Escherichia coli*,[13,14] *Pseudotuberculosis*,[15] *Pasteurella*,[16] and porcine thyroid tissue.[17] The dTDP-D-glucose-4,6-dehydratase from *Salmonella typhimurium* LT2 overexpressed in *Escherichia coli* strain BL21 is a homodimer in solution. In order to elucidate the three-dimensional structure of dTDP-D-glucose-4,6-dehydratase and to shed light on the structure and function of this enzyme, we have crystallized this enzyme. We report here the preliminary crystallographic results on this enzyme and discuss about the crystallization procedures.

MATERIAL and METHODS

General : The *rfb* gene from *Salmonella typhimurium* LT2, which encodes for the dTDP-D-glucose-4,6-dehydratase, was cloned in pT7-6 and expressed in *Escherichia coli* strain BL21 (Piepersberg, BGHU-wuppertal, Germany). The pT7-6 contains β-lactamase gene and also ampicillin resistance gene. The inserted dTDP-D-glucose-4,6-dehydratase gene is under the strong control of transcription and translation of bacteriophage T7. The expression is released by the T7-RNA polymerase. The gene of dTDP-D-glucose-4,6-dehydratase is encoded in chromosome of *E.coli* BL21. The addition of IPTG induces the T7 RNA polymerase. This expression is blocked by the addition of rifampicin.

Bacterial strains : *Escherichia coli* strain BL21, containing dTDP-D-glucose-4,6-dehydratase gene was kindly provided by Dr. Elling from IET, Jülich, Germany.

Growth condition : *Escherichia coli* strain BL21, which harbors the gene of dTDP-D-glucose-4,6-dehydratase was grown in 2% peptone, 1% yeast extract, 0.5% NaCl, 0.2% glucose and 100 μg/ml ampicillin(pH 7.5) at 30 ℃ overnight. This overnight preculture was innoculated into the main culture and grown at 37 ℃ with shaking at 180 r.p.m. When the OD_{600} of the culture solution reached between 0.4 and 0.6, IPTG (0.4 mM) was added to the main culture to the gene of dTDP-D-glucose-4,6-dehydratase. After 2 hours of shaking, rifampicin (0.36 mM) was added to hamper the gene, and the incubation was continued for 3 more hours at 30 ℃. The cells were then harvested by centrifugation at 15,000 g for 20 min at 4 ℃ and washed twice with the buffer A (50 mM Tris/HCl, pH 7.5, 10 mM $MgCl_2$ and 1 mM EDTA) and stored at -20 ℃ until use.

Purification of dTDP-D-glucose-4,6-dehydratase : The enzyme was isolated from the dTDP-D-glucose-4,6-dehydratase over-producing *E.coli* strain BL21, according to the modified method of Thompson et al.[18] 65 gram of the harvested *E. coli* cells were usually disrupted with a French press at 15,000-16,000 p.s.i., 4 ℃. Unbroken and cell walls were pelleted by centrifugation at 25,000 g for 20 min. The supernatant was filtered by 0.2 μm filter and the filtrate was loaded on a Q-sepharose FPLC column (2.6 × 5 cm), which had been equilibrated with the buffer A containing 200 mM KCl. After washing the column with 150 ml of equilibration buffer, the enzyme was eluted with a linear gradient of 200 mM - 500 mM KCl in buffer A (pH 7.5). The active

fractions were pooled and concentrated by Amicon YM30. The concentrated pooled enzyme solution was further purified through a Superdex G-200 gel filtration column (1.6 × 95 cm). The elution buffer thereby was 50 mM Tris/HCl (pH 8.0) containing 150 mM NaCl.

The purity and activity of dTDP-D-glucose-4,6-dehydratase were determined by SDS-PAGE[19] and spectrophotometric assay, respectively, as described by Thompson et al.[15]

Crystallization : To crystallize this enzyme, purified protein was dialyzed thoroughly against 100 mM Tris/HCl buffer (pH 8.0) containing 400 mM NaCl, and concentrated to 20 mg/ml by ultrafiltration using a YM30 membrane (Amicon). A rapid initial screening of crystallization conditions was conducted using a sparse matrix of 50 conditions as initially described by Jancrik and Kim[20]. The most promising conditions were then optimized using sitting drop vapor diffusion to obtain large crystals. In this experiment 5 μl of the concentrated enzyme solution (20 mg/ml) was mixed on a plastic bridge with an equal volume of reservoir solution containing the precipitating agents and was set for equilibration against 1 ml of reservoir solution in a Linbro tissue culture plate sealed with fat grease.

X-ray diffraction studies : Crystals were 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 data were collected with a Mar-Research image plate scanner, using graphite-monochromated CuKα, radiation from a GX-21 rotating anode generator operating at 45 kV and 50 mA. The data set was evaluated with REFIX[21] and MOSFLM[22].

RESULTS and DISCUSSION

The enzyme, dTDP-D-glucose-4,6-dehydratase, which has been cloned in *Escherichia coli* strain BL21 was purified to homogeneity through ion exchange and gel filtration as determined by SDS-PAGE.

Single crystals were grown by sitting drop vapor diffusion with 1.6 M Na,K phosphate as a precipitating agent in 100 mM Tris/HCl (pH 8.0) containing 400 mM NaCl at room temperature. These tiny microcrystals appear within a week, but they were not suitable for X-ray analysis.

The cocrystallization with substrate (dTDP-D-glucose) resulted much better crystals. In order to investigate the effect of the enzymatic activity on the cocrystallization with substrate of this enzyme, a systematic study in the time range of one day to a month was carried out by keeping the purified sample at 4 ℃. In a series of crystallization batches with the time, large single crystals were obtained only in the batches where the relative activity of the enzyme was beyond 80 %. The cocrystals with substrate appear usually within a day. Fully grown crystals typically have dimensions of 0.2 × 0.2 × 0.8 mm (Figure 2).

The crystals have a hexagonal form, belonging to the space group $P6_1$ with cell parameters of a=b=168.54 Å, c=81.08 Å. The crystal diffract to 2.5 Å. A X-ray data set to 2.7 Å of native enzyme has been collected. On the basis of a relative molecular weight of 80,000 for the dimer of this enzyme, there is one dimer molecule per asymmetric unit, with V_M=2.4 Å^3/Da and V_{sol}=64 % (v/v) of mother liquor. These values are within the

Fig. 2 : Crystals of dTDP-D-glucose dehydratase grown with 1.6 M Na,K-phosphate buffer (pH 8.0). The approximate dimensions of the largest crystal complexed with substrate (dTDP-D-glucose) are 0.2 × 0.2 × 0.8 mm.

commonly observed range.[22] The collection of the heavy atom derivative data is under way.

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REFERENCES

1. David, S., Auge, C., and Gautheron, C., *Adv. Carbohydr. Chem. Biochem.* 49 (1991), 175-237.
2. Kragl, U., Vasic-Racki, D. and Wandrey, C., *Chem. Ing. Technol.* 69 (6), (1992), 499-509.
3. Matern, H., Brillinger, G. U. and Pape, H., *Archiv für Mikrobiologie* 88 (1973), 37-48.
4. Grisebach, H., *Adv. Carbohydr. Chem. Biochem.* 35 (1978), 81-126.
5. Snipes, C. E., Chang, C.-J. and Floss, H. G., *J. Am. Chem. Soc.* 101 (1979), 701-706.
6. Vara, J. A. and Hutchinson, C. R., *J. Biol. Chem.* 263 (1988), 14992-14995.
7. Fujiwara, A. and Hoshino, T., *Critical reviews in Biotechnology* 3 (1983), 133-157.
8. Garbriel, O., *Adv. Chem. Ser.* 117 (1973), 387-410.
9. Glaser, L. and Zarkowsky, H., *Enzymes* 5 (1971), 465-480.
10. Melo, A., Elliott, W. H. and Graser, L., *J. Biol. Chem.* 243 (7), (1968), 1467-1474.
11. Garbriel, O. and Lindquist, L. C., *J. Biol. Chem.* 243 (1968), 1479-1484.
12. Bevill, R. D., *Biochem. Biophys. Res. Commun.* 30 (6), (1968), 595-599.
13. Wang, S. -F. and Garbriel, O., *J. Biol. Chem.* 244 (1969), 3430-3437.
14. Zarkowsky, H. and Glaser, L., *J. Biol. Chem.* 244 (17), (1969), 4750-4756.
15. Gonzalez-Porque, P. and Strominger, J. L., *J. Biol. Chem.* 247 (21), (1972), 6748-6756.
16. Liao, T.-H and Barber, G. A., *Biochim. Biophys. Acta* 276 (1972), 85-93.
17. Broschet, K. O., Chang, S. and Serif, G., *Eur. J. Biochem.* 153 (1985), 397-401.
18. Thompson, M. W., Strohl, W. R. and Floss, H. G., *J. General Microbiol.* 138(1992), 779-786.
19. Laemmli, U. K., Nature 227 (1970), 680-685.
20. Jancarik, J. and Kim S.-H., *J. Acta. Crystallogr.* 24 (1991), 409-411.
21. Kasch, W., *J. Appl. Cryst.* 24 (1991), 409-411.
22. Leslie, A. G. W., *Newsletters on protein*

crystallography 26 (1992).
23. Mathews, B. W., *J. Mol. Biol.*, 33 (1968), 491-497.