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Recapture of [S]-allantoin, the product of the two-step degradation of uric acid, by urate oxidase

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Abstract **Urate oxidase from *Aspergillus flavus* catalyzes the degradation of uric acid to [S]-allantoin through 5-hydroxyisourate as a metastable intermediate. The second degradation step is thought either catalyzed by another specific enzyme, or spontaneous. The structure of the enzyme was known at high resolution by X-ray diffraction of *I*222 crystals complexed with a purine-type inhibitor (8-azaxanthin). Analyzing the X-ray structure of urate oxidase treated with an excess of urate, the natural substrate, shows unexpectedly that the active site recaptures [S]-allantoin from the racemic end product of a second degradation step.**

Keywords: Urate oxidase; Uricase; Allantoin; 8-Azaxanthin; 5-Hydroxyisourate; *Aspergillus flavus*

1. Introduction

Urate oxidase (uricase; EC 1.7.3.3; or UOX) is a 135 kDa tetrameric enzyme of therapeutic interest implicated in the catalysed degradation of uric acid (Scheme 1). It catalyzes the first step of the degradation of uric acid **1** to 5-hydroxyisourate (5-HIU) **2**. The second degradation step leads to [S]-allantoin **3** (or [S]-ALN). UOX from *Aspergillus flavus* gene, cloned in a *Saccharomyces cerevisiae* strain, is produced by Sanofi-Aventis according to a patented process.

The first three-dimensional structure of extracted UOX was solved at a resolution of 2.05 Å in complex with 8-azaxanthin (8-AZA), a stabilizing inhibitor [1] in the *I*222 orthorhombic form. Following this first report and the preparation of recombinant UOX, several more accurate structures, in complex with 9-methyl urate or oxonate became available [2]. High-diffraction properties of the resulting diamond-shaped *I*222 crystals confirmed that these molecules were good analogues. Moreover, the structure of the ligand-free enzyme solved from a monoclinic form ($P2_1$) was also described as well as other polymorphs using the less analogous uracil or guanine as ligands [3] but diffracting at lower resolution. All these purine-type inhibitors share the same –NH(3)–CO(2)–NH(1)–CO(6)– motif in their six-membered ring. Other close compounds like cafein, theobromin or theophyllin whose NH are methylated, do not interact with UOX [4]. When ligand-free UOX crystals are soaked in a urate-containing solution, they immediately crack and dissolve, as a result of conformational changes during the in situ catalytic reaction. To get more information about the catalytic pathway and about the end of the reaction, the enzyme was first set to react in solution with urate, then set to crystallize afterwards. In all cases, the complex leads to the formation of the high resolution *I*222 orthorhombic crystals, an indication that stabilization occurs within the active site at the end of the reaction prior to crystallization. The present study aims at characterizing the content of the protein at that time.

2. Materials and methods

2.1. Crystallizations

Purified recombinant UOX from *A. flavus*, expressed in *S. cerevisiae*, was supplied by Sanofi-Aventis. All additives and chemicals used in this study (uric acid, polyethyleneglycol, etc.) were purchased from Sigma–Aldrich. The enzyme was incubated at a concentration of 15 mg/mL at pH 8.5 with an excess of uric acid (0.5–2 mg/mL). The solution was immediately set to crystallize following identical crystal growth conditions as previously described using the sitting-drop vapor diffusion method at room temperature [5]. 5–10 mg/mL protein in 50 mM Tris/HCl pH 8.5 in the presence of 5–8% w/v PEG 8000 and NaCl 0.05 M, led to orthorhombic crystals within 24–48 h. They grow to their full extension (~0.6 mm) within a week.

2.2. X-ray data collection and data processing

X-ray data collections were carried out at the ESRF BM14 beamline, at a wavelength of 0.972 Å and operating in a 16 bunch mode, using a MAR CCD detector; temperature was set to 277 K. Data were integrated by DENZO and scaled independently using SCALEPACK, both software are parts of the HKL package [6]. Cell parameters were refined by the post-refinement technique implemented in SCALEPACK. The merged intensity data were converted to structure-factor amplitudes by TRUNCATE [7] and put on an approximate absolute scale using the scale factor taken from a Wilson plot computed with data in the resolution range of 5 Å – d_{min} (see statistics in Table 1).

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Abbreviations: UOX, urate oxidase; 8-AZA, 8-azaxanthin; [S]-ALN, [S]-allantoin; 5-HIU, 5-hydroxyisourate; RMSD, root mean squares deviation

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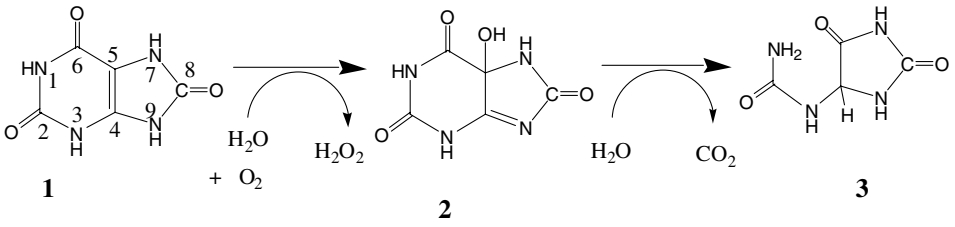

Scheme 1.

2.3. Protein structure solution

The structure was solved by Fourier synthesis using the calculated phases derived from the isomorphous structure taken from the Protein Data Bank (code 1R51 or 1R4S) after removing all the water molecules and the ligand.

Table 1
Data collection and refinements statistics

Data collection	
Space group	*I*222
Parameters (Å)	
a	80.36
b	96.09
c	105.37
Number of unique reflections	39454
Data redundancy	4.8
All data (10.9–1.75 Å)	
R_{sym} (%)	6.4
Mean $I/\sigma(I)$	18.7
Overall completeness (%)	98.1
Highest resolution shell (2.1–2.0 Å)	
R_{sym} (%)	28.7
Mean $I/\sigma(I)$	5.1
Completeness (%)	97.6
Refinements (SHELXL)	
Number of atoms	
UOX	2384
[S]-ALN	11
Solvent (H_2O)	168
Resolution range (Å)	10–1.76
Number of parameters/restraints	10259/9887
R factor (%), for 4σ observed data (number)	19.1 (33270)
R factor (%), for all data (number)	20.8 (39227)
Free *R* factor (%) for 7% of obs. data (number)	23.0 (2758)

	Value
Mean isotropic $\langle B \rangle$ values (Å^2)	
Main chain	18.7
Side chain	25.6
Water molecules	35.9
[S]-allantoin	28.0
Residuals in final Fourier map (min/max) (e$^-$)	−0.25/+0.40

RMS deviations	Number	Target/value
1–2 Bond length (Å)	2451	0.03/0.034
1–3 Bond-distance angles (Å)	3321	0.08/0.077
Planes (Å)	790	0.4/0.35
Non-zero chiral volumes (Å^3)	369	0.1/0.045

The structure refinements were carried out with the program SHELXL [8]. Throughout the course of refinements, 7% of the data was left out for cross validation (*R*-free).

The first SIGMAA 2$|F_o-F_c|$ map revealed the presence of an elongated density in the active site accurate enough to be readily shaped as [S]-allantoin. Subsequent maps were checked to remove significant errors in the model, and manual rebuildings were performed using the program O [9]. Alternations of rounds of positional plus restrained *B*-factor refinements followed by manual rebuilding using O were performed until model completion, including successive locations and refinements of water molecules. Final statistics on the model parameter refinements are given in Table 1. Coordinates are deposited with the Protein Data Bank (PDB code: 2FXL). Fig. 1 typically represents a $2F_o-F_c$ map around the ligand, located in the active site.

3. Results and discussion

Strategy for structural studies of an enzymatic mechanism usually aims at trapping the enzyme both in the ground- and transition states, using in the latter case inhibitors that mimic the expected geometry. Co-crystallization with the substrate analogs becomes a mandatory stage to line out the enzyme active site in its stable ground state. For that purpose, analogues that closely resemble uric acid, the natural substrate were pre-

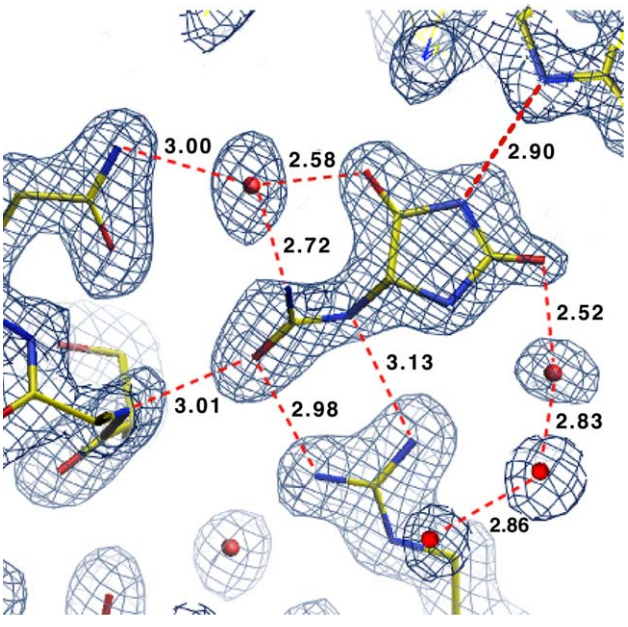

Fig. 1. Weighted electron density map of the active site cavity, calculated with theoretical phases, contouring is at 1.8 sigma above the background level. The [S]-ALN molecule is strongly H-bonded to both the side chains lining the active site and the water molecules that build a long spine in the access channel. Almost all the polar atoms of allantoin are involved in its binding scheme.