Acta Crystallographica Section F
Structural Biology and Crystallization Communications
ISSN 1744-3091

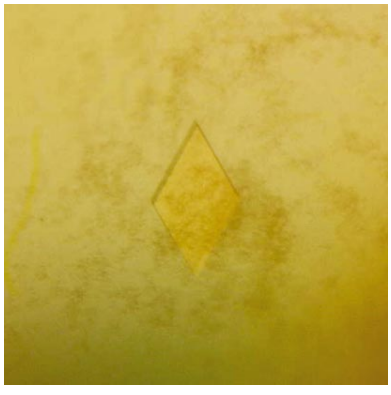
Crystallization and preliminary crystallographic analysis of bacterial fructosyl amino acid oxidase

Ryoichi Sakaue,[a] Toru Nakatsu,[b,c] Yoko Yamaguchi,[b] Hiroaki Kato[b,c] and Naoki Kajiyama[a]*

[a]Research and Development Division, Kikkoman Corporation, 399 Noda, Noda City, Chiba 278-0037, Japan, [b]Kinetic Crystallography Research Team, Membrane Dynamics Research Group, RIKEN Harima Institute at SPring-8, 1-1-1 Kouto, Mikazuki-cho, Sayo-gun, Hyogo 679-5148, Japan, and [c]Graduate School of Pharmaceutical Sciences, Kyoto University, 46-29 Yoshida, Shimoadachi-cho, Sakyo-ku, Kyoto City, Kyoto 606-8501, Japan

Correspondence e-mail: rsakaue@mail.kikkoman.co.jp

Received 10 November 2004
Accepted 28 December 2004
Online 20 January 2005



Bacterial fructosyl amino acid oxidase [fructosyl α-L-amino acid:oxygen oxidoreductase (defructosylating); EC 1.5.3] has been crystallized by the hanging-drop vapour-diffusion technique using sodium citrate as the precipitant. Two types of crystals were grown: one type are rhombic prismatic yellow crystals that belong to space group $C2$ with unit-cell parameters a = 101.08, b = 63.36, c = 83.07 Å, β = 108.80° and diffract to at least 1.8 Å resolution, while the second type are rod-like crystals that belong to space group $P4_122$ or $P4_322$ with unit-cell parameters $a = b$ = 119.09, c = 164.66 Å and diffract to 2.7 Å resolution.

1. Introduction

Fructosyl amino acid oxidase [fructosyl-α-L-amino acid:oxygen oxidoreductase (defructosylating); EC 1.5.3] is a flavoprotein that catalyzes the oxidation of fructosyl amino acids to generate glucosone, amino acid and hydrogen peroxide,

$$\text{fructosyl amino acid} + O_2 \rightarrow \text{amino acid} + \text{glucosone} + H_2O_2.$$

Fructosyl amino acid oxidase is a candidate for the enzymatic detection of nonenzymatically glycated proteins. This nonenzymatic reaction is called glycation in order to distinguish it from the enzymatic glycosylation of proteins. The glycation of protein has been implicated in the development of diabetic complications and in the aging process (Bunn *et al.*, 1976; Chiou *et al.*, 1981; Monnier & Cerami, 1982). The glycation of blood proteins such as haemoglobin and albumin is enhanced in diabetics with high blood glucose. The amount of these glycated proteins reflects the level of blood glucose in periods corresponding to the half-life of the protein (14–20 d for albumin and 1–2 months for haemoglobin). Since the glycation of blood proteins is not affected by transient increases in blood glucose, the levels of glycated proteins are good indices for monitoring diabetes mellitus patients during therapy.

Horiuchi *et al.* (1989) discovered fructosyl amino acid oxidase from *Corynebacterium* sp. 2-4-1 (FAOX-C) and we have succeeded in cloning the FAOX-C gene and expressing it in *Escherichia coli* (Sakaue *et al.*, 2002). FAOX-C showed high activity towards D-fructosyl-L-valine (FV), but no activity toward N^{ε}-fructosyl lysine (εFLys) and N^{ε}-fructosyl N^{α}-formyl lysine (εFfLys). Hirokawa & Kajiyama (2002) reported that an AgaE-like protein in *Agrobacterium* sp. has FAOX-C-like activity. In addition, we succeeded in the thermostablization of FAOX-C (FAOX-TE) without loss of specificity and activity by directed evolution (Sakaue & Kajiyama, 2003). On the other hand, many investigators have discovered fungal FAOXs (Horiuchi & Kurokawa, 1991; Sakai, Yoshida, Isogai *et al.*, 1996; Sakai, Yoshida, Tani *et al.*, 1996; Takahashi *et al.*, 1997; Wu *et al.*, 2000; Yoshida *et al.*, 1995, 1996), but these enzymes from fungi showed activity toward εFLys and εFfLys. We consider there to be two types of FAOXs in terms of substrate specificities. These two types have a low homology at the amino-acid sequence level. Fungal FAOXs have a high homology with sarcosine oxidases. Recently, Hirokawa and coworkers (Hirokawa, Gomi, Bakke *et al.*, 2003; Hirokawa, Gomi & Kajiyama, 2003) screened and cloned fungal fructosyl peptide oxidases, the sequences of which were similar to those of fungal FAOXs. We expect to obtain much information on the mechanism of

catalysis and substrate specificity from crystallographic studies of FAOX. In this paper, we report the crystallization and preliminary X-ray analysis of bacterial FAOX.

2. Materials and methods

2.1. Expression and purification

Recombinant FAOX-TE was overexpressed in *Escherichia coli* and purified as described previously (Sakaue & Kajiyama, 2003). The purified protein was centrifuged to remove insoluble extracts and sterilized by Ultrafree-MC (Amicon). The protein was concentrated to 5–50 mg ml^{-1} in 20 m*M* potassium phosphate buffer pH 8.0 and stored at 253 K. In optimized crystallizations, we dialyzed FAOX-TE to 0.1 *M* MOPS pH 7.0 (for rhombic shaped crystals; Fig. 1*a*) or 0.1 *M* Tris–HCl pH 8.0 (for rod-shaped crystals; Fig. 1*b*).

2.2. Crystallization

All crystallization trials were performed using the hanging-drop vapour-diffusion method at 277 and 298 K. Initial experiments to determine the crystallization conditions were undertaken using Crystal Screen 1 (Hampton Research) by mixing 5 µl protein solution (5–50 mg ml^{-1} protein) with 5 µl reservoir solution and equilibrating the drops over 1 ml of their respective reservoirs. Initial trials showed that sodium citrate was an effective precipitant for the crystallization of FAOX-TE. Conditions were further optimized by varying the concentrations of protein and additives, as well as the pH of the buffer.

2.3. Data collection and processing

For the X-ray diffraction experiment, the FAOX-TE crystals were flash-cooled in a cryosolution composed of crystallization components with a higher concentration of precipitating reagent: 1.6 *M* sodium citrate and 0.1 *M* MOPS pH 7.0 for rhombic shaped crystals and 1.6 *M* sodium citrate and 0.1 *M* Tris–HCl pH 8.0 for rod-shaped crystals. X-ray diffraction data were collected at 100 K with synchrotron radiation of wavelength 1.0 Å at BL44B2 of SPring-8 (Hyogo, Japan) on an MAR CCD 165 detector. The oscillation angle was 1.0° and the exposure time was 10 s. The crystal-to-detector distances for the rhombic shaped and rod-shaped crystals were 90 and 180 mm, respectively. The data were integrated and scaled with *CrystalClear* (Pflugrath, 1999).

3. Results and discussion

Two types of crystals of FAOX-TE, rhombic shaped and rod-shaped crystals, were obtained in initial trials with sodium citrate as the precipitant in HEPES buffer pH 7.0–8.0 at 298 K. We optimized the crystallization conditions by varying the concentration of sodium citrate, the buffer and the pH. Rhombic shaped crystals were grown using 1.5 *M* sodium citrate and 0.1 *M* MOPS pH 7.0 and rod-shaped crystals were grown using 1.5 *M* sodium citrate and 0.1 *M* Tris–HCl pH 8.0. The rhombic shaped crystals diffracted to better than 2.0 Å resolution, but the crystals were stacked. Microseeding was effective in reducing the crystal stacking. X-ray diffraction data were collected at 1.8 Å with an $R_{\rm merge}$ of 4.4% and a completeness of 98.4%. The crystals belong to space group *C*2, with unit-cell parameters $a = 101.08$, $b = 63.36$, $c = 83.07$ Å, $\beta = 108.80°$. Assuming one FAOX-TE molecule to be present in the asymmetric unit, the Matthews coefficient $V_{\rm M}$ is 3.2 Å^3 Da^{-1}, corresponding to a solvent content of 62% (Matthews, 1968). The rod-shaped crystals diffracted to 2.7 Å resolution and belong to space group $P4_122$ or $P4_322$, with unit-cell parameters $a = b = 119.09$, $c = 164.66$ Å. Assuming two molecules to be present in the asymmetric unit, the Matthews coefficient $V_{\rm M}$ is 3.6 Å^3 Da^{-1}, corresponding to a solvent content of 66%. The diffraction data had an $R_{\rm merge}$ of 12.4% and a completeness of 100.0%. The data-collection statistics of each crystal are summarized in Table 1. Both types of crystal were redissolved in the buffer and subjected to SDS–PAGE. The results showed that these crystals did not consist of degradation fragments of FAOX-TE (data not shown).

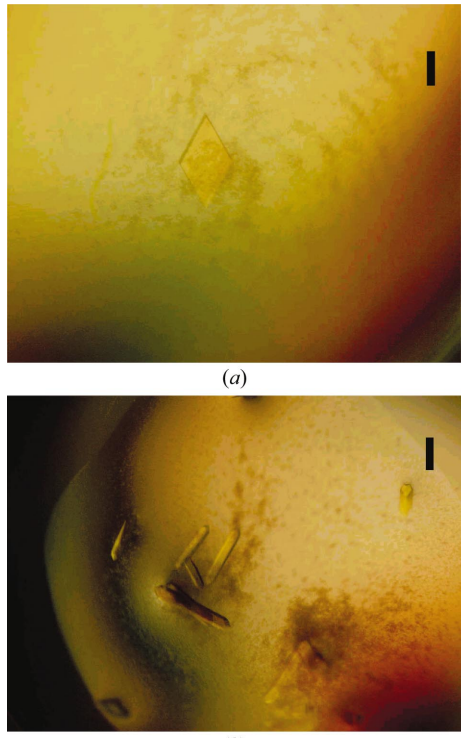
Table 1
Data-collection statistics.

Values in parentheses are for the highest resolution shell.

	Rhombic shaped	Rod-shaped
Space group	*C*2	$P4_122$ or $P4_322$
Unit-cell parameters (Å, °)	$a = 101.08$, $b = 63.36$, $c = 83.07$, $\beta = 108.80$	$a = b = 119.09$, $c = 164.66$
Resolution range (Å)	48–1.8 (1.86–1.8)	45–2.7 (2.8–2.7)
No. observed reflections	165367	482519
No. unique reflections	45347	33249
Completeness (%)	98.4 (95.3)	100 (99.9)
Multiplicity	3.65 (3.58)	14.51 (14.74)
$R_{\rm merge}$† (%)	4.4 (21.2)	12.4 (39.8)
$I/\sigma(I)$	16.7 (5.0)	15.9 (6.7)

† $R_{\rm merge} = \sum_h \sum_i |I(h)_i - \langle I(h)\rangle| / \sum_h \sum_i I(h)_i$, where $I(h)_i$ is the intensity of reflection h, $\sum_h$ is the sum over all measured reflections and $\sum_i$ is the sum over I measurements of a reflection.

Several similar structures have been deposited in the PDB, of which sarcosine oxidase (SAOX; PDB code 1el5) showed 27% sequence identity. However, we could not solve the structure of FAOX-TE by the molecular-replacement (MR) method using SAOX as a search model. Therefore, the structure will be solved by multi-



(*a*)

(*b*)

Figure 1
Two types of yellow crystals of FAOX-TE in a hanging drop. (*a*) shows rhombic prismatic crystals and (*b*) shows rod-like yellow crystals. The bar corresponds to 0.5 mm.

wavelength anomalous dispersion (MAD) methods with a Hg derivative using synchrotron X-ray radiation at SPring-8.

We thank N. Yamaji and Y. Imai for continuous support, encouragement and valuable discussions.

References

Bunn, H. F., Haney, D. N., Kamin, D. S., Gabbay, K. H. & Gallop, P. M. (1976). *J. Clin. Invest.* **57**, 1652–1659.
Chiou, S. H., Chylack, L. T., Tung, W. H. Jr & Bunn, H. F. (1981). *J. Biol. Chem.* **256**, 5176–5180.
Hirokawa, K., Gomi, K., Bakke, M. & Kajiyama, N. (2003). *Arch. Microbiol.* **180**, 227–231.
Hirokawa, K., Gomi, K. & Kajiyama, N. (2003). *Biochem. Biophys. Res. Commun.* **311**, 104–111.
Hirokawa, K. & Kajiyama, N. (2002). *Biosci. Biotechnol. Biochem.* **66**, 2323–2329.
Horiuchi, T. & Kurokawa, T. (1991). *Agric. Biol. Chem.* **55**, 333–338.
Horiuchi, T., Kurokawa, T. & Saito, N. (1989). *Agric. Biol. Chem.* **53**, 103–110.
Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
Monnier, H. F. & Cerami, A. (1982). *Clin. Endocrinol. Metab.* **11**, 431–452.
Pflugrath, J. W. (1999). *Acta Cryst.* D**55**, 1718–1725.
Sakai, Y., Yoshida, N., Isogai, A., Tani, Y. & Kato, N. (1996). *Biosci. Biotechnol. Biochem.* **59**, 487–491.
Sakai, Y., Yoshida, N., Tani, Y. & Kato, N. (1996). *Biosci. Biotechnol. Biochem.* **60**, 150–151.
Sakaue, R., Hiruma, M., Kajiyama, N. & Koyama, Y. (2002). *Biosci. Biotechnol. Biochem.* **66**, 1256–1261.
Sakaue, R. & Kajiyama, N. (2003). *Appl. Environ. Microbiol.* **69**, 139–145.
Takahashi, M., Pischetsrieder, M. & Monnier, V. M. (1997). *J. Biol. Chem.* **272**, 3437–3443.
Wu, X., Takahashi, M., Chen, S. G. & Monnier, V. M. (2000). *Biochemistry*, **39**, 1515–1521.
Yoshida, N., Sakai, Y., Isogai, A., Fukuya, H., Yagi, M., Tani, Y. & Kato, N. (1996). *Eur. J. Biochem.* **242**, 499–505.
Yoshida, N., Sakai, Y., Serata, M., Tani, Y. & Kato, N. (1995). *Appl. Environ. Microbiol.* **61**, 4487–4489.