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# Purification, crystallization and preliminary X-ray diffraction studies of xanthine dehydrogenase and xanthine oxidase isolated from bovine milk

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Xanthine dehydrogenase catalyzes the oxidation of hypoxanthine to xanthine and the further oxidation of xanthine to uric acid. The enzyme is the target of the anti-gout drug allopurinol and its involvement in postischemic reperfusion injury is presently being defined. Each subunit of the homodimeric 290 kDa enzyme contains four cofactors: one Mo-pterin, two [2Fe–2S] clusters and one FAD. Both the dehydrogenase (XDH) and the proteolytically modified oxidase form (XO) of the enzyme from bovine milk have been crystallized. XO crystals belong to space group $C222_1$, with unit-cell parameters $a = 116.3$, $b = 164.4$, $c = 153.2$ Å at room temperature and $a = 117.8$, $b = 165.4$, $c = 154.5$ Å when flash-frozen. They allow data collection to 3.3 and 2.5 Å, respectively. In addition, a data set was collected from frozen XDH crystals and processed to 2.1 Å. These crystals belong to space group $C2$, with unit-cell parameters $a = 169.9$, $b = 124.8$, $c = 148.6$ Å, $\beta = 90.9^\circ$. The unit-cell volumes and Matthews parameters are similar for the two crystal forms. There is one monomer per asymmetric unit in the XO crystals and a complete native dimer per asymmetric unit in the XDH crystals.

## 1. Introduction

Xanthine oxidase (XO) is an archetypal enzyme which was originally described as aldehyde oxidase by Schardinger (1902) and was subsequently identified as xanthine oxidase by Morgan *et al.* (1922). It is now clear that the enzyme can catalyze the oxidation of a wide range of substrates including purines, pyrimidines and aldehydes. Xanthine oxidoreductases have been isolated from a wide range of organisms from bacteria to man (Hille & Nishino, 1995), with sequence identities ranging from 98% to the low 40%. So far, no full-length homologues have been found in archaeal organisms. All of the enzymes have similar molecular weights and bind the same series of redox centers: Mo-pterin, two iron–sulfur centers and FAD. The bovine milk xanthine oxidoreductase is still the best characterized of the molybdenum-containing flavoproteins and work on the enzyme is at the forefront of physicochemical and kinetic studies of this important class of biocatalysts.

Mammalian xanthine oxidoreductases are synthesized in their dehydrogenase forms (XDH), but can be converted to their oxidase forms (XO) either reversibly by oxidation of sulfhydryl residues to disulfides or irreversibly by proteolysis, most notably by trypsin or pancreatin (Hille & Nishino, 1995). The XO and XDH forms of the enzyme differ in the accessibility of some cofactor sites to priming from external electron sources (Walker *et al.*, 1991) and the preferred substrate used as the ultimate electron source during the oxidative half-reaction (Saito & Nishino, 1989). In both forms, the oxidation of xanthine takes place at the Mo-pterin center (Bray *et al.*, 1964) and the electrons thus introduced are rapidly distributed to other centers (Olson *et al.*, 1974). The reduction of the oxidants, $NAD^+$ in the case of XDH and oxygen in XO (Saito & Nishino, 1989), occurs through FAD, which is located at a distance from the Mo center (Komai *et al.*, 1969).

The active form of mammalian or avian XDH is a homodimer of molecular weight 290 kDa, with each of the subunits acting independently in catalysis. Each monomer of xanthine oxidoreductase (XDH) consists of three stable domains, which can be cleaved by proteases into a 20 kDa domain containing the two non-identical [2Fe–2S] centers (Massey *et al.*, 1969; Johnson *et al.*, 1989), a 40 kDa domain containing the FAD center and an 85 kDa domain containing the molybdopterin center (Hille & Nishino, 1995). The cleavage occurs after residues 184 and 551 in the rat XDH sequence (Amaya *et al.*, 1990). The site of cleavage that is responsible for the irreversible conversion is most probably on an external loop which connects the 40 kDa and the 85 kDa domains (Nishino & Nishino, 1997). Following proteolysis, which results in the formation of the irreversible oxidase form,

the three domains remain associated under non-denaturing conditions (Amaya *et al.*, 1990).

Also there is medical interest in xanthine oxidoreductase. The enzyme is the target of the widely used anti-gout drug allopurinol (Emmerson, 1996) and has been proposed to be involved in postischemic reperfusion injury (McCord, 1985).

Small crystals of bovine XO were obtained during purification of the enzyme from bovine milk (Avis *et al.*, 1954). These crystals, however, were not of sufficient quality to allow any structural analysis. In the absence of such structural information, partial correlations about structure and function for XO have been inferred from the distantly related enzymes aldehyde oxidoreductase from *Desulfovibrio gigas* (Romao *et al.*, 1995; 23% sequence identity for the FeS- and Mo-binding domains; this enzyme lacks a flavin domain) and CO dehydrogenase from *Oligotropha carboxidovarans* (Dobbek *et al.*, 1999; 17% sequence identity). To provide a structural framework for the enormous amount of data collected on xanthine oxidoreductases, we are now pursuing crystal structure analyses of both the dehydrogenase and proteolytically produced oxidase forms of the bovine enzyme.

## 2. Materials and methods

### 2.1. Purification and crystallization

XO was purified from bovine milk according to the method of Ball (1939) and was further purified by affinity chromatography using a folate affinity gel (Nishino *et al.*, 1981) followed by DEAE cellulose chromatography.

XDH was purified from bovine milk using a modification of the previous method. 1 g $l^{-1}$ of *Candida rugosa* type VII lipase (Sigma) replaced porcine pancreatin. After lipase treatment, the protein solution was brought to 30%(*w*/*v*) saturation in ammonium sulfate and stirred for 30 min. The mixture was centrifuged at 10 000*g* for 10 min at 277 K and the precipitate was discarded. Additional ammonium sulfate was added to a final concentration of 60%. The mixture was centrifuged again at 10 000*g* for 10 min at 277 K and the supernatant was discarded. After resuspending the pellet in 10 m*M* Tris–HCl pH 7.4, 1 m*M* salicylate and 0.2 m*M* EDTA (buffer *A*), the sample was dialyzed extensively against buffer *A*. The dialysate was applied to a DE-53 column and the brown-colored fractions of the eluate were collected. XDH was concentrated by ammonium sulfate precipitation at 60% saturation and was then resuspended in buffer *A*. The protein was again loaded onto a DE-53 column and was eluted with a linear gradient of 0–0.2 *M* KCl in buffer *A*. The colored fractions were further purified by the folate-gel chromatography column previously described (Nishino *et al.*, 1981). The purified XDH was concentrated and dialyzed against 50 m*M* Tris–HCl initial pH 7.8, 0.1 *M* pyrophosphate initial pH 8.5, 1 m*M* salicylate and 0.2 m*M* EDTA. The enzyme was concentrated to ∼125 mg $ml^{-1}$ and stored at 277 K without loss of any of its bound cofactors or its catalytic activity.

The batch method of crystallization (McPherson, 1999) proved to be by far the most suitable method for crystallizing bovine XO or XDH. Initial screenings for crystallization conditions were all carried out on the XO form of the enzyme. The conditions that resulted in the most promising crystalline material all contained PEG as precipitant. Based on these hits, narrower refinement screens using PEGs of varying molecular sizes and varying pHs yielded crystals in many of the setups. The protein and precipitant samples were filtered using 0.1 µm cutoff spin cartridges. The protein was incubated with fresh DTT for 1 h before an equal volume of the precipitant solution was added and about 40 µl of the solution was placed into batch slides at 295 K. The glass surface of the batch slides had been coated with silicone before the crystallizations.

The final conditions for growing the best XO crystals, not containing the cryoprotectant, were as follows. The XO stock solution was diluted with 40 m*M* Tris initial pH 7.5, 20 m*M* pyrophosphate initial pH 8.5, 5 m*M* DTT, 1 m*M* salicylate and 0.2 m*M* EDTA (buffer *B*) to a concentration of 24 mg $ml^{-1}$. The precipitant solution contained 50 m*M* potassium phosphate pH 6.5, 5 m*M* DTT, 1 m*M* sodium salicylate and 0.2 m*M* EDTA (buffer *C*) and a final concentration of 18%(*w*/*v*) PEG 4000.

In addition, crystals of both XO and XDH could be produced in crystallization solutions which contained a glycerol concentration sufficient to allow flash-freezing of the crystals. For the enzyme solution, the stored enzyme stock was diluted with buffer *B* to a final concentration of 20% glycerol and 18 mg $ml^{-1}$ enzyme for XO or 30% glycerol and 10 mg $ml^{-1}$ enzyme for XDH. The optimized conditions for the precipitant solution were buffer *C* containing either 23% PEG 4000 and 20% glycerol for the XO crystals or 22% PEG 4000 and 30% glycerol for the XDH crystals. Protein and precipitant solutions were filtered and equal volumes (20 µl each) of both were mixed to start crystallization.

### 2.2. Data collection and processing

Initially, data sets for XO were collected from three different crystals at 295 K and $\lambda$ = 1.000 Å at beamline X8C, NSLS, Brookhaven National Laboratories on a mosaic 4K CCD detector (ADSC, San Diego, CA, USA) and on beamline BL6B (TARA-Sakabe project) at the Photon Factory, KEK, Tsukuba, Japan, using imaging plates and a Weissenberg camera (Sakabe, 1991).

A complete data set from a single flash-frozen XO crystal was collected at 100 K on beamline BM14C, BioCARS, Argonne National Laboratory using a mosaic 4K ADSC CCD detector and $\lambda$ = 1.000 Å.

XDH diffraction data were collected from a single crystal that was broken away from one of the clusters produced with this enzyme form. After flash-freezing, the crystal was held at a temperature of 100 K on beamline X8C, NSLS, Brookhaven National Laboratory, again using a 4K CCD and the X-ray wavelength set to 1.000 Å for data collection.

All data sets were processed and scaled together using the program suite *HKL* (Otwinowski & Minor, 1997).

## 3. Results and discussion

### 3.1. Purification and crystallization

Attempts to produce diffraction-quality crystals using enzyme purified following published procedures (Ball, 1939) met with no success. The inclusion of lipase treatment, essential for the removal of trace amounts of contaminating lipids, as an integral part of the purification protocol, aided by an additional ion-exchange column, provided enzyme of improved purity at still very good yields. This improvement in the purification procedure proved to be indispensable for the production of crystals.

It was very important to carefully filter all solutions to remove any particulate matter and to keep the enzyme under reducing conditions through the repeated addition of fresh DTT in order to ensure the production of quality crystals. In addition, the enzyme showed a pronounced temperature dependence in its crystallization behavior. The crystallization conditions were optimized for 295 K.

XO crystallizations with glycerol resulted in discrete crystals of dimensions up to

**Table 1**
Data-collection statistics.

Values in parentheses refer to the outermost resolution shell (3.38–3.30 Å for the RT XO data set, 2.59–2.50 Å for the 100 K XO data set and 2.14–2.10 Å for the 100 K XDH data set, respectively).

| | XO form (273 K) | XO form (100 K) | XDH form (100 K) |
|---|---|---|---|
| Resolution (Å) | 40.0–3.3 | 30.0–2.5 | 25.0–2.1 |
| Unique reflections | 22359 | 51299 | 154427 |
| Multiplicity | 3.2 | 3.5 | 2.8 |
| Completeness (%) | 95.2 (67.7) | 97.9 (97.5) | 87.2 (67.8) |
| $R_{\rm merge}$† (%) | 9.2 (19.3) | 5.9 (35.4) | 8.1 (40.3) |
| $I/\sigma(I)$ | 11.1 (4.0) | 11.2 (3.4) | 7.8 (2.2) |

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

400 × 400 × 75 µm (Fig. 1*a*). In contrast to the XO crystals, which are of reasonable thickness, the XDH crystals are very thin plates, with typical dimensions of 300 × 300 × 10 µm. They usually grow together in a fan-like shape (Fig. 1*b*).

### 3.2. Data collection and processing

The crystals of XO were initially grown without cryoprotectant in the mother liquor. As XO crystals proved to be extremely sensitive to any change in their chemical environment, it was not too surprising to find that all attempts at soaking cryoprotectant into the XO crystals yielded diffraction patterns of 5 Å resolution or worse. Similar results were obtained for rapid washing of the crystals with cryoprotectant. Therefore, an initial native data set was collected to 3.3 Å resolution using three crystals at 295 K and synchrotron radiation (Table 1). The initial image from each crystal showed diffraction to better than 2.5 Å resolution (data not shown). Owing to the extensive radiation damage during data collection, however, the crystals had to be translated several times and data from the three crystals had to be merged together to obtain a 95.2% complete data set with an overall $R_{\rm sym}$ of 9.2%. The unfrozen XO crystals belong to space group $C222_1$, having unit-cell parameters $a$ = 118.6, $b$ = 165.3, $c$ = 156.4 Å.

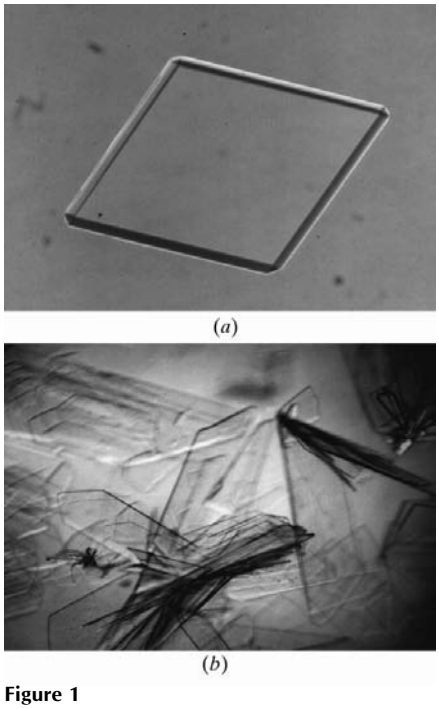

**Figure 1**
The two crystal forms of xanthine oxidoreductase: (*a*) 75 µm thick plate of XO, (*b*) 10 µm thick plates of XDH. The crystallization conditions are given in the text. Both crystalline enzyme forms contained all their cofactors, FAD, Mo-pterin and the two FeS clusters; when redissolved they showed specific catalytic activities comparable with those of the enzyme stock solutions.

Once flash-freezing conditions for the XO crystals had been established, a 97.9% complete data set was collected on a single crystal and was processed to 2.5 Å, with an $R_{\rm sym}$ of 5.9%. This crystal also belonged to space group $C222_1$, but its unit-cell parameters were slightly changed to $a$ = 117.8, $b$ = 167.7, $c$ = 154.5 Å.

Bovine XDH crystals belong to space group $C2$, but show pseudo-orthorhombic symmetry. Their unit-cell parameters are $a$ = 169.9, $b$ = 124.8, $c$ = 148.6 Å, $\beta$ = 90.9°. The resolution extends to 2.1 Å, with an overall $R_{\rm sym}$ of 8.1%.

The monoclinic space group of the XDH crystals may be generated by slight changes in the unit-cell axes and a shift in one unit-cell angle from 90 to 90.9° from the XO crystals. The Matthews parameters are 2.6 and 2.7 Å$^3$ Da$^{-1}$ for XO and XDH, respectively (Matthews, 1968), assuming one subunit per asymmetric unit in the XO crystals and a complete dimer per asymmetric unit in the XDH crystals (Table 1).

We are in the process of combining molecular replacement, multi-wavelength anomalous dispersion, multiple isomorphous replacement and multi-crystal averaging techniques in an effort to determine the crystal structures of the dehydrogenase as well as oxidase forms of bovine xanthine oxidoreductase, the most studied complex flavoprotein.

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