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Crystallization and diffraction data of 1*H*-3-hydroxy-4-oxoquinoline 2,4-dioxygenase: a cofactor-free oxygenase of the α/β-hydrolase family

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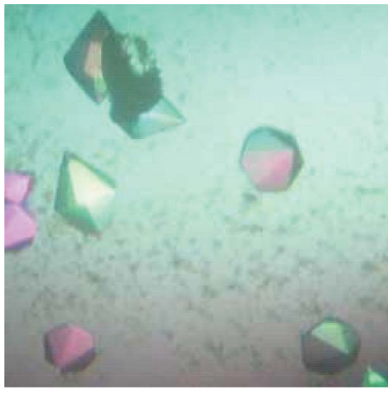
1*H*-3-Hydroxy-4-oxoquinoline 2,4-dioxygenase (QDO) from *Pseudomonas putida* 33/1 catalyses the oxygenolysis of 1*H*-3-hydroxy-4-oxoquinoline to form *N*-formylanthranilic acid and carbon monoxide without the aid of cofactors. Both N-terminally His_6-tagged and native QDO were overexpressed in *Escherichia coli* and purified by conventional chromatographic procedures. Untagged QDO, but not His_6-tagged QDO, was crystallized by the vapour-diffusion method, giving hexagonal bipyramid crystals belonging to space group $P6_122$. Selenomethionine-containing native QDO was prepared and crystallized under identical conditions. The unit-cell parameters were $a = b = 90.1$, $c = 168.6$ Å, $\alpha = \beta = 90$, $\gamma = 120^\circ$. Using synchrotron radiation, these crystals diffract to 2.5 Å. The expression, purification and crystallization of QDO are reported here.

1. Introduction

Monooxygenases and dioxygenases catalyse the incorporation of oxygen into organic compounds in several aerobic metabolic pathways. 1*H*-4-Oxoquinoline is degraded by *Pseudomonas putida* 33/1 *via* the anthranilate pathway. An intermediate step in this pathway is the ring oxygenolysis of 1*H*-3-hydroxy-4-oxoquinoline to form carbon monoxide and *N*-formylanthranilate. This step is catalysed by 1*H*-3-hydroxy-4-oxoquinoline 2,4-dioxygenase (QDO; EC 1.13.11.47). *Arthrobacter nitroguajacolicus* Rü61a (formerly *A. ilicis* Rü61a) possesses an enzyme, 1*H*-3-hydroxy-4-oxoquinaldine 2,4-dioxygenase (HOD; EC 1.13.11.48; Fischer *et al.*, 1999), that catalyzes an analogous reaction (Fig. 1).

Most oxygenases contain metal ions (usually copper or iron) or flavins to activate oxygen. QDO and HOD have no requirement for such cofactors (Fetzner, 2002). Kinetic analysis of HOD indicates that the enzyme operates by a compulsory-order ternary-complex mechanism, with binding of the heterocyclic substrate preceding that of oxygen (Frerichs-Deeken *et al.*, 2004).

QDO is a 264-amino-acid protein of molecular weight 30 kDa. Analysis of the amino-acid sequence of QDO indicates that it is a member of the α/β-hydrolase superfamily of enzymes (Fischer *et al.*, 1999). The canonical fold has a central β-sheet with strand $\beta2$ antiparallel to the rest. The connection of strand order is +1, +2, −1x, +2x, +1x, +1x (Heikinheimo *et al.*, 1999). These enzymes contain a catalytic triad consisting of a nucleophile, a histidine and an acidic residue. The nucleophile can be a serine, cysteine or aspartate residue. In QDO, the catalytic histidine has been identified (Fischer & Fetzner, 2000). Site-directed mutagenesis of QDO indicates that this



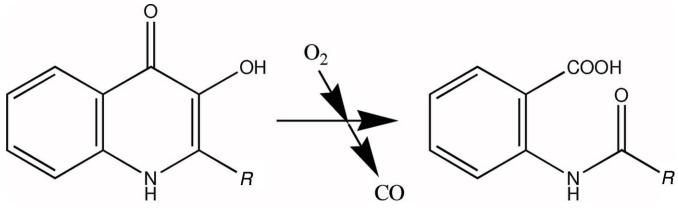


Figure 1
The reactions catalysed by QDO (R = H) and HOD (R = CH_3). The substrates are 1*H*-3-hydroxy-4-oxoquinoline (R = H) and 1*H*-3-hydroxy-4-oxoquinaldine (R = CH_3).

Table 1
Crystallographic parameters and Se-MAD data-collection statistics.

Values in parentheses are for the highest resolution bin.

Protein	SeMet	SeMet	Native
Space group	$P6_122$	$P6_122$	$P6_122$
Unit-cell parameters (Å,°)	$a = b = 90.1$, $c = 168.6$, $\alpha = \beta = 90.0$, $\gamma = 120.0$		$a = b = 90.2$, $c = 168.9$, $\alpha = \beta = 90.0$, $\gamma = 120.0$
Wavelength (Å)	0.97929 (peak)	0.98220 (inflection)	0.97929
Resolution (Å)	50.0–2.7 (2.8–2.7)	50–2.7 (2.8–2.7)	50–2.5 (2.59–2.5)
$\langle I/\sigma(I)\rangle$	24.2 (2.1)	34.2 (3.2)	23.1 (2.89)
R_{sym}† (%)	5.1 (33.9)	6.2 (36.3)	9.4 (43.5)
Completeness (%)	99.2 (92.3)	99.7 (97.3)	98.8 (98.2)
Unique reflections	20929 (1933)	21039 (2038)	25905 (2324)
Average redundancy	3.8 (3.1)	7.5 (6.2)	9.3 (5.4)

† $R_{sym} = \sum_{hkl}\sum_i |I_{hkl,i} - \langle I_{hkl}\rangle| / \sum_{hkl}\sum_i I_{hkl,i}$, where $\langle I_{hkl}\rangle$ is the mean intensity of a set of equivalent reflections and $I_{hkl,i}$ is the ith measurement of the reflection with Miller indices hkl.

residue is absolutely required for catalytic activity (Fischer & Fetzner, 2000).

In order to understand the catalytic mechanism of QDO, we have undertaken to solve the structure of this enzyme. Here, we report the expression, purification, crystallization and preliminary diffraction data for QDO. The crystallization of HOD is described in the accompanying paper (Steiner *et al.*, 2007).

2. Materials and methods

2.1. Cloning, expression and protein purification

The QDO gene was obtained in vector pQE50-QDO (Fischer & Fetzner, 2000). Initial attempts to express the protein in this vector were unsuccessful. We therefore set about cloning the gene into new vectors for protein expression. The QDO gene was PCR-amplified with Turbo *Pfu* Taq polymerase (Stratagene) using the primer set RSC968 d(AACTATGAGAGGACATATGCATTCG) and RSC979 d(ATTAACCATGGCTGCAGGTCGACCAG) and pQE50-QDO as the template. The PCR product was digested with restriction enzymes *Nde*I and *Nco*I and cloned into linearized vectors pRSC722 (for untagged QDO) and pRSC774 (N-terminally His_6-tagged QDO), respectively. The tagged protein contained only an N-terminal methionine residue plus six histidine residues. Selection was made in Luria–Bertani (LB) medium supplemented with ampicillin (100 µg ml^{-1}). Recombinant plasmid DNA was prepared with a QIAprep Spin Miniprep kit (Qiagen).

Escherichia coli strain BL21(DE3)recA was used to express QDO in all cases. Cells harbouring pRSC774-QDO or pRSC722-QDO were cultured for 12 h in LB medium supplemented with 100 µg ml^{-1} ampicillin. Stationary phase culture (2 ml) was transferred into 200 ml LB media and incubated for 7 h at 310 K with agitation at 200 rev min^{-1}. Overexpression of QDO was achieved by sixfold dilution of the culture in the same medium supplemented with 100 µg ml^{-1} ampicillin and 0.5 m*M* (final concentration) isopropyl *β*-D-thiogalactopyranoside (IPTG) and overnight culture at room temperature in a water bath with 200 rev min^{-1} agitation. The culture was then centrifuged at 8000 rev min^{-1} in an SLA 3000 rotor (Sorvall) and the cells were resuspended in 20 m*M* sodium phosphate buffer pH 7.5 for N-terminal His_6-tagged QDO cells or in 50 m*M* Tris pH 7.6 for non-His-tagged QDO. The cells were lysed using a French Press (SLM Instruments) at 4.9 MPa in the presence of 0.5 m*M* phenylmethanesulfonyl fluoride (PMSF). Cell debris was removed by centrifugation at 15 000 rev min^{-1} using an SS34 rotor (Sorvall).

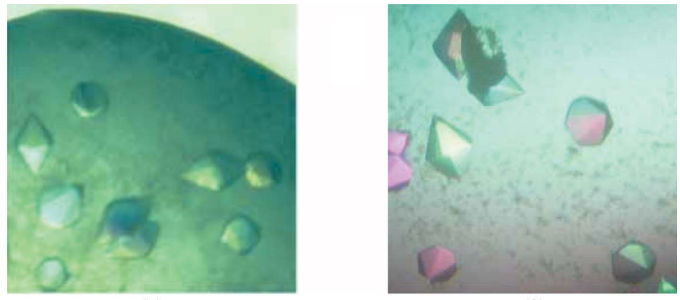
His_6-tagged QDO was purified using a 5 ml prepacked HiTrap chelating column (Amersham) charged with Ni^{2+} connected to an Äkta liquid-chromatography system (Pharmacia). The cell lysate was loaded onto the prepacked 5 ml Ni–NTA column pre-equilibrated with buffer containing 20 m*M* sodium phosphate, 0.5 *M* sodium chloride pH 7.4, followed by washing with five column volumes of the same buffer. Bound recombinant His_6-tagged QDO was eluted with a 0–250 m*M* imidazole gradient in 20 m*M* sodium phosphate buffer containing 0.5 *M* sodium chloride pH 7.4 at a flow rate of 1 ml min^{-1}. Pure fractions were pooled, dialysed against 50 m*M* Tris buffer pH 7.6 to remove the imidazole and concentrated.

Nontagged QDO was purified using a DEAE Fractogel column followed by a G75 gel-filtration column using an Äkta liquid-chromatography system (Pharmacia). QDO was precipitated by the addition of ammonium phosphate (0.25–0.50 g ml^{-1}). The precipitate was centrifuged at 13 000 rev min^{-1} for 30 min using an SS34 rotor. The pellet was resuspended in 50 m*M* Tris pH 7.6 and dialysed against the same buffer. Crude QDO fractions were obtained using a 0–300 m*M* linear gradient of sodium chloride on a Toyopearl DEAE-650M column (Tosoh) with the aid of an Äkta liquid-chromatography system. This fraction was dialysed against two changes of 50 m*M* Tris pH 7.6 in an at least 100-fold dilution volume and concentrated to less than 3 ml using Amicon Ultra-15 centrifugal filter devices (10 000 NMWL, Millipore). Concentrated crude sample was loaded onto a 300 ml G-75 column (Pharmacia) and pure QDO was separated from other proteins at a flow rate of 0.5 ml min^{-1} over a 300 ml volume. Protein purity was monitored by SDS–PAGE.

Selenomethionine labelling of the recombinant QDO protein was conducted according to Van Duyne *et al.* (1993). 50 ml stationary phase BL21(DE3)recA cells harbouring pRSC722-QDO plasmid were spun down, washed once with MilliQ water and transferred to 1.0 l minimal medium containing 50 m*M* Na_2HPO_4, 50 m*M* KH_2PO_4, 25 m*M* $(NH_4)_3PO_4$, 0.4% glucose, 2 m*M* $MgSO_4$ plus 0.2% trace elements. Amino acids supplemented per litre culture were as follows: selenomethinine (50 mg), threonine (100 mg), phenylalanine (100 mg), lysine (100 mg), leucine (50 mg), isoleucine (50 mg) and valine (50 mg). The inoculated culture was incubated at 310 K with 200 rev min^{-1} agitation until the OD_{595} reached 0.7. Induction of the protein took place by the addition of 0.5 m*M* IPTG (final concentration) and culture at room temperature with 200 rev min^{-1} shaking. Labelled QDO was purified in an identical manner to native QDO as described above.

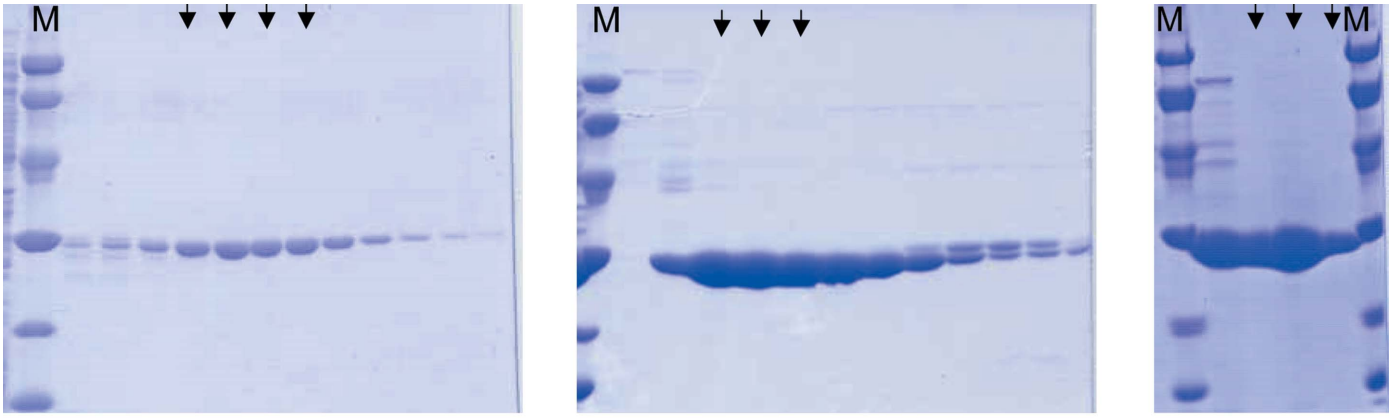
2.2. Crystallization

Initially, a search for potential crystallization conditions was conducted using the 96-solution Index Screen (Hampton Research).



(*a*) (*b*)

Figure 2
Drops containing crystals of (*a*) native and (*b*) selenomethionine-labelled QDO. The largest crystals in both cases are about 275 µm from tip to tip.



(*a*) (*b*) (*c*)

Figure 3
Coomassie-stained SDS–PAGE gels showing (*a*) His_6-tagged QDO eluted from nickel resin, (*b*) native and (*c*) selenomethionine QDO fractions eluted from a G75 column (final step). Arrows indicate fractions pooled for the final concentration step prior to crystallization. M indicates the column containing molecular-weight markers. These are, in descending order, 97, 66, 45 30 20.1 and 14.4 kDa.

Art Robinson IntelliPlates were used for sitting-drop vapour-diffusion experiments. Equal volumes (1 µl) of protein (15 mg ml^{-1} in 50 m*M* Tris pH 7.6 in all cases) and crystallization reagents were mixed and then allowed to equilibrate against the well solution (100 µl) at 277 K. For optimization of crystallization conditions, the hanging-drop technique in 24-well plates (Linbro) was used. Drops containing 3 µl protein solution and 3 µl reservoir solution were suspended on plastic cover slips above 1 ml reservoir solution.

2.3. X-ray diffraction

X-ray data were collected on beamline 19-ID at the Advanced Photon Source. Multiple-wavelength anomalous dispersion (MAD) data were collected using a Quantum-315 area detector (ADSC) for native and selenomethionine QDO. A crystal-to-detector distance of 290 mm and an exposure time of 15 s per 0.25° frame were used in all cases. Diffraction images were indexed, integrated and scaled using the *HKL*-3000 package (Minor *et al.*, 2006). Crystals were prepared for freezing by transferring them into artificial mother liquor containing increasing concentrations of sodium formate. At each step, the sodium formate concentration was increased by 0.25 *M* and the crystals were equilibrated in the solutions for 3 min each. The maximum concentration of sodium formate used was 6 *M*. Crystals were flash-cooled by transferring them into liquid nitrogen. All data were collected at 100 K using an Oxford Cryostream. Data-collection statistics are given in Table 1.

3. Results

His_6-tagged QDO did not yield crystals under any of the conditions in the Index Screen. Untagged native QDO gave crystals in conditions Nos. 19 (0.056 *M* sodium dihydrogen phospate, 1.344 *M* dipotassium hydrogen phosphate), 24 (2.8 *M* sodium acetate), 25 (3.5 *M* sodium formate), 29 (60% Tacsimate pH 7.0) and 30 (0.1 *M* sodium chloride, 0.1 *M* bis-Tris pH 6.5, 1.5 *M* ammonium sulfate). The best crystals, as judged by size and morphology, grew as hexagonal bipyramids from condition No. 25 and appeared after 9 d. Optimization using the hanging-drop vapour-diffusion technique resulted in good crystals from 4.1–4.6 *M* sodium formate pH 7.8. Crystals appeared after 2 d and grew to maximum size in one week. The largest crystals used for X-ray data collection were about 275 µm long by 200 µm wide at the equator (Fig. 2). Untagged selenomethionine-containing QDO gave crystals with a similar appearance under identical conditions (Fig. 2). Initial phasing was based on three (out of a theoretical six) selenium sites. The correct hand was indicated by a clear difference in contrast between the original and inverted-hand enantiomorphs. Initial electron-density maps revealed helices of the correct hand in a protein that appears to adopt the predicted α/β-hydrolase topology. The asymmetric unit appears to contain one monomer, giving a Matthews coefficient of 3.25 $Å^3$ Da^{-1} and a solvent content of 62% for the crystal.

It is noteworthy that the His_6-labelled QDO did not crystallize in screens, whereas untagged native and selenomethionine-derived QDO did. Even when hanging-drop vapour-diffusion experiments with His_6-tagged QDO and sodium formate were performed under identical conditions to those used for native QDO, no crystals resulted. Since the purity of all the samples used in crystallization experiments was close to 100% as judged by SDS–PAGE (Fig. 3) and the final buffer composition for all protein samples subjected to crystallization trials was identical, we propose that the His_6 tag itself directly interfered with crystallization. Such tags do not always interfere with crystallization. The current release of the PDB (Berman *et al.*, 2007) contains 457 proteins with His tags solved by protein crystallography. In a systematic study of the effect of tags on the crystallization of *Pyrococcus furiosus* maltodextrin-binding protein, His_6-tagged protein produced crystals with similar crystallographic characteristics to the native protein (Bucher *et al.*, 2002). Conversely, the removal of a His tag was required for the formation of high-quality crystals of phosphotransfer protein ZmHP1 from maize (Sugawara *et al.*, 2005).

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