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Crystallization and preliminary X-ray analysis of formate oxidase, an enzyme of the glucose–methanol–choline oxidoreductase family

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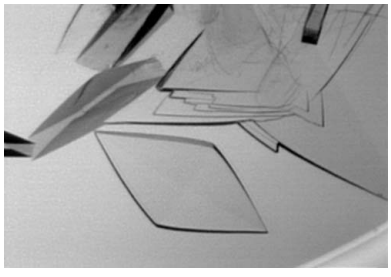

Formate oxidase (FOD), which catalyzes the oxidation of formate to yield carbon dioxide and hydrogen peroxide, belongs to the glucose–methanol–choline oxidoreductase (GMCO) family. FOD from *Aspergillus oryzae* RIB40, which has a modified FAD as a cofactor, was crystallized at 293 K by the hanging-drop vapour-diffusion method. The crystal was orthorhombic and belonged to space group $C222_1$. Diffraction data were collected from a single crystal to 2.4 Å resolution.

1. Introduction

The enzymes of the glucose–methanol–choline oxidoreductase (GMCO) family exhibit little sequence similarity in their catalytic sites. However, either a His–Asn or a His–His pair is conserved in the catalytic site of all enzymes belonging to the GMCO family. The crystal structures of choline oxidase from *Arthrobacter globiformis* (Quaye *et al.*, 2008), glucose oxidase from *Aspergillus niger* (Hecht *et al.*, 1993), cholesterol oxidase from *Brevibacterium sterolicum* (Vrielink *et al.*, 1991; Lario *et al.*, 2003), the flavin domain of cellobiose dehydrogenase from *Phanerochaete chrysosporium* (Hallberg *et al.*, 2002) and pyranose 2-oxidase from *Peniophora* sp. (Bannwarth *et al.*, 2004) show that they all share a highly conserved catalytic site.

Enzymes that are capable of oxidizing formate to carbon dioxide are classified as formate dehydrogenases and formate oxidases (FODs). Formate dehydrogenases from various microorganisms and higher plants have been well characterized (Boyington *et al.*, 1997; Moura *et al.*, 2004; Tishkov & Popov, 2006; Schirwitz *et al.*, 2007; Shabalin *et al.*, 2009). In contrast, there are only a few reports on FODs. FOD from *Aspergillus nomius* IRI013 was the first FOD to be purified and characterized (Kondo *et al.*, 2002). Recently, we purified and characterized FOD from *Debaryomyces vanrijiae* MH201 and subsequently cloned and expressed three FOD genes from *D. vanrijiae* MH201 in *Escherichia coli* (Uchida *et al.*, 2007; Maeda *et al.*, 2008). An FOD gene from *A. oryzae* RIB40 was also expressed in *E. coli* (Maeda *et al.*, 2009). The native and recombinant enzymes are all dimeric proteins with subunit molecular masses of about 64 kDa. In the amino-acid sequences deduced from their nucleotide sequences, three FAD-binding site motifs and a His residue are conserved throughout the GMCO family (Dym & Eisenberg, 2001; Fan *et al.*, 2004; Ghanem & Gadda, 2005).

The amino-acid sequence deduced from the nucleotide sequence of the FOD gene from *A. oryzae* RIB40 shows 28.9 and 25.7% similarity to those of choline oxidase from *Arthrobacter globiformis* and glucose oxidase from *Aspergillus niger*, respectively (Fig. 1). A His511–Arg554 pair might exist in the catalytic site of the recombinant *A. oryzae* FOD (FOD_{AO}), instead of the His–Asn and His–His pairs that exist in the catalytic sites of the choline and glucose oxidases, respectively. Also, His508 and Arg551, which correspond to His511 and Arg554, respectively, are found in FOD from *D. vanrijiae* MH201. The choline and glucose oxidases contain FAD that is covalently bound to His97 *via* the 8α-position of the isoalloxazine

ring and noncovalently bound, respectively (Hecht *et al.*, 1993; Quaye *et al.*, 2008). Spectroscopic analyses of FOD_{AO} and an extract that was obtained by boiling the enzyme indicated that FOD_{AO} has a modified noncovalently bound FAD with a molecular mass of 799, which was expected to be 8-formyl-FAD (Maeda *et al.*, 2009). The spectroscopic properties of the extract derived from wild-type FOD purified from *D. vanrijiae* MH201 were identical to those of FOD_{AO}. The modification of FAD as well as the presence of the His–Arg pair in the catalytic site might be characteristic of FOD. In this report, we describe the crystallization and preliminary X-ray diffraction studies of FOD_{AO} in order to obtain insight into the structures of its catalytic site and modified FAD.

2. Experimental procedure

2.1. Protein expression and purification

E. coli BL21 (DE3) cells harbouring plasmid pET-*FOD*$_{AO}$ were grown and induced with 0.1 m*M* isopropyl β-D-1-thiogalactopyranoside as described previously (Maeda *et al.*, 2009). The cells were harvested by centrifugation, suspended in 10 m*M* acetate buffer pH 6.0 and disrupted using an ultrasonic generator. Cell debris was removed by centrifugation. The supernatant was applied onto a column of Ni Sepharose 6 Fast Flow (bed volume 10 ml; GE Healthcare, UK) equilibrated with 10 m*M* acetate buffer pH 6.0. The column was washed with the equilibration buffer and FOD_{AO} was eluted with 500 m*M* imidazole buffer pH 6.2. Fractions showing FOD activity were combined and dialyzed against 10 m*M* phosphate buffer pH 7.0. The dialyzate was applied onto a HiTrap QHP column (5 ml; GE Healthcare, UK) that had been equilibrated with 10 m*M* phosphate buffer pH 7.0. After washing the column with the equilibration buffer, FOD_{AO} was eluted with a linear gradient of 0–0.5 *M* NaCl in 10 m*M* phosphate buffer pH 7.0. The active fractions were pooled and applied onto a HisTrap FF column (1 ml; GE Healthcare, UK) equilibrated with 10 m*M* phosphate buffer pH 7.0. The column was washed with the equilibration buffer and FOD_{AO} was eluted with 500 m*M* imidazole buffer pH 6.2. The active fractions were collected and dialyzed against 10 m*M* acetate buffer pH 6.0. The dialyzate was concentrated by ultrafiltration using Ultrafree-MC (Millipore Co., Tokyo, Japan) and used for crystallization.

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FOD             ----------------------------------MATDGSHF DFVIVGGGTAGNTVAGRL   26
Choline oxidase ----------------------------MHIDNIENLSDREF DYIVVGGGSAGAAVAARL   32
Glucose oxidase MQTLLVSSLVVSLAAALPHYIRSNGIEASLLTDPKDVSGRTV DYIIAGGGLTGLTTAARL   60
                                                .. . *:::.*** :* :.*.**

FOD             AENPNVTVLIVEAG IGNPEDIPEITTPSSAMDLRNSKYDWAYKTTMVRRDDYERIEKPNT   86
Choline oxidase SEDPAVSVALVEAG PDD-RGVPEVLQLDRWMELLESGYDWDYP---IEPQENGNSFMRHA   88
Glucose oxidase TENPNISVLVIESG SYESDRGPIIEDLNAYGDIFGSSVDHAYETVELATNNQTALIRS-   118
                :*:* ::* ::*:*   :   * :   .   :: * * *   :  ::

FOD             RGKTLGGSSSLNYFTWVPGHKATFDQWEEFGGKE-WTWDPLVPYLRKSATY-----HDDP   140
Choline oxidase RAKVMGGCSSHNSCIAFWAPREDLDEWEAKYGATGWNAEAAWPLYKRLETN-----EDAG   143
Glucose oxidase -GNGLGGSTLVNGGTWTRPHKAQVDSWETVFGNEGWNWDNVAAYSLQAERARAPNAKQIA   177
                 .: :**.:  *       :  .*.**   *   *. :   .   :          .:

FOD             RLYSPELEKIGGGGPIPISHAELIDEMAPFRENLTKAWKSMGQPLIEN------IYDGEM   194
Choline oxidase ----PDAPHHGDSGPVHLMNVPPKDPTG---VALLDACEQAGIPRAKFNTGTTVVNGANF   196
Glucose oxidase AGHYFNASCHGVNGTVHAGPRDTGDDYSPIVKALMSAVEDRGVPTKKDFG-CGDPHGVSM   236
                     :   * .*.:       *  .    * .* :. * *  :         . .:

FOD             DGLTHCCDTIYRGQRSGSFLFVKNKPNITIVPEVHSKRLIINEADRTCKGVTVVTAAGN-   253
Choline oxidase FQINRRADGTRSSSSVSYIHPIVEQENFTLLTGLRARQLVF-DADRRCTGVDIVDSAFG-   254
Glucose oxidase FPNTLHEDQVRSDAAREWLLPNYQRPNLQVLTGQYVGKVLLSQNGTTPRAVGVEFGTHKG   296
                 .  *    .    :    :: *: ::     :::: : .   .* :  .:

FOD             ELNFFADR-E VILSQGVFETPKLLMLSGIG PTRELSRHGINTIVDSRHVGQNLMDHPGVP 312
Choline oxidase RTHRLTARNE VVLSTGAIDTPKLLMLSGIG PAAHLAEHGIEVLVDSPGVGEHLQDHP--- 311
Glucose oxidase NTHNVYAKHE VLLAAGSAVSPTILEYSGIG MKSILEPLGIDTVVDLP-VGLNLQDQT--- 352
                . : .  : * *:*: *   :*.:*  ****     *   **:.:** ** :*   *:.

FOD             FVLRVKDGFGMDDVLLRHGPKRDAVVSAYNKNRSGPVGSGLLELVGFPRIDKYLEKDAEY   372
Choline oxidase ----------------------EGVVQFEAKQPMVAESTQWWEIGIFTPTEDGLDR----   345
Glucose oxidase ----------------------TATVRSRITSAGAGQGQAAWFATFNETFGDYSEKAHEL   390
                                     ..*   ..   .        .  ::

FOD             RKAKAANGGKDPFSP---LGQPHFELDFVCMFGTAFQWHFPTPKTGDHLTVVVDLVRP--   427
Choline oxidase ---------PDLMMH---YGSVPFDMN--------TLRHGYPTTENGFSLTPNVTHAR---   383
Glucose oxidase LNTKLEQWAEEAVARGGFHNTTALLIQYENYRDWIVNHNVAYSELFLDTAGVASFDVWDL   450
                      : .     .  : ::       ..   . .:    :  .

FOD             -ISDPGEVTLNSAD-PFQQPNINLNFFAN--DLDIIAMREGIRFSYDLLFKGEGFKDLVE   483
Choline oxidase ---SRGTVRLRSRD-FRDKPMVDPRYFTDPEGHDMRVMVAGIRKAREIAAQP-AMAEWTG   438
Glucose oxidase LPFDRGYVHILDKDPYLHHFAYDPQYFLN--ELDLLGQAAATQLARNISNSG-AMQTYFA   507
                 . * * : . *   .:   : .:* :   *:     . : : :: .  .:

FOD             SEYPWEMPLDSDKEM---HRAVLDRCQTAFHPTGTARLSKNIDQG-VVDPKLK VHGIKKL 539
Choline oxidase RELSPGVEAQTDEEL---QDYIRKTHNTVYHPVGTVRMGAVEDEMSPLDPELR VKGVTGL 495
Glucose oxidase GEILPGDNLAYDADLSAWTEYIPYHFRPNYHDVGTCSMMP-KEMGSVVDNAAR VYGVRGL 566
                 *        * ::      :    .. :* ,** :    :    :*   : * *:  *

FOD             RVADASVIP IIPDCRIQNSVYAVGEKCADMIKAEHKDLY----------- 578
Choline oxidase RVADASVMP EHVTVNPNITVMMIGERCADLIRSARAGETTTADAELSAALA 546
Glucose oxidase RVIDGSIPP TQMSSHVMTVFYAMALKISDAILEDYASMQ----------- 605
                ** *.*: *      .   .  :. : :* *     .
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Figure 1
Sequence alignment of FOD_{AO} with choline oxidase from *Arthrobacter globiformis* (NCBI accession No. AAS99880) and glucose oxidase from *Aspergillus niger* (AAF59929). The alignment was constructed using *ClustalW*2. Gaps were introduced into the sequences to maximize the homology and are indicated by dashes. Identical residues are labelled with asterisks (*). Homologous and semi-homologous residues are labelled with ':' and '.', respectively. The three FAD-binding motifs are indicated by boxes. The two active-site residues are indicated in bold.

2.2. Crystallization

Initial screening for FOD_{AO} crystallization was performed at 293 K by the sitting-drop vapour-diffusion method using Crystal Screen, Crystal Screen 2 and Crystal Screen Lite from Hampton Research (Laguna Niguel, California, USA) with 96-well plates. Low-quality crystals of the enzyme were obtained from condition Nos. 37 [0.1 *M* sodium acetate trihydrate pH 4.6, 4%(*w*/*v*) PEG 4000] and 40 [0.1 *M* sodium citrate tribasic dyhydrate pH 5.6, 10%(*v*/*v*) 2-propanol, 10%(*w*/*v*) PEG 4000] from Crystal Screen Lite. Further screening was carried out at 277 and 293 K by modification of the PEG concentration and by replacement of the buffer. After improvement of the conditions, final crystallization was performed by the hanging-drop vapour-diffusion method using 24-well plates at 293 K. The hanging drop contained 2 µl enzyme solution (35 mg ml^{-1}) and 2 µl reservoir solution [0.1 *M* sodium acetate buffer pH 4.6 and 6%(*w*/*v*) PEG 4000] and was equilibrated against 1 ml reservoir solution.

2.3. X-ray data collection

A crystal of FOD_{AO} was picked up from a droplet and transferred into cryoprotectant solution [0.1 *M* sodium acetate buffer pH 4.6, 6%(*w*/*v*) PEG 4000 and 30%(*w*/*v*) 2-methyl-2,4-pentanediol]. The crystal was mounted on a nylon loop (Hampton Research, Laguna Niguel, California, USA) and immediately flash-cooled in a cold nitrogen-gas stream at 100 K. X-ray diffraction images were checked using an in-house Bruker High-Star multi-wire detector and a rotating-anode X-ray generator. The final diffraction data for the selected crystal were collected on a Rigaku R-AXIS V detector using

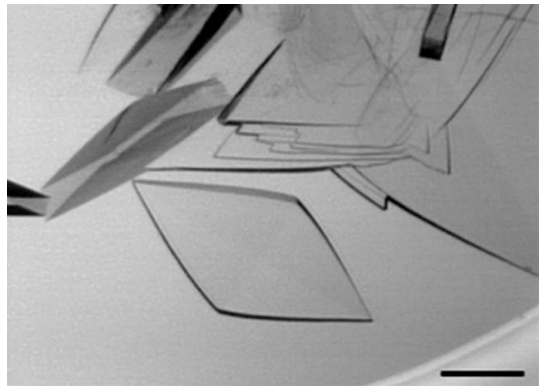

Figure 2
Crystal of FOD_{AO}. The scale bar is 0.1 mm in length.

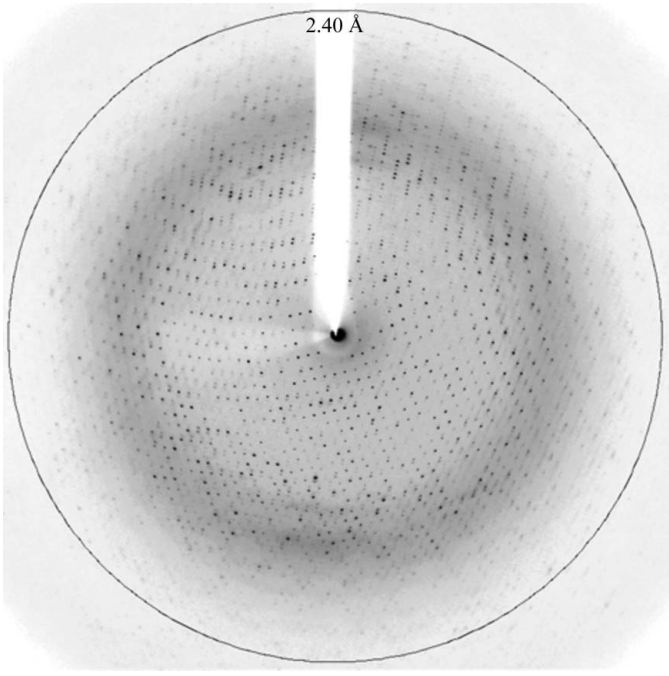

Figure 3
X-ray diffraction pattern from a crystal of FOD. The ring indicates a resolution of 2.4 Å.

Table 1
Data-collection and processing statistics.

Beamline	SPring-8 BL-26B1
Wavelength (Å)	1.00
Detector	R-AXIS V
Crystal-to-detector distance (mm)	450
Rotation range per image (°)	0.80
Total rotation range (°)	106
Exposure time per image (s)	15.0
Size of crystal used for data collection (mm)	0.3 × 0.3 × 0.1
Resolution range (Å)	50–2.40 (2.49–2.40)
Space group	$C222_1$
Unit-cell parameters (Å)	a = 155.36, b = 159.97, c = 184.66
Mosaicity (°)	0.578
Total No. of measured intensities	357244 (34120)
No. of independent reflections	86521 (8530)
Mean $I/\sigma(I)$	13.5 (3.7)
Multiplicity	4.1 (4.0)
Completeness (%)	97.5 (97.5)
$R_{\rm merge}$† (%)	6.7 (50.3)
Overall B factor from Wilson plot (Å^2)	46.9
No. of molecules per asymmetric unit	3
Matthews coefficient (Å^3 Da^{-1})	2.92
Solvent content (%)	57.2

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the intensity of the ith measurement of reflection hkl and $\langle I(hkl)\rangle$ is the mean value of $I(hkl)$ for all i measurements.

synchrotron radiation of wavelength 1.00 Å at the BL-26B1 station of SPring-8 (Hyogo, Japan; Ueno *et al.*, 2006). The collected data were processed with *HKL*-2000 (Otwinowski & Minor, 1997).

3. Results and discussion

A diamond-shaped crystal of FOD_{AO} appeared in two weeks (Fig. 2). The crystal yielded good diffraction images as shown in Fig. 3. The crystal parameters and diffraction data statistics are summarized in Table 1. The space group of the crystal was determined to be $C222_1$, with unit-cell parameters a = 155.36, b = 156.97, c = 184.66 Å. 86 521 independent reflections were obtained from the total of 357 244 measured reflections, with an $R_{\rm merge}$ value of 6.7%. The data set was 97.5% complete at the resolution limit of 2.4 Å. The crystal contained three subunits of the enzyme in the asymmetric unit. The $V_{\rm M}$ value (the crystal volume per unit protein molecular weight; Matthews, 1968) was determined to be 2.92 Å^3 Da^{-1} and the solvent content was 57.2% assuming the presence of three molecules of the subunit in the asymmetric unit. The $V_{\rm M}$ value and solvent content lie within the range usually found for protein crystals.

All FODs isolated to date are dimeric proteins. The crystal of FOD_{AO} used in this report contains three subunits in the asymmetric unit. In order to obtain information about its catalytic site and modified FAD, structure determination by molecular replacement using choline oxidase (PDB code 2jbv; Quaye *et al.*, 2008) and glucose oxidase (PDB code 1cf3; Wohlfahrt *et al.*, 1999) is now in progress.

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