Acta Crystallographica Section D
Biological Crystallography
ISSN 0907-4449

Crystallization and preliminary crystallographic analysis of the terminal oxygenase component of carbazole 1,9a-dioxygenase of *Pseudomonas resinovorans* strain CA10

Jeong-Won Nam,[a] Zui Fujimoto,[b] Hiroshi Mizuno,[b] Hisakazu Yamane,[a] Takako Yoshida,[a] Hiroshi Habe,[a] Hideaki Nojiri[a] and Toshio Omori[a]*

[a]Biotechnology Research Center, The University of Tokyo, Yayoi 1-1-1, Bunkyo-ku, Tokyo 113-8657, Japan, and [b]Department of Biochemistry, National Institute of Agrobiological Sciences, 2-1-2 Kannondai, Tsukuba, Ibaraki 305-8602, Japan

Correspondence e-mail: aseigyo@mail.ecc.u-tokyo.ac.jp

Received 7 February 2002
Accepted 17 May 2002

The terminal oxygenase component (CarAa) of carbazole 1,9a-dioxygenase from *Pseudomonas resinovorans* strain CA10 was crystallized at 293 K using the sitting-drop vapour-diffusion method under the following conditions: 0.1 *M* sodium citrate pH 5.6 in the presence of 0.5 *M* ammonium sulfate and 1.0 *M* lithium sulfate. By using additive reagents with the crystallizing condition, improved diffraction was obtained from the crystals. Preliminary X-ray diffraction analysis indicated that CarAa crystals are hexagonal and belong to space group $P6_2$ or $P6_4$, with unit-cell parameters $a = b = 244.5$, $c = 65.7$ Å, $\alpha = \beta = 90.0$, $\gamma = 120.0^\circ$. Diffraction data were collected to 3.0 Å resolution. The V_M value is 2.16 Å^3 Da^{-1}, which indicates a solvent content of 43.0%. This is the first report of crystallization of the terminal oxygenase component of an angular-type dioxygenase.

1. Introduction

In recent years, various kinds of xenobiotics have been produced and released into the environment as a consequence of the advance of industry. Most of these xenobiotics are chemically stable and thus recalcitrant and are of considerable concern owing to their accumulation in the food chain. This is particularly the case for dioxins, the chlorinated congeners of dibenzofuran and dibenzo-*p*-dioxin, and some molecules such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin are extremely toxic and have become a public concern.

P. resinovorans strain CA10 was isolated from an activated sludge and has the ability to utilize carbazole as a sole source of carbon, nitrogen and energy (Ouchiyama *et al.*, 1993). We cloned the genes encoding the carbazole 1,9a-dioxygenase (CARDO) system from strain CA10 and analyzed the characteristics of CARDO as a multicomponent enzyme system which consists of a terminal oxygenase (CarAa), ferredoxin (CarAc) and ferredoxin reductase (CarAd) (Sato *et al.*, 1997). From the amino-acid sequence homology and phylogenetic analysis of CarAa, CarAa is known to be a unique type of oxygenase that shares low homology with other dioxygenases (Sato *et al.*, 1997; Nam *et al.*, 2001). Furthermore, from the studies on the substrate specificity, CARDO can catalyze diverse oxygenations with a broad substrate range, including angular dioxygenation, lateral dioxygenation and monooxygenation (Nojiri *et al.*, 1999), and can transform some chlorinated dioxins (Habe *et al.*, 2001).

Considering these unique properties of the CARDO system, the substrate oxygenation mechanism is of great interest. We purified and characterized the proteins involved in the CARDO system (Nam *et al.*, 2002) and the functions of each component were revealed as shown in Fig. 1. Though CarAa is known to be the catalytic component of CARDO, a detailed understanding of the mechanism by which CarAa performs the substrate oxygenation is limited by the lack of three-dimensional information for this protein. Here, we report the crystallization and preliminary X-ray diffraction studies of CarAa.

2. Experimental results and discussion

A thorough description of the bacterial strains, plasmids, genetic manipulations and procedures for protein purification is provided elsewhere (Nam *et al.*, 2002). In brief, the *carAa* gene from *P. resinovorans* strain CA10 (Sato *et al.*, 1997) was expressed from a plasmid in *Escherichia coli* and the protein was purified by anion-exchange and gel-filtration chromatography. The protein was concentrated and buffer-exchanged using an Amicon Centriprep-10 membrane. Protein concentrations were estimated using Protein assay kit (Bio-Rad; Bradford, 1976) using BSA as the standard. For crystallization experiments, a solution of the protein in 5 m*M* Tris–HCl pH 7.5 with a protein concentration of 10–20 mg ml^{-1} was used.

Crystallization was performed using the hanging-drop or sitting-drop vapour-diffusion method at 278 or 293 K. Drops containing 3 µl of protein and 3 µl of mother liquor were equilibrated against 800 µl of reservoir solution. Sparse-matrix screens (Jancarik & Kim, 1991; Cudney *et al.*, 1994) for initial trials were

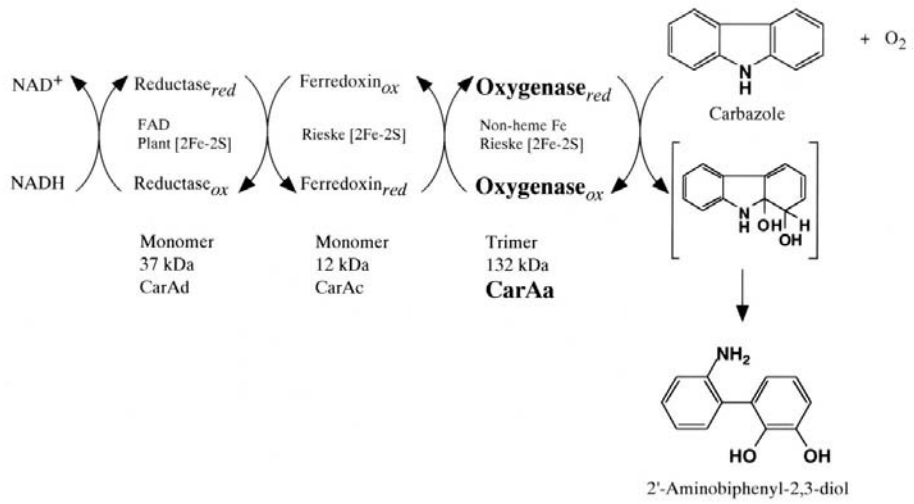
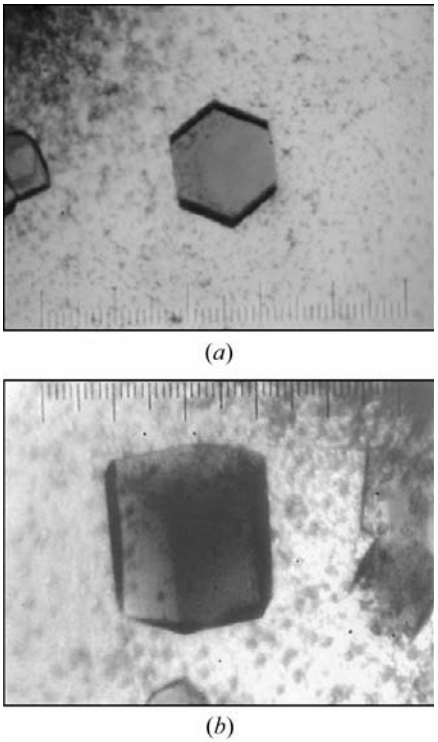


Figure 1
Components and functions of the carbazole 1,9a-dioxygenase system. The proposed electron-transfer reactions and the conversion of carbazole to 2′-aminobiphenyl-2,3-diol are illustrated.



(*a*)

(*b*)

Figure 2
Photographs of hexagonal crystals of CarAa of *P. resinovorans* strain CA10. (*a*) Crystals grown in 0.5 *M* ammonium sulfate, 1.0 *M* lithium sulfate and 0.1 *M* sodium citrate pH 5.6. The dimensions of the crystal are approximately 0.25 × 0.25 × 0.03 mm. (*b*) Crystals grown in the presence of 0.02 *M* strontium chloride hexahydrate in the reagents for (*a*). The dimensions of the crystal are approximately 0.33 × 0.33 × 0.35 mm.

performed using Hampton Research Crystal Screen kits I and II (Hampton Research, California, USA). Initial crystallization trials showed that the protein crystallizes under a variety of different crystallization conditions, forming red–brown hexagonal plate-shaped crystals (Fig. 2*a*) or clusters of very thin crystals. Crystallization parameters were optimized for the hexagonal plate-shaped crystals using Hampton Research Additive Screen kit I (Hampton Research). Some additives improved the growth of crystals as thick plates. The best crystals were obtained with 0.5 *M* ammonium sulfate and 1.0 *M* lithium sulfate in 0.1 *M* sodium citrate pH 5.6 in the presence of 0.02 *M* strontium chloride hexahydrate or urea as additives in sitting-drop vapour-diffusion experiments at 293 K using Cryschem Plates (Hampton Research). With 800 µl reservoir solution and drops consisting of 5 µl protein solution, 1 µl additive solution and 4 µl reservoir solution, crystals appeared after 2 d and grew to maximum dimensions of 0.33 × 0.33 × 0.35 mm in a week (Fig. 2*b*).

Owing to the sensitivity of the crystals to X-ray radiation, cryocooling was a necessary procedure during data collection in order to eliminate crystal decay at room temperature. The crystals were transferred directly into a cryoprotectant solution containing 20% glycerol and 52% mother-liquor solution, mounted in a nylon loop and flash-frozen in a nitrogen stream at 100 K.

Diffraction measurements of the frozen crystals were carried out using a Quantum CCD X-ray detector (ADSC, USA) at the Photon Factory BL6A at the High Energy Accelerator Research Organization, Tsukuba, Japan. A total range of 91° was covered with a 1.0° oscillation and a 5 min exposure per frame. Crystals diffracted to 2.8 Å resolution, but a native data set was collected to 3.0 Å resolution because of anisotropy (Table 1). The determination of unit-cell parameters, integration of reflection intensities and data scaling were performed using the programs *MOSFLM* and *SCALA* from the *CCP*4 program suite (Collaborative Computational Project, Number 4, 1994).

Table 1
Crystal parameters and data-collection statistics.

The data set was collected on BL6A at the PF, Tsukuba, Japan. Values in parentheses are for the highest resolution shell.

Space group	$P6_2$ or $P6_4$
Unit-cell parameters (Å, °)	$a = b = 244.5$, $c = 65.7$, $\alpha = \beta = 90.0$, $\gamma = 120.0$
Resolution range (Å)	48.80–3.00 (3.16–3.00)
Total No. of reflections	237939 (34842)
No. of unique reflections	40457 (5960)
Completeness (%)	88.5 (90.3)
Average $I/\sigma(I)$	5.9 (1.9)
R_{sym} (%)	10.4 (38.8)
Multiplicity	5.9 (5.8)

The space group was determined to be either $P6_2$ or $P6_4$, with unit-cell parameters $a = b = 244.5$, $c = 65.7$ Å, $\alpha = \beta = 90.0$, $\gamma = 120.0°$.

Assuming six monomers per asymmetric unit, the Matthews coefficient V_M (Matthews, 1968) is 2.16 Å^3 Da^{-1} (corresponding to a solvent content of 43.0%); assuming four monomers per asymmetric unit, V_M is 3.24 Å^3 Da^{-1} and corresponds to 62.0% solvent. Both values are in the range generally found for proteins. Previously, the molecular weight of the protein was calculated to be 132 kDa from gel-filtration chromatography and the monomer is approximately 44 kDa from SDS–PAGE (Nam *et al.*, 2002). Consequently, the protein is more likely to be a trimer than a tetramer. In the case of the trimer, V_M is 2.16 Å^3 Da^{-1} and the asymmetric unit contains two trimeric terminal oxygenase molecules.

Attempting to solve the structure by the molecular-replacement method using the known structure of the terminal oxygenase component of naphthalene 1,2-dioxygenase (Kauppi *et al.*, 1998) has given no useful results so far owing to the low sequence homology and the structural differences expected from sequence alignment. The amino-acid sequence homology score was 9% from *CLUSTALW* (Thompson *et al.*, 1994) over the entire amino-acid range (CarAa, 384 amino acids; NahAc, 449 amino acids). Instead, it is intended to solve the structure using the multiple-wavelength anomalous dispersion method, taking advantage of the [2Fe–2S] cluster and the Fe^{2+} in the catalytic site of the protein. There are approximately three irons per 44 kDa

monomer (Nam *et al.*, 2002). The preparation of a SeMet derivative is also in progress.

The anticipated CarAa structure will provide the first three-dimensional structure of an angular dioxygenase and will provide a fundamental framework for understanding the molecular mechanism of angular dioxygenation. Furthermore, the solution of the structure will explain the broad substrate specificity of CARDO and will provide clues to the structural basis of the electron-transport mechanism between the components

Synchrotron radiation was used for this work with the approval of the Photon Factory Advisory Committee and High Energy Accelerator Research Organization, Tsukuba (proposal number 01G158). This work was supported by the Program for Promotion of Basic Research Activities for Innovative Bioscience (PROBRAIN) in Japan.

References

Bradford, M. M. (1976). *Anal. Biochem.* **72**, 248–254.

Collaborative Computational Project, Number 4 (1994). *Acta Cryst.* D**50**, 760–763.

Cudney, R., Patel, S., Weisgraber, K., Newhouse, Y. & McPherson, A. (1994). *Acta Cryst.* D**50**, 414–423.

Habe, H., Chung, J.-S., Lee, J.-H., Kasuga, K., Yoshida, T., Nojiri, H. & Omori, T. (2001). *Appl. Environ. Microbiol.* **67**, 3610–3617.

Jancarik, J. & Kim, S.-H. (1991). *J. Appl. Cryst.* **24**, 409–411.

Kauppi, B., Lee, K., Carredano, E., Parales, R. E., Gibson, D. T., Eklund, H. & Ramaswamy, S. (1998). *Structure*, **6**, 571–586.

Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.

Nam, J.-W., Nojiri, H., Yoshida, T., Habe, H., Yamane, H. & Omori, T. (2001). *Biosci. Biotechnol. Biochem.* **65**, 254–63.

Nam, J.-W., Nojiri, H., Yoshida, T., Habe, H., Yamane, H. & Omori, T. (2002). Submitted.

Nojiri, H., Nam, J.-W., Kosaka, M., Morii, K., Takemura, T., Furihata, K., Yamane, H. & Omori, T. (1999). *J. Bacteriol.* **181**, 3105–3113.

Ouchiyama, N., Zhang, Y., Omori, T. & Kodama, T. (1993). *Biosci. Biotechnol. Biochem.* **57**, 455–460.

Sato, S., Nam, J.-W., Kasuga, K., Nojiri, H., Yamane, H. & Omori, T. (1997). *J. Bacteriol.* **179**, 4850–4858.

Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994). *Nucleic Acids Res.* **22**, 4673–4680.