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Supporting Material

Structure-function perturbation and dissociation of tetrameric urate oxidase by high hydrostatic pressure

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MATERIAL AND METHODS

Enzyme assays and kinetics

Activity was measured at atmospheric pressure after overnight incubation at high pressure. Apo-UOX was incubated for 20 hours at specified pressures up to 200 MPa. After decompression, samples were centrifuged to remove the precipitate and the remaining soluble protein fraction was estimated by UV absorbance at 280 nm, using an extinction coefficient of 1.69 $mg^{-1}.cm^{-1}$ (1). UOX activity was determined spectrophotometrically by measuring the initial consumption rate of uric acid (2). UOX (1 μg/ml) was mixed with 36 μM uric acid in 50 mM Tris-HCl pH 8 buffer. The variation in uric acid concentration was followed at 292 nm (extinction coefficient of uric acid solution at 292 nm, ε_{292} = 12,200 $mol^{-1}.cm^{-1}$). The initial consumption rate, expressed in $mol.min^{-1}$, was then normalized by the amount of UOX introduced (in mg) to obtain the specific activity of the enzyme. These measurements were repeated with a substrate concentration of 150 μM.

Activity measurements under high pressure, using home-built stopped-flow apparatus (3), were carried out from 0.1 to 190 MPa at 298 K. Equal volumes of enzyme and substrate solutions were mixed in a high-pressure stopped-flow cell connected to a MOS-200 rapid-kinetics optical system from BioLogic (Grenoble, France). All experiments were performed at least in triplicate, with different concentrations of UOX (1-2 μg/ml) and substrate (36, 54, 72, 110 and 150 μM).

Native molecular mass determination

The molecular mass of UOX after a 20-hour incubation under pressure (Fig. S2) was determined by gel filtration using a Superose 12 FPLC column (Pharmacia) at 4°C

equilibrated with 50 mM Tris-HCl pH 8 buffer containing 150mM KCl at a rate flow of 0.5ml/min. Samples were centrifuged at 16,000 g for 40 min in order to remove aggregates, and 100µl were loaded onto the column. For calibration, the following molecular mass standards (Sigma) were used: (1) β-amylase (200,000), (2) alcohol dehydrogenase (150,000), (3) albumin (66,000), (4) carbonic anhydrase (29,000), (5) cytochrome C (12,400) and (6) aprotinin (6,500). The void and total volumes of 8 and 20.8 ml, respectively, were determined with cytidine and dextran blue dye for the calculation of the distribution coefficient K_{av}. As shown in Fig. S2, the molecular mass of pressure-treated UOX, 134 kDa, is not significantly different from that of native UOX (135 kDa).

SAXS experiments

Data were collected using a FReLoN (Fast-Readout, Low-Noise) CCD detector based on the Kodak KAF-4320 image sensor (512x512 pixels after 4x4 binning). The sample to detector distance was set at 1.73 m. The exposure time per frame was 0.3 s. Under each condition, 4 frames were recorded for the protein and 20 frames for the buffer. No radiation damage was observed. Data reduction is described in (4).

Various experiments were done at three protein concentrations: 17, 8.5 and 4.25 $mg.ml^{-1}$, in 10 mM sodium phosphate buffer, pH 8, either without inhibitor or with 8-aza in saturating concentrations.

HPMX experiments and structure comparisons

Data were integrated using *XDS* (5). Integrated intensities were scaled and merged using *SCALA* and refinements were carried out with *REFMAC* (6) both from the *CCP4* package (7). The graphics program *O* (8) was used to visualize the (*2Fobs – Fcalc)* and (*Fobs – Fcalc)* electron-density maps and for manual refitting.

REFERENCES

1. Conley, T. G. and D. G. Priest. 1980. Thermodynamics and stoicheiometry of the binding of substrate analogues to uricase. Biochem. J. 187:727-732.

2. Amounas, M., V. Magne, C. Innocent, E. Dejean, and P. Seta. 2002. Elaboration and chemical reactivity of enzyme modified ion exchanging textiles. Enzyme and Microbiol. Technol. 31:171-178.

3. Balny, C., J. L. Saldana, and N. Dahan. 1984. High-pressure stopped-flow spectrometry at low temperatures. Anal Biochem 139:178-189.

4. Bösecke, P. 2007. Reduction of two-dimensional small- and wide-angle X-ray scattering data. J. Appl. Cryst. 40:s423-s427.

5. Kabsch, W. 1993. Automatic processing of rotation diffraction data from crystals of initially unknown symmetry and cell constants. J. Appl. Cryst. 26:795-800.

6. Murshudov, G. N., A. A. Vagin, and E. J. Dodson. 1997. Refinement of macromolecular structures by the Maximum-Likelihood method. Acta Crystallogr. D53:240-255.

7. Collaborative Computational Project Number 4. 1994. The *CCP4* suite : programs for protein crystallography. Acta Crystallogr. D50:760-763.

8. Jones, T. A., J. Y. Zou, S. W. Cowan, and M. Kjeldgaard. 1991. Improved methods for building protein models in electron density maps and the location of errors in these models. Acta Crystallogr. A47:110-119.

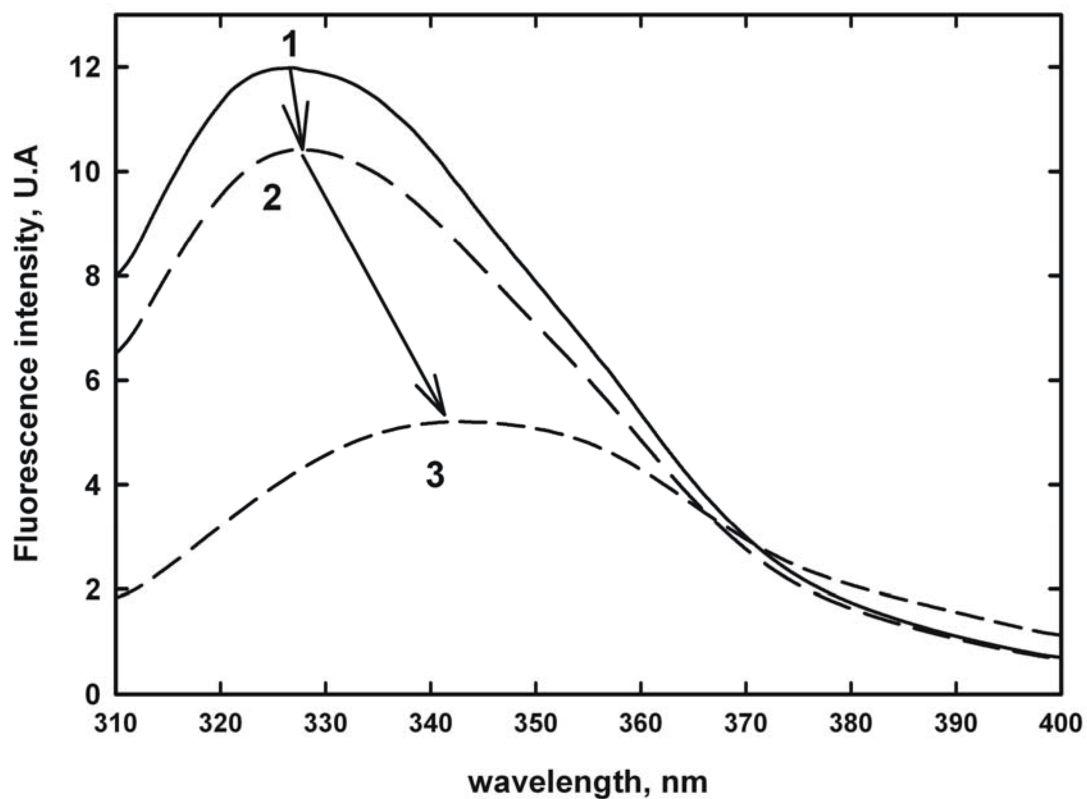

Figure S1. Representative view of apo-UOX fluorescence emission upon excitation at 295 nm as a function of hydrostatic pressure: (1) 1 MPa, (2) 150 MPa, (3) 350 MPa.

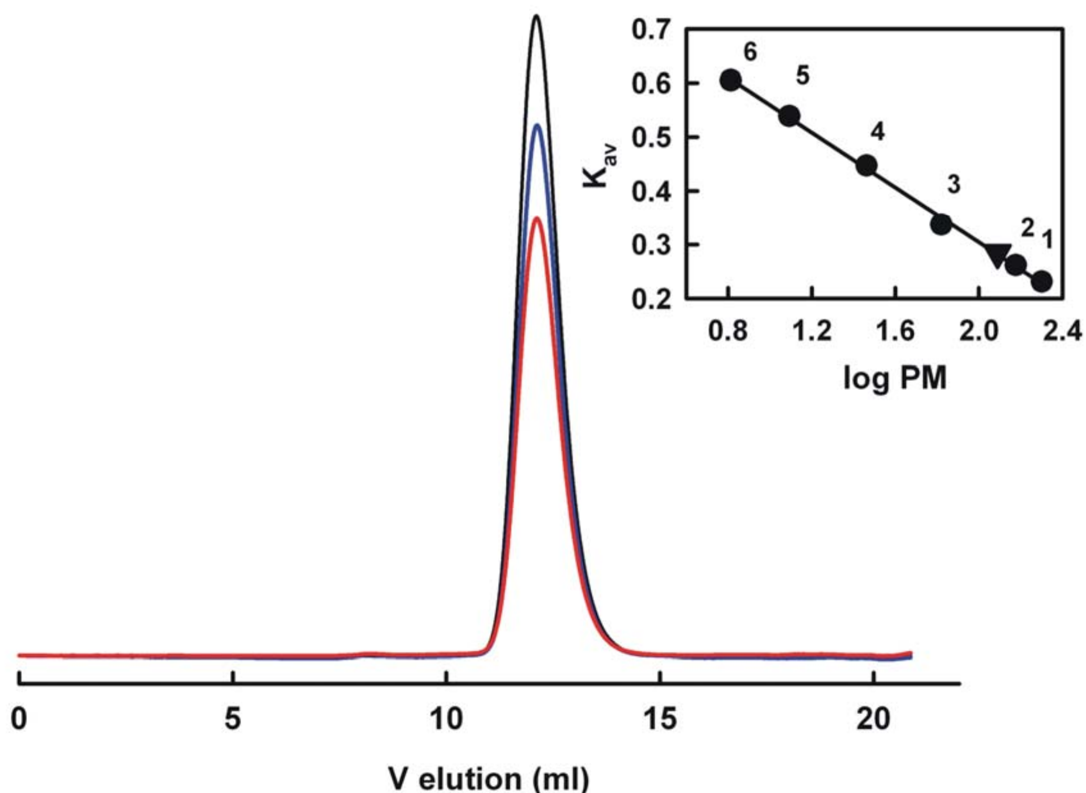

Figure S2. Evidence of the tetrameric state of the soluble UOX after pressure treatment. After 20h incubation under pressure (150 MPa (black), 175 MPa (blue), 185 MPa (red)), a sample of 100 µl UOX was applied on a gel-filtration Superose 12 10/300 GL column (Pharmacia). Chromatography was performed at a flow rate of $0.5 ml.min^{-1}$. (*Inset*) The molecular mass of the soluble pressure treated protein was determined using β-amylase, alcohol dehydrogenase, albumin, carbonic anhydrase, cytochrome C and aprotinin as calibration standards (●). From the measured distribution coefficient K_{av} of UOX (▼), a molecular mass of 134 kDa was determined.

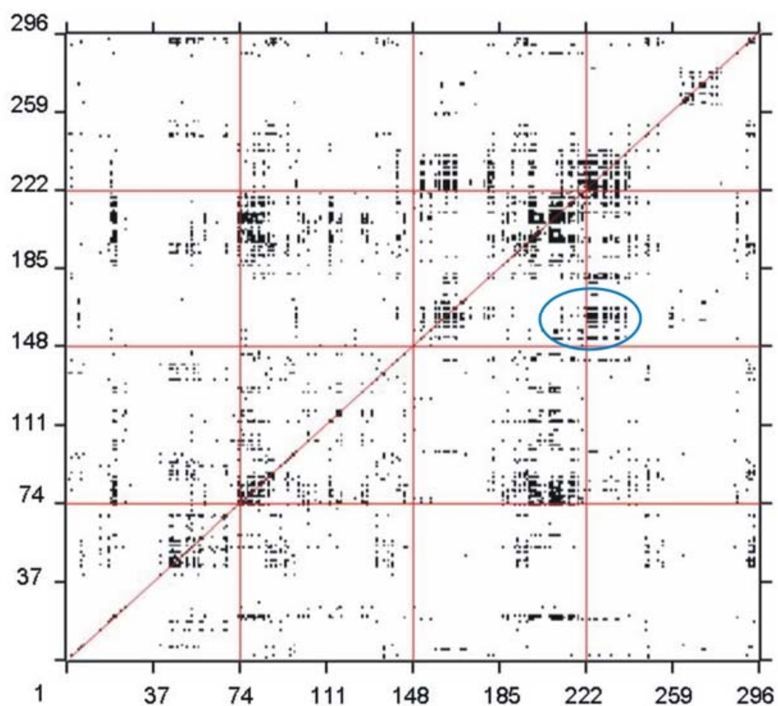

Figure S3. Correlation matrix between HP and AP structure, where the correlation factor is higher than 0.9 (very high correlation). A circle highlights correlated displacements between residues belonging to the active site (S154, T155, F159, F162, S226, V227, Q228) and residues belonging to the hydrophobic cavity (V151, L178, T180, V182, T215, F219, V227, T230, M234).

Table S1. Crystallographic data collection and refinement statistics.

Protein	Urate oxidase:8-aza complex	
Data collection		
Pressure (MPa)	1	150
Temperature (K)	277	277
Unit cell parameters (Å)		
a	80.00	79.70
b	96.24	95.87
c	105.57	104.99
Resolution range (Å)	14.90 - 1.85 (1.95 – 1.85)	19.94-1.80 (1.90 – 1.80)
No of unique reflections	30808 (4535)	36047 (5232)
R_{merge} (%) overall *	6.4 (33.1)	5.2 (26.2)
Completeness (%)	88.4 (89.3)	96.4 (96.6)
$I/\sigma(I)$	8.1 (2.0)	7.4 (2.5)
Redundancy	2.7 (2.6)	2.7 (2.6)
Refinements		
Resolution range (Å)	14.77 – 1.85	14.82 – 1.80
R_{work} (%)†	17.47	17.80
R_{free} (%)‡	20.46	21.78
Nb of atoms	2524	2519
Protein	2370	2368
Ligand/ion	12	12
Water	142	139
Thermal B factor (Å)^2	19.64	24.53
Protein	19.24	24.17
Ligand/ion	14.19	18.56
Water	26.91	31.27
r.m.s.d. from ideality		
Bond length (Å)	0.015	0.16
Bond angle (°)	1.608	1.669

Both structures have an orthorhombic unit cell; space group I222. The highest resolution shell is shown in parentheses. *R_{merge} is defined as $\sum_{h,k,l}\sum_i \left| I_i(h,k,l) - \overline{I_i(h,k,l)} \right| / \sum_{h,k,l}\sum_i I_i(h,k,l)$ where I_i(h,k,l) is the ith observation of reflection h,k,l and <I(h,k,l)> is the weighted mean of all observations (after rejection of outliers). $^{\dagger}R_{work}$ is defined as $\sum |F_o| - |F_c| / \sum |F_o|$ and indicates the accuracy of the model. $^{\ddagger}R_{free}$ is a cross-validation residual calculated using 5% of the native data which were randomly chosen and excluded from the refinement.