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Functional relevance of the internal hydrophobic cavity of urate oxidase

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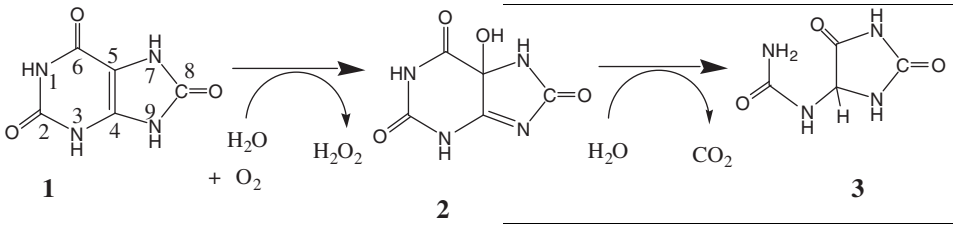
ABSTRACT

Urate oxidase from *Aspergillus flavus* is a 135 kDa homo-tetramer which has a hydrophobic cavity buried within each monomer and located close to its active site. Crystallographic studies under moderate gas pressure and high hydrostatic pressure have shown that both gas presence and high pressure would rigidify the cavity leading to an inhibition of the catalytic activity. Analysis of the cavity volume variations and functional modifications suggest that the flexibility of the cavity would be an essential parameter for the active site efficiency. This cavity would act as a connecting vessel to give flexibility to the neighboring active site, and its expansion under pure oxygen pressure reveals that it might serve as a transient reservoir on its pathway to the active site.

1. Introduction

Urate oxidase (uricase; EC 1.7.3.3; UOX) is a tetrameric enzyme belonging to the purine degradation pathway which catalyzes the oxidation of uric acid (1) to a metastable intermediate, 5-hydroxy-isourate (5-HIU) (2). The reaction needs molecular oxygen and releases hydrogen peroxide. 5-HIU is further transformed to S-allantoin (3) through a catalytic cascade.



The exact catalytic mechanism is still not fully understood, there is no cofactor nor metallic ion, the optimal pH is basic (between 8.5 and 9), and it is known for a long time that the oxygen atoms in H_2O_2 derive from those of O_2 [1–5].

UOX from *Aspergillus flavus* in presence of a competitive inhibitor like 8-azaxanthine crystallizes in the orthorhombic space group I222 with one monomer of 301 residues per subunit. The functional homotetrameric enzyme is built around the 2-fold crystallographic axes. The active site is located at the interface between two subunits folded around a tunnel. 8-azaxanthine is locked in the active site by a Arg 176-Gln 228 molecular tweezers [6,7].

The peroxohole located above the active site is usually filled by a catalytic water molecule W1 hydrogen-bounded to the side

Abbreviation: UOX, urate oxidase

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chains of Asn 254 and Thr 57* of a symmetric subunit. When UOX crystals are soaked in NaCl or in cyanide solution, the peroxohole exchanges this water molecule for a chloride ion or a cyanide [8,9].

A large internal cavity completely buried inside each monomer is located very close to the active site, with the residue Val 227 in frontier between the active site and the cavity. Indeed, Val 227 side chain lines the internal cavity while its main chain nitrogen is hydrogen-bonded to the ligand (either the inhibitor 8-azaxantine or the natural substrate uric acid). This internal cavity is void, meaning that no water can be seen in the electron density map. It is very hydrophobic, lined with almost 90% of carbon atoms. It is well known that internal cavities within proteins are crucial for conformational flexibility and domain motion [10,11] and accessibility for small ligands can only be granted through thermal motion [12]. Flexibility is thought to be necessary for functional efficiency.

To unravel the functional relevance of UOX internal cavity, we used a structural approach combining crystallography at room temperature under moderate anesthetic gas pressure and under high hydrostatic pressure. These two complementary approaches allow to determine the most flexible part of the protein in relation with internal cavity modifications. Indeed, the analysis of protein structures determined under moderate inert gas pressure have revealed that the main gas-induced structural modifications occur on the volume of cavities which bind the inert gas, their volumes expanding with the applied gas pressure [13,14]. On the other hand, the analysis of protein structures determined under high hydrostatic pressure have revealed that the main pressure-induced structural effects occur on the volumes of cavities which are reduced [15,16]. Activity assays in presence of inert gas and under high hydrostatic pressure have also been performed on UOX to establish the biological significance of these crystallographic findings. We also used crystallography under dioxygen pressure in order to visualize the oxygen binding site.

2. Methods

2.1. Crystallization and data collections

Purified recombinant UOX from *A. flavus*, expressed in *Saccharomyces cerevisiae*, was a gift from Sanofi-Aventis (Montpellier, France). UOX crystals were grown either by batch and hanging-drop methods using a 10–15 mg/ml solution of UOX, with an excess of 8-azaxanthine (purchased from Sigma–Aldrich, Lyon, France), in 50 mM Tris/acetate (pH 8) in the presence of 5–8% PEG 8000. This led to crystal in space group I222 with 1 monomer per asymmetric unit [7].

Crystallography under Xe and N_2O pressure and activity assays in presence of gas have been described in [17]. Crystallography under O_2 pressure has been described in [8] and uses the same experimental devices. Crystallography under high hydrostatic pressure and activity assays under high pressure have been described in [18]. All diffraction data (gas-less, under pressurized gas, ambient pressure and high hydrostatic pressure) have been collected at room temperature.

Diffraction data for two additional structures of UOX in complex with 8-azaxanthin, gas-less and under 4 MPa of dioxygen, were collected at room temperature at the BM14 beamline at the European Synchrotron Radiation facility (ESRF, Grenoble, France). Detector used was a MAR CCD detector. Data were indexed and integrated by *DENZO* and scaled independently and reduced using *SCALEPACK*, both programs from the HKL package [19]. Intensities were converted in structure factor amplitudes using *TRUNCATE* and structure refinements were carried out using *REFMAC* [20], all from the CCP4 package [21]. PDB entry 2IBA in which heteroatoms and alternate side-chain positions were removed was used as starting model for rigid body refinement. The graphics program *COOT* [22] was used to visualize electron density maps and for manual rebuilding. A summary of the X-ray data collection and refinement statistics is given in Table 1. Atomic coordinates and structure factors have been deposited at the PDB (entry code 4OP9 for gas-less UOX and 4OP6 for UOX under 4 MPa oxygen).

Table 1
Data collection and refinement statistics.

	Native	O_2 4 MPa
PDB entry	4OP9	4OP6
Parameters (Å)		
a	80.11	79.92
b	96.18	96.17
c	105.41	105.40
Resolution (Å)	1.60	1.65
Completeness (%)	98.8 (95.6)	100 (100)
Redundancy	5 (4.6)	5 (5)
Unique reflections	55375 (2674)	49104 (2417)
Rsym*	4.2 (36.4)	4.4 (37.3)
I/σ (I)	33.4 (2.9)	33.6 (3.4)
Rwork§	16.78	16.46
Rfree#	19.06	18.85
Mean B factor (Å^2)	22.78	22.62
r.m.s. ideality		
Length (Å)	0.009	0.009
Angle (°)	1.342	1.361

Data between parentheses correspond to the highest resolution shell. *Rsym = $\sum h,k,l \sum i|Ii(h,k,l) - \langle Ii(h,k,l)\rangle|/\sum h,k,l \sum i\ Ii(h,k,l)$, where $Ii(h,k,l)$ is the intensity of observed reflections and $\langle Ii\ (h,k,l)\rangle$ the weighted mean of all observations after rejection of outliers. §Rwork = $\sum|Fo| - |Fc|/\sum|Fo|$; indicates accuracy of the model. #Rfree is a cross validation residual using 5% of the native data which were randomly chosen and excluded from the refinement.

2.2. Cavity volumes calculations

Several programs are available for that purpose, they give slightly different results as the method is very sensitive to the geometric parameters but whatever the program in use, accurate comparative results are obtained as long as the same protocol is used throughout. In the present study, calculations of cavities volume were performed using *CASTp* with a sphere radius of 1.4 Å [23], and *VOIDOO* [24] with the parameters as described in [25]: primary grid spacing of 0.6 Å, grid spacing of 0.6 Å, and probe radius of 1 Å. The cavities volume in the different structures are given in Table 2. Room mean squares deviations (rmsd's) derived from bond lengths, angles, and chiral volumes rmsd's obtained from X-ray structures were found in all cases within the range 5–8% of the calculated volumes. Figures were prepared using *PYMOL* (DeLano Scientific, CA, USA).

3. Results

3.1. Crystallography and activity assays under inert gas pressure

Metabolically inert gases like Xe or N_2O were bound within the internal void cavity of UOX located close to the active site. The main structural modification in UOX-gas complexes was the expansion of the volume of the cavity, which increased with the applied pressure (Fig. 1A). The volume of the internal cavity in the gas-less UOX structure expanded with the presence of gas. The expansion induced by the mixture Xe:N_2O was greater than the expansion induced by Xe alone, itself greater than the expansion induced by N_2O alone (Table 2). In parallel, activity assays performed in presence of air, Xe, N_2O and Xe:N_2O in physiological conditions showed that the presence of gas within the internal