Large crystal growth by thermal control allows combined X-ray and neutron crystallographic studies to elucidate the protonation states in *Aspergillus flavus* urate oxidase

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Urate oxidase (Uox) catalyses the oxidation of urate to allantoin and is used to reduce toxic urate accumulation during chemotherapy. X-ray structures of Uox with various inhibitors have been determined and yet the detailed catalytic mechanism remains unclear. Neutron crystallography can provide complementary information to that from X-ray studies and allows direct determination of the protonation states of the active-site residues and substrate analogues, provided that large, well-ordered deuterated crystals can be grown. Here, we describe a method and apparatus used to grow large crystals of Uox (*Aspergillus flavus*) with its substrate analogues 8-azaxanthine and 9-methyl urate, and with the natural substrate urate, in the presence and absence of cyanide. High-resolution X-ray (1.05–1.20 Å) and neutron diffraction data (1.9–2.5 Å) have been collected for the Uox complexes at the European Synchrotron Radiation Facility and the Institut Laue-Langevin, respectively. In addition, room temperature X-ray data were also collected in preparation for joint X-ray and neutron refinement. Preliminary results indicate no major structural differences between crystals grown in H₂O and D₂O even though the crystallization process is affected. Moreover, initial nuclear scattering density maps reveal the proton positions clearly, eventually providing important information towards unravelling the mechanism of catalysis.

Keywords: urate oxidase; neutron and X-ray crystallography; crystal growth; phase diagram; H–D exchange; protonation states

1. INTRODUCTION

Urate oxidase (uricase or Uox, EC 1.7.3.3) is an enzyme involved in purine metabolism. It catalyses the oxidation of uric acid to 5-hydroxyisourate, a metastable intermediate, which is further degraded to allantoin. Humans and other primates lack a functional Uox, and therefore the final product of purine metabolism is uric acid. It has been speculated that this confers an evolutionary advantage to long-lived animals like primates owing to the anti-oxidant properties of urate, but the downside is that in certain conditions high urate levels (hyperuricaemia) may lead to crystallization of uric acid, as in gout.

Uox from *Aspergillus flavus* has been commercialized by Sanofi-Aventis as a protein drug to treat

hyperuricemic conditions such as the tumour lysis syndrome, but the catalytic mechanism of Uox is still poorly understood. Several X-ray structures have been solved for Uox in the complex with various uric acid analogues (Colloc'h *et al.* 1997, 2007; Retailleau *et al.* 2004, 2005; Gabison *et al.* 2006, 2008), and a putative mechanism for the oxidation of uric acid has been proposed. However, many of the key questions are related to the protonation state of the substrate during the reaction. Uric acid has four potentially acidic protons; this gives rise to four different monoanions and six dianions. To be able to describe the mechanism, it is necessary to know which of the anions is the real substrate and how the hydrogen bonding network in the active site is arranged. This information is to date unavailable.

The most common method to study enzymatic mechanisms from a structural point of view is X-ray crystallography. As it is based on the scattering of

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X-rays from electrons, hydrogen atoms with only one electron are particularly difficult to observe. Even in the highest resolution X-ray structures of proteins determined thus far, not all the hydrogens are visible in the electron density maps. For example, in the 0.66 Å resolution X-ray structure of human aldose reductase (Howard *et al.* 2004), around 54 per cent of the hydrogen atoms were identified. Whether a hydrogen atom is observed or not is highly dependent on the atomic displacement factor (or B-factor) of the atom it is bound to, which in practice means that many of the biologically more interesting polar hydrogens in the side chains are not observed, even at subatomic resolution. The same limitation applies to non-covalently bound ligands in which the polar hydrogen atoms are rarely as ordered as, for example, main-chain amide hydrogens.

The protonation states of some side chains, such as glutamate or aspartate, can be inferred by comparing the C–O bond lengths in the carboxyl moiety using high-resolution X-ray data (Ahmed *et al.* 2007; Fisher *et al.* 2008). A protonated carboxyl group has a clearly shorter bond length to one of the oxygens, while in an ionized carboxylate the lengths are equal. Engh & Huber (1991) quote 1.249 (19) Å for the COO[−] bond distance and 1.208 (23) and 1.304 (22) Å for the C=O and C–OH bond distances, respectively. In order to observe this difference reliably, the data-to-parameter ratio has to be sufficient for unrestrained refinement to avoid biasing the atomic coordinates by bond-length restraints (e.g. 6 : 1 in the case of concanavalin A structure solved to 0.94 Å resolution by Deacon *et al.* (1997)). It is also necessary to perform a full matrix inversion to estimate the standard deviations of the refined coordinates in order to judge whether the bond-length differences are significant (Cruickshank 1999). This method can also be applied to histidines, albeit with less reliability owing to the more complicated π -bond system. This approach is not very helpful in the case of Uox because the relation between the protonation state of the ligand and the bond lengths is not known with certainty. The changes are also likely to be even smaller than in the case of histidine.

One method to observe the protons more directly is nuclear magnetic resonance (NMR) spectroscopy. The ¹³C resonances of carboxylates or ¹⁵N resonances of histidine change as a function of protonation, so an NMR titration in which these resonances are observed can determine the pK_as of individual residues and hence their protonation states at the pH of interest. The major limitation of NMR is that the molecule has to tumble sufficiently rapidly in solution, which imposes a practical size limitation. As the size of the molecule increases, the spectra grow more complicated and elaborate labelling schemes are required to resolve and interpret them. Uox is a tetramer in solution, with a total molecular weight of approximately 137 kDa; a size certainly very challenging for NMR.

The most unambiguous method for determining the proton positions in the protein is neutron crystallography (Blakeley *et al.* 2008; Niimura & Bau 2008). As neutrons are scattered from the atomic nuclei rather than the electrons, the scattering lengths of hydrogen and the other elements commonly found in proteins

(i.e. carbon, nitrogen, oxygen, sulphur) are of roughly equal magnitude. This ‘high visibility’ of hydrogen with neutrons allows the positions of the protons to be determined at much lower resolutions (less than 2.5 Å) than are necessary with X-rays. Despite this clear benefit of neutron crystallography, currently only around 30 neutron macromolecular structures have been deposited in the Protein Data Bank (<http://www.rcsb.org/pdb>). This is due to various difficulties associated with neutron crystallography that make a successful experiment more demanding than its X-ray counterpart. The major limitation is that even the most intense neutron sources are very weak compared with laboratory X-ray sources, not to mention synchrotrons. As a consequence, large and well-ordered crystals are required in order to gain sufficiently high reflection intensities; the actual crystal volume required being dependent on the unit-cell volume as the total scattering of an individual reflection is proportional to the inverse third power of the unit-cell volume.

A further complication in neutron crystallography is the large incoherent scattering from hydrogen (¹H) (80.27 barns (8.027 × 10^{−23} cm²)). This scattering does not produce any interference effects or Bragg peaks but rather a uniform background radiation. Consequently, for macromolecules, in which around 50 per cent of the atoms are hydrogens, an extremely large incoherent signal is observed that severely limits the signal-to-noise ratio of the data, and hence the resolution limit. On the other hand, deuterium (²H) has a much lower incoherent scattering cross section (2.05 barns (2.05 × 10^{−24} cm²)) and so it is advantageous to exchange the hydrogen atoms in the crystal for deuterium. In practice, this has been achieved by growing crystals from, or by soaking the crystals in, D₂O solutions. This has the effect of exchanging solvent-accessible groups (e.g. amino and hydroxyl groups) that typically amounts to 15–20% of the solvent-accessible hydrogen content of the macromolecule (Myles 2006). Hydrogen atoms attached to carbon atoms remain unexchanged. The only method to replace all the hydrogen atoms is to produce a completely perdeuterated macromolecule *in vivo* (Berns 1963; Gamble *et al.* 1994; Shu *et al.* 2000; Hazemann *et al.* 2005). To achieve this, the macromolecule must be expressed in cells grown in deuterated media, so that perdeuterated macromolecules can be synthesized. This is most convenient in bacterial systems such as *Escherichia coli*. Even though yeasts such as *Pichia pastoris* can be adapted to deuterated conditions, the level of perdeuteration is lower (up to 81% of the non-exchangeable protons in Morgan *et al.* (2000)). This is not always possible for non-recombinant proteins, as higher eukaryotic organisms cannot survive in fully deuterated conditions.

Whether the sample is hydrogenated or perdeuterated, optimization of crystal volumes remains a critical step towards the success of neutron protein crystallographic studies. For hydrogenated proteins, soaked or grown in D₂O, H–D exchange alters the physicochemical properties of protein solutions and affects the crystallization process in a significant way

(Budayova-Spano *et al.* 2000). For instance, it has been found that hydrogenated lysozyme, BPTI and α -amylase have a lower solubility in D₂O than in H₂O and our solubility measurements with hydrogenated recombinant Uox complexed with 8-azaxanthine show the same tendency (Budayova-Spano *et al.* 2007). It has been suggested that stronger attractive protein–protein interactions and lower protein solubility observed in D₂O (Gripon *et al.* 1997; Budayova-Spano *et al.* 2000) result from an enhanced hydrophobic effect in heavy water compared with light water (Kresheck *et al.* 1965; Bonneté *et al.* 1994).

During crystallization, the parameters characterizing the solution may change with time, and the system will follow some path on the phase diagram in order to reach the conditions for nucleation—the initial act of phase separation. The rate at which changes occur is important: if high supersaturation is reached too rapidly, many nuclei are formed before the concentration of protein is depleted significantly (Luft *et al.* 1994). The remaining protein is then insufficient to sustain the growth of all these nuclei to a usable size. The possible remedies are to approach supersaturation more slowly, to use a micro- or macrocrystal as a seed in a less supersaturated solution or to actively change solution conditions after the formation of the first few nuclei. Fast growth also risks the formation of crystals with poor morphology (De Mattei *et al.* 1992). This suggests that the growth of large macromolecular crystals suitable for neutron diffraction analysis should benefit from a systematic study of the phase diagram under deuterated crystallization conditions.

Even though the electronic structure of the protein remains unchanged upon isotopic substitution, the vibrational frequencies of chemical bonds do change. Most notably, the hydrogen bonds, which are crucial for protein structure and function, effectively become stronger. The acid dissociation constants (pK_a s) of functional groups also change. This may, in principle, cause changes in the structure of the protein (Liu *et al.* 2007), particularly if the effective pH changes. Therefore, it is important to solve also the X-ray structure of the crystals used for neutron crystallography.

This is not the only reason to collect X-ray data from the same crystal used for the neutron experiment. Neutron crystallographic refinement is plagued by a low data-to-parameter ratio because there are almost twice as many atoms to refine for a given number of reflections compared with X-ray crystallography. As the resolution of the neutron diffraction data is usually limited to approximately 2 Å, combining the information from the X-ray amplitudes to the refinement target function (Adams *et al.* 2002), as implemented, for example, in the program suite PHENIX, significantly improves the quality of the neutron maps. The heavier atoms contribute to both the neutron and X-ray amplitudes, which means that the X-ray map can be used to ‘bootstrap’ the heavy atom positions in the neutron map. It is imperative that the X-ray and neutron data are collected from the same crystal to ensure that the unit cell is the same and at the same temperature so that the B-factors are the same.

X-ray data collection at bright synchrotron sources is difficult at ambient temperature, especially at atomic resolution, because of radiation damage. Even though

atomic resolution data are hardly necessary for the purposes of joint refinement, it is certainly helpful for identifying subtle changes caused by deuteration, as well as the characterization of possible defects of the crystalline order, as discussed below.

2. MATERIAL AND METHODS

2.1. Sample preparation

Recombinant hydrogenated Uox from *A. flavus* expressed in *Saccharomyces cerevisiae* (or rasburicase; EU trade name Fasturtec, USA trade name Elitek) was purified and supplied to us by Sanofi-Aventis. The protein solution was exchanged with buffered D₂O (50 mM Tris–DCl pD 8.5, 100 mM NaCl) containing the substrate of interest in large excess (0.5–2 mg ml^{−1}). The excess substrate was then eliminated by gel-filtration chromatography, using the same D₂O buffer. The different protein complexes were then concentrated to 10 mg ml^{−1}. The substrate analogues 8-azaxanthine and 9-methyl uric acid as well as the natural substrate, uric acid, and all the buffers, salts, poly(ethylene glycol)s (PEGs) and additives used in this study were purchased from Sigma-Aldrich. Prior to dissolution, proper amounts of salts, PEGs, buffers and additives were dissolved in heavy water (Euriso-top, 99.92% D₂O) to obtain solutions with the concentrations required for crystallization. The pD of the buffers was adjusted with NaOD (Euriso-top, 99% D) and DCl (Euriso-top, 99.8% D) according to the formula $pD = pH_{meas} + 0.3314n + 0.0766n^2$, where $n = \%D_2O$ (Lumry *et al.* 1951). In all the crystal-growth experiments, initial crystallization mixtures were obtained using batch and/or dialysis techniques (Ducruix & Giegé 1992). Before starting the experiment, crystallization mixtures were centrifuged and filtered to remove all solid particles (precipitate, dust or nuclei).

Finally, crystals of about a few tens of micrometres in size, which had been nucleated previously in crystallization batch containing 5 per cent PEG 8000, 100 mM NaCl, 10 mg ml^{−1} protein and 100 mM Tris–HCl pD 8.5, were used as seeds and their size and quality were further improved using a temperature-control device developed at EMBL Grenoble (Budayova-Spano *et al.* 2007). Therefore, large crystals of Uox, cocrystallized with its substrate analogues 8-azaxanthine and 9-methyl uric acid as well as with the natural substrate in the presence of cyanide (0.5–2 mg ml^{−1}), and soaked with the natural substrate in the absence of cyanide, diffracting to high resolutions were obtained via knowledge of the phase diagram described previously (Budayova-Spano *et al.* 2007).

2.2. Crystal growth through phase-diagram investigation

The growth of crystals from a protein solution requires the existence of a phase transition, which allows the protein state to be manipulated between at least two thermodynamic phases: soluble and crystalline. Crystal nucleation and growth arise on the boundary between these two phases and are governed by subtle physico-chemical effects. The different zones of a two-dimensional phase diagram are illustrated schematically

for the case of a Uox complex that has direct solubility (protein solubility increases with temperature) in figure 1. Even though the division into these zones is qualitative, the behaviour of the system upon temperature change can be described by the following scenarios, which also serve as a guide for the appropriate conditions to grow seeded Uox crystals.

- (i) The seeded crystals grow and no spontaneous nucleation is observed. This corresponds to the metastable zone, where the supersaturation level is too low for nucleation, so that no new crystals form in any reasonable amount of time (figure 1).
- (ii) The seeded crystals dissolve. This corresponds to the zone of undersaturation (bottom right in figure 1). The temperature is reduced to increase the supersaturation until the dissolution of seeds is stopped and saturation and crystal growth are attained.
- (iii) The seeded crystals grow and further nuclei form in the crystallization solution. This corresponds to the zone of spontaneous nucleation, where the supersaturation is large enough that spontaneous nucleation is observable (figure 1). The temperature is increased to decrease the supersaturation until the formation of new nuclei is stopped and the growth of seeds is maintained.
- (iv) Disordered structures such as aggregates or precipitates form, which may prevent the growth of seeded crystallites as well as the formation of new nuclei. This corresponds to the precipitation zone, where the supersaturation is so large that aggregates and precipitates form faster than crystals (top left in figure 1). We could demonstrate the reversibility of the formation of these precipitates by their dissolution after decreasing the supersaturation level using temperature as a variable under the studied crystallization conditions.

The principle of the method for promoting crystal growth of different Uox complexes is schematized in figure 1. The crystallization solution with a volume of 200 μl containing 5 per cent PEG 8000, 100 mM NaCl, 8 mg ml⁻¹ Uox complex, 100 mM Tris-HCl pH 8.5 is seeded with corresponding Uox-complex crystals (usually 5–50 μm in size) at some point in the metastable zone or on the solubility curve. The corresponding seeds are nucleated in the apparatus (§2.3) by a cocrystallization approach (§3.3). By using only one seed, the size of the seed increases during growth and the result is a single large crystal. The growth of the crystals is maintained within the metastable zone for as long as possible by temperature variations just after the crystal–solution equilibrium is achieved (shown by blue arrows representing changes in temperature in figure 1).

2.3. Crystallization set-up

A semiautomated protein crystal-growth system for the investigation of the phase diagram and controlled crystal

Figure 1. Phase diagram illustrating the control process for crystal growth in the metastable zone in the case of a protein with direct solubility.

growth has been described previously (Budayova-Spano *et al.* 2007). It includes an inverted Leica DM IRB HC microscope, a Leica DFC 280 digital video camera, a computer (Intel Pentium 4 2.6 GHz processor, 512 MB RAM), a proportional–integral–derivative electronic temperature controller and a crystal-growth apparatus produced in-house. The crystal-growth apparatus is incorporated onto the stage of the microscope. The crystallization solution (25–1000 μl) is poured into a specially designed quartz cell with an optical bottom covered with a quartz air-tight cap, which is attached to a brass support incorporating a single well or several wells maintained at the same temperature. The accessible temperature range is from 233 to 353 K, with the temperature controlled by Peltier elements to an accuracy of 0.1 K. The cooling system of the Peltier elements helps to improve temperature regulation. In order to prevent condensation, particularly at low temperatures, a circuit of dry air is included. The crystal-growth apparatus is mounted onto the microscope table in such a way that the digital video camera of the microscope can view the different wells. The computer is equipped with the software program LEICA IM500, which allows visualization and measurement of crystals, image acquisition, processing and storage. The temperature is controlled by a program developed in-house and written with LABVIEW, which allows step-by-step, gradient or custom temperature variation. The temperature controller, as well as the corresponding software, was conceived to simultaneously control two crystal-growth experiments (i.e. with two crystal-growth apparatuses). In this way, one can work simultaneously at two different temperatures, for example. To facilitate the extraction of protein crystals after growth without causing any mechanical damage to the protein crystal, a micromanipulator (Eppendorf TransferMan NK2) has been added to the apparatus. The main part of the micromanipulator, the digital box with a joystick, enables the quartz capillary to be displaced inside the quartz cell containing the crystallization solution and the crystals with a precision of approximately 40 nm along each of the three axes.

Table 1. Data-processing statistics for the neutron Laue datasets collected from the different Uox complexes at ambient temperature on LADI-III at the ILL. (The values in bold type indicate the resolution limit of the dataset in question.)

	8-azaxanthine	9-methyl urate	urate + cyanide	urate
λ_{range} (Å)	3.25–4.35	3.2–4.25	3.2–4.2	3.25–4.15
λ_{centre} (Å)	3.83	3.76	3.77	3.79
crystal volume (mm ³)	4	2	1.5	1
number of images/number of crystal settings/ exposure time	35/3/4 h	5/1/12 h + 2/1/8 h	21/2/6 h	24/3/24 h + 5/1/6 h
resolution (Å)	61.43– 1.9 (2.00–1.9)	34.84– 2.5 (2.64–2.5)	38.32– 2.2 (2.32–2.2)	40.06– 2.3 (2.42–2.3)
R_{merge}	0.141 (0.236)	0.125 (0.174)	0.117 (0.204)	0.133 (0.190)
R_{pim}	0.049 (0.108)	0.096 (0.167)	0.065 (0.134)	0.060 (0.125)
number _{observable}	135 680 (6189)	14 274 (833)	45 636 (3514)	52 171 (2888)
number _{unique}	23 362 (2069)	8089 (686)	14 554 (1600)	13 179 (1328)
I/σ	9.2 (2.6)	5.4 (2.0)	8.3 (1.8)	7.9 (2.2)
completeness (%)	73.2 (45.2)	28.7 (17.0)	70.6 (53.8)	72.2 (50.6)
multiplicity	5.8 (3.0)	1.8 (1.2)	3.1 (2.2)	4.0 (2.2)

2.4. Data collection

The 100 K X-ray diffraction data were collected at the European Synchrotron Radiation Facility in Grenoble, using beamlines ID14-1 (urate, urate with CN[−]), ID14-2 (8-azaxanthine) and ID23-1 (9-methyl urate). The ambient temperature X-ray data were collected with a Xenocs Genix copper anode sealed tube source and an MAR345 image-plate detector at the European Molecular Biology Laboratory Grenoble Outstation. The X-ray data were processed with the XDS package (Kabsch 1993). Refinement against the X-ray data was performed in REFMAC5 (Murshudov *et al.* 1997) and manual model building in COOT (Emsley & Cowtan 2004).

The neutron data were collected at 293 K using the Laue diffractometer LADI-III at the Institut Laue-Langevin (ILL) in Grenoble (Blakeley *et al.* 2008). LADI-III is a recent replacement (March 2007) for the LADI-I instrument that has been used to collect data from perdeuterated crystals as small as 0.15 mm³ (Hazemann *et al.* 2005). The LADI-III instrument is equipped with a large neutron image-plate detector mounted on a cylindrical camera, which completely encircles the sample. An improved reading system located internally provides a threefold gain in neutron detection with respect to the LADI-I instrument (Wilkinson *et al.* 2007). Crystals were mounted in quartz capillaries and sealed with wax. Data were then collected using quasi-Laue methods in order to provide a rapid survey of reciprocal space while reducing background scattering and reflection overlap compared with the use of the full white beam. An Ni/Ti multi-layer wavelength band-pass filter was used to select the wavelength range ($\delta\lambda/\lambda_{\text{centre}} = 25\%$) and wavelength (λ_{centre}) best suited to the sample. The crystals were mounted on a goniometer head along the cylindrical drum axis and were rotated around this axis by 7° for each successive image. The neutron beam, which enters and leaves via opposed holes in the cylinder, interacts with the crystal to produce Bragg reflections, which are recorded on the neutron image plates mounted on the inside cylindrical surface. The

wavelength ranges with corresponding wavelengths (λ_{centre}) best suited to the sample, the number of diffraction images recorded from a given number of crystal settings in order to fill in the blind region, as well as the exposure time per image for each Uox complex studied are summarized in table 1.

The observed Bragg reflections were indexed and integrated using LAUEGEN (Campbell *et al.* 1998). The program LSCALE (Arzt *et al.* 1999) was used to derive the wavelength-normalization curve using the intensities of symmetry-equivalent reflections measured at different wavelengths. The data were then scaled and merged using SCALA (Collaborative Computational Project, Number 4 1994). Full statistics for processing of the neutron and X-ray datasets are provided in tables 1 and 2, respectively. Figures were prepared with PyMOL (DeLano Scientific LLC, Palo Alto, CA, USA).

3. RESULTS AND DISCUSSION

3.1. Deuteration

As discussed above, replacing H by D, known as deuteration, is a powerful method for changing the scattering contrast of specific parts of a macromolecule and also for enhancing its scattering properties. Water and exchangeable H atoms in proteins can be substituted by soaking crystals in D₂O mother liquor. To substitute the remaining H atoms (perdeuteration), the protein has to be expressed in a deuterated growth medium. Perdeuteration offers several advantages, such as the use of smaller crystal volumes, the ability to collect data from larger and more complex systems, shorter data collection times and potentially higher resolution data. Perdeuteration, however, is not always a possibility, especially for non-recombinant proteins.

In the case of Uox, hydrogenated protein was used to grow crystals under deuterated crystallization conditions. By growing the crystals directly in D₂O, the percentage deuteration at exchangeable hydrogen positions in the protein is higher (approaching 80% of accessible protons) than by the vapour-diffusion

Table 2. Data-processing statistics for the X-ray datasets collected from the different Uox complexes at cryo- and ambient temperatures. (The values in bold type indicate the resolution limit of the dataset in question.)

	8-azaxanthine, ID 14-2 (100 K)	8-azaxanthine, GeniX (298 K)	9-methyl urate, ID 23-1 (100 K)	9-methyl urate, GeniX (298 K)	urate+cyanide, ID 14-1 (100 K)	urate+cyanide, GeniX (298 K)	urate, ID 14-1 (100 K)	urate, GeniX (298 K)
resolution (Å)	50– 1.1 (1.13–1.10)	50– 1.92 (2.04–1.92)	50– 1.2 (1.23–1.20)	20– 2.0 (2.14–2.0)	50– 1.19 (1.22–1.19)	20– 2.0 (2.14–2.0)	50– 1.06 (1.09–1.06)	20– 2.0 (2.14–2.0)
R_{merge}	0.049 (0.413)	0.042 (0.144)	0.065 (0.496)	0.158 (0.534)	0.043 (0.671)	0.040 (0.184)	0.037 (0.369)	0.040 (0.145)
I/σ	11.94 (2.93)	19.88 (8.53)	10.86 (2.62)	10.74 (3.11)	14.65 (2.20)	21.54 (7.33)	15.71 (2.77)	23.56 (9.54)
completeness (%)	99.1 (99.7)	96.5 (84.2)	99.7 (99.7)	96.9 (84.3)	98.9 (90.0)	95.7 (82.5)	98.3 (83.4)	97.5 (87.0)
multiplicity	3.6 (3.5)	3.5 (3.2)	3.6 (3.4)	3.8 (3.3)	3.9 (3.4)	3.5 (3.1)	3.7 (2.4)	4.2 (3.6)
R_{work}	0.138 (0.211)	0.166 (0.242)	0.183 (0.365)	0.174 (0.34)	0.142 (0.258)	0.196 (0.250)	0.135 (0.238)	0.153 (0.200)
R_{free}	0.152 (0.215)	0.201 (0.356)	0.210 (0.355)	0.225 (0.45)	0.160 (0.279)	0.236 (0.310)	0.149 (0.258)	0.182 (0.280)
FOM	0.925	0.872	0.836	0.833	0.912	0.835	0.921	0.886

method (Habash *et al.* 1997). This improves the signal-to-noise ratio of the data and hence the resolution limit by significantly reducing the hydrogen incoherent scattering contribution to the background.

In the presence of different inhibitors, the crystal form of Uox has a body-centred orthorhombic symmetry (*I*222) and one of the largest primitive unit-cell volumes ($a = 80$, $b = 96$, $c = 106$ Å) and molecular weights (137 kDa for the homotetramer) so far successfully studied with neutrons. The preliminary refinements against the X-ray data, both the high-resolution data at 100 K and the ambient temperature data, indicated that the changes owing to D₂O exchanges were minimal. For example, the $C\alpha$ root mean square deviation (r.m.s.d.) between the 8-azaxanthine structure in H₂O (PDB-ID 2IBA; Colloc'h *et al.* 2007) and our structure in D₂O was 0.06 Å.

3.2. Combined use of the seeding and temperature control in crystallization

The knowledge of the phase diagram of Uox with different substrate analogues allowed large crystals to be obtained, thanks to a device and a methodology that combine the use of temperature control with seeding to drive the process of crystallization that has been developed at the EMBL Grenoble (Budayova-Spano *et al.* 2007). They allow the manipulation of the kinetics of the crystallization process, taking advantage of generic features of the phase diagram. Crystal growth is promoted by keeping the crystallization solution metastable (figure 1). This is achieved by regulating the temperature of the crystallization solution using control parameters determined *in situ* during the growth process. Knowledge of the phase diagram and the ability to control the temperature to drive the process of the crystallization allow us to tailor crystallization experiments to search for conditions that lead to crystals of a desired crystalline form, quality and size. The final crystal volumes obtained in this way for Uox-8-azaxanthine complex, 9-methyl urate complex, urate complex with cyanide (figure 2*a*) and urate complex were 4, 2, 1.5 and 1 mm³, respectively, and yielded high-quality neutron and X-ray diffraction data (tables 1 and 2). Figure 2*b* shows a typical neutron Laue diffraction pattern obtained from a 4 mm³ crystal of urate complex with 8-azaxanthine, which diffracted to 1.9 Å resolution.

The main advantage of the employed method was that the volume and composition of the crystallization solution remained constant during the entire process of phase-diagram investigation and crystal growth. The only variable in our system was the temperature, which is often ignored as an optimization variable and/or is poorly controlled. The use of temperature as an optimization variable requires more precise and rapid control than, for example, an incubator set-up can offer. The temperature within incubators or cold rooms is rarely uniform and the significant heat capacity of an incubator compared with the crystallization vessel does not allow well-controlled temperature gradients. In addition, the described instrument allows for simultaneous *in situ* regulation of the

temperature and observation by optical microscopy of the crystallization solution. In our scheme, the use of temperature control and seeding were combined to drive the process of the crystallization. Both of these techniques individually are powerful tools for the separation of nucleation and growth.

Seeding is a powerful tool for controlled crystal growth, in which previously nucleated crystals are introduced into a crystallization solution equilibrated at lower levels of supersaturation, thus favouring slow ordered growth of large crystals. Seeding has been critical for obtaining diffraction-quality crystals for many structures (Bergfors 2003). On the other hand, temperature is also an important variable in biological macromolecule and small-molecule crystallization (Boistelle & Astier 1988; Ducruix & Giegé 1992; Lorber & Giegé 1992; Christopher *et al.* 1998) as it governs the balance between enthalpy and entropy effects on free energy, which are typically comparable in magnitude. Depending on whether crystallization is enthalpy-driven or entropy-driven, proteins may become either more soluble at higher temperatures (direct solubility) or less soluble at higher temperatures (reverse solubility). In addition, temperature change can provide precise, quick and reversible control of the relative supersaturation levels of crystalline solutions. Temperature control is non-invasive and can be used to manipulate sample solubility and crystallization without altering reagent formulation. Temperature influences nucleation and crystal growth by affecting the solubility and supersaturation of the sample and so can be used to carefully manipulate crystal nucleation and crystal growth. Temperature can affect different phases and growth mechanisms and then induce solution-mediated phase transitions (Boistelle & Astier 1988; Lorber & Giegé 1992; Budayova 1998; Veessler *et al.* 2003). On the other hand, temperature also affects the quantity, size and quality of the crystals. It can be used to dissolve smaller crystals for the benefit of larger ones. By analogy with what is known from the small-molecule field, the Ostwald ripening mechanism was proposed to account for this phenomenon (Ng *et al.* 1996; Boistelle & Astier 1988). In contrast to phase transitions, it concerns only crystals of the same composition and structure, i.e. crystals of the same phase. Finally, temperature can also be used to etch or partially dissolve and then grow back the crystal in an attempt to improve the crystal volume and quality (Budayova-Spano *et al.* 2007).

3.3. CocrySTALLIZATION versus soaking

CocrySTALLIZATION and soaking are two common approaches used to produce crystals of protein–inhibitor complexes of interest.

In the soaking approach, the compound is incubated with preformed crystals of the target protein. This method commonly employs crystals of a form of the protein that has been crystallized in the absence of any added inhibitor (apocrystal form), but may also involve the displacement of an originally cocrySTALLIZED inhibitor with a second one. The soaked compound is expected to bind at a functional binding site of the

protein, such as the enzyme active site or a functional regulatory site.

In the cocrySTALLIZATION approach, the inhibitor is mixed with the protein prior to crystallization and the complex is crystallized. This process is then repeated for each new inhibitor.

In this study, the cocrySTALLIZATION of Uox with its substrate analogues 8-azaxanthine and 9-methyl uric acid, as well as with the natural substrate in the presence of cyanide, was used successfully to grow crystals that diffracted X-rays at high resolution (table 2). In the case of the natural substrate in the absence of cyanide, a variation of the soaking method was successfully used: in the first stage, we cocrySTALLIZED Uox with 8-azaxanthine, which has been bound in the active site of the enzyme, and then soaked the Uox-8-azaxanthine complex with urate. In comparison with the previous studies when ligand-free Uox crystals soaked in a urate-containing solution immediately cracked and dissolved (Gabison *et al.* 2006), our soaking approach did not result in crystal cracking. These results should be considered as discussed below.

When compared with small-molecule crystals, protein crystals are loosely packed, typically containing from 30 to 80 per cent solvent (Matthews 1968, 1974). The network of lattice interactions determines the size and configuration of channels traversing the crystal, which average typically from 20 to 100 Å in diameter (Vilenchik *et al.* 1998). These channels contain the bulk solvent bathing the crystals in addition to a shell of ‘bound water’ interacting with the protein molecules and provide considerable access for small ligands to the protein molecules in the lattice. Soaking inhibitors into preformed crystals is often used in iterative structure-aided drug design and can provide certain advantages. The ability to stockpile a large number of crystals of known structure and diffraction quality and then soak them in compounds of interest can provide speed, convenience and reproducibility. Importantly, the crystals used in these experiments must be compatible with inhibitor binding. For example, if inhibitor binding induces conformational changes in the protein that are not compatible with the crystal lattice-packing interactions, crystal cracking or dissolution may occur and the crystals become of no use for diffraction experiments. Alternatively, if lattice packing in the apocrystal form includes interactions that inhibit access to the binding site, the crystals may also not be of use for soaking studies. Where possible, it is useful to obtain multiple crystal forms so that any limitations of one form may be overcome in a second packing arrangement.

A variation of the soaking method is to cocrySTALLIZE with an initial inhibitor bound in the site of interest and then to soak a new inhibitor into that site. Again, crystal lattice interactions must be compatible with any protein solubility or conformational changes that occur during the binding of a new structurally different compound.

If, in the preformed crystal, steric hindrance or incompatible conformational changes upon inhibitor binding occur and if a new crystal form compatible with binding cannot be found, soaking may not be

Figure 2. (a) Typical crystal habits of a Uox complex, here a crystal of urate complex with cyanide with approximate dimensions $1.2 \times 1.2 \times 1$ mm. Scale bar, 0.5 mm. (b) Typical neutron Laue diffraction pattern collected on LADI-III (ILL) from a 4 mm^3 crystal of recombinant Uox complexed with 8-azaxanthine, which diffracted to $1.9 \text{ \AA}$ resolution.

successful. In addition, in some cases, there may be concern that the binding mode observed in a soaked structure may not accurately represent the solution binding mode (Zhu *et al.* 1999; Hiller *et al.* 2006).

Adding an inhibitor to the target protein to form a complex in solution prior to crystallization (cocrySTALLIZATION) may circumvent these issues and should be considered. One should keep in mind that in cocrySTALLIZATION the solubility and/or conformation of each new complex formed may differ from that of the apoenzyme or of other inhibitor complexes. This may lead to an inability to grow crystals of a given complex even when apoenzyme crystals or other complexes were successful. When this is the case, the individual protein–inhibitor complex should be screened for crystallization conditions as a unique crystallization problem and a crystal form compatible with the inhibitor-bound complex may be found.

In the case of Uox, the binding of ligands causes negligible changes in the main-chain conformation, so the cracking of crystals upon soaking is probably due to subtle changes in the quaternary structure. The enzyme tetramer is formed by crystallographic two-folds, and very slight movements are sufficient to destroy the crystal contacts. The neutron crystallographic experiment depends on large and well-ordered crystals and the Laue geometry is particularly sensitive to the crystal mosaicity (Ren *et al.* 1999), so even if the crystal does not completely crack, soaking can disturb the lattice order of a large crystal, rendering it useless for neutron experiments. Different ligands may induce

lattice heterogeneities like pseudo-symmetry as discussed below, even when cocrySTALLIZED, but cocrySTALLIZATION and a combination of cocrySTALLIZATION with soaking are still the methods of choice for producing large ordered crystals of a given complex.

3.4. Pseudo-symmetry

While symmetry in protein crystals usually helps structure determination, various deviations from the ‘standard’ description of symmetry, such as twinning or pseudo-symmetry, can significantly complicate structure solution and refinement, especially when not detected. Pseudo-symmetry occurs when non-crystallographic symmetry (NCS) elements are found close to a crystallographic symmetry element. The pseudo-symmetry can concern either rotational or translational symmetry elements (Zwart *et al.* 2008). In the case of translational pseudo-symmetry, some reflections become systematically weak, the limiting case being crystallographic centring, where those reflections are completely absent. The crystal form of Uox, in the presence of different inhibitors, possesses body-centred orthorhombic symmetry ($I222$). Such centring is always an approximation, so the choice of lattice in indexing critically depends on the selection threshold of spot intensity for indexing. If weak X-ray sources such as in typical laboratory diffractometers are used, the weak spots may remain completely undetected, whereas intense synchrotron radiation is likely to help in detecting these weak reflections (Oksanen *et al.* 2006). Neutron sources are even weaker than laboratory X-ray sources, so it is crucial to characterize the crystals at a synchrotron beam for defects like this. In the crystals of Uox in heavy water, in the presence of 9-methyl urate and urate, indications of translational pseudo-symmetry were observed (table 3). The data were indexed in a primitive orthorhombic cell with the same cell dimensions as previously observed. In both cases, the peak in the Patterson map was positioned exactly at (0.5, 0.5, 0.5) in fractional coordinates as expected for I-centring. Despite the original indexing, the data were also scaled in the space group $I222$ with similar statistics. No indications of pseudo-symmetry could be detected in either the data collected using a laboratory X-ray source or the LADI-III neutron diffractometer, although the significantly higher R_{merge} values for the 9-methyl urate room temperature dataset probably result from the pseudo-symmetry. This is as expected based on the signal-to-noise ratio of the weak reflections compared with the strong ones.

Refinement in both the primitive ($P2_12_12$) and centred ($I222$) space groups behaved very similarly and essentially no differences were observed between the two independently refined molecules in the primitive group. Presumably, the pseudo-symmetry results from some variability of the packing within the crystal owing to the inhibitor used in cocrySTALLIZATION as discussed above. As the tetramer is formed by crystallographic symmetry operations, changes or inhomogeneities in the quaternary structure could cause translational pseudo-symmetry.

Table 3. Indications of translational pseudo-symmetry.

ligand	resolution (Å)	height of Patterson peak (% of origin peak)
9-methyl urate	1.2	78.7
urate	1.05	94.9

3.5. Preliminary X-ray and neutron crystallographic analysis

Uox complexed with its substrate analogues (8-azaxanthine, 9-methyl uric acid) as well as with the natural substrate (in the presence and absence of cyanide) has one of the largest primitive unit-cell volumes ($I222$, $a = 80$, $b = 96$, $c = 106$ Å) and molecular weights (137 kDa for the homotetramer) so far successfully studied with neutrons. The data-processing statistics for the neutron Laue and X-ray datasets collected from the different Uox complexes are summarized in tables 1 and 2.

These ambient temperature datasets are currently being used for joint X-ray and neutron refinement of the different Uox complexes. The quality of the nuclear density maps calculated from the joint refinements is clearly superior compared with previous refinements against neutron data only (Budayova-Spano *et al.* 2006). Even though the data statistics for the 8-azaxanthine complex are quite similar to those reported previously, the data reported here were collected in a significantly shorter time, demonstrating the benefit of using larger crystals and the improved LADI-III instrument. There was also a clear advantage in using the models from our high-resolution X-ray structures in D_2O . This may seem slightly surprising at first because the r.m.s.d. between the H_2O and D_2O structures was so low, but it is probably explained by subtle differences in the quaternary structure. These high-resolution X-ray diffraction datasets will probably give additional hints on the mechanism of Uox, even if the nuclear scattering maps are not adequate to see all of the interesting protonation states. Two examples of typical neutron Fourier maps for the data are shown in figure 3 for Uox-8-azaxanthine complex. These maps clearly show the protonation states of, for example, histidines (figure 3*a*) or the orientations of hydroxyl protons that are, in principle, free to rotate (figure 3*b*). Most of the ordered solvent molecules are not spherical (figure 3*b*), but rather have a banana-shaped density that defines the direction of hydrogen bonding. The refinements and the interpretation of the structures in terms of the catalytic mechanism are still in progress, but we are confident that the fully refined structures will contribute significantly to the understanding of the mechanism.

4. CONCLUSIONS

This work illustrates the high quality of X-ray and neutron diffraction data collected from crystals grown by careful control and optimization of crystallization conditions via knowledge of the phase diagram. A

Figure 3. (a) Neutron scattering density map ($2F_o - F_c$ at 1.5σ) superposed on the current model of Uox-8-azaxanthine for His93; an $F_o - F_c$ map at 2σ calculated without the D-atoms in the model is represented in green. Note the clear density for the D-atoms. (b) Neutron scattering density map ($2F_o - F_c$ at 1.5σ) superposed on the current model of Uox-8-azaxanthine for Ser145 with three D_2O water molecules. Note the clear orientations of the D_2O water molecules.

method and a device for the promotion of crystal growth by keeping the crystallization solution metastable during the growth process that find application in the growth of large high-quality crystals for neutron crystallography were described. Therefore, this work contributes to the development of automated crystal-growth optimization methods that help to remove the main bottleneck in the application of neutron crystallography to structural biology. An important point is that the crystals diffracted to high resolutions even though they were obtained with hydrogenated protein (previously exchanged with buffered D_2O) and possessed relatively small crystal volumes in comparison with other neutron structural projects (Coates *et al.* 2001; Bau 2004; Hanson *et al.* 2004; Maeda *et al.* 2004). It is noteworthy that for a hydrogenated D_2O -exchanged protein with such a large primitive unit-cell volume ($407\,040$ Å³), this neutron diffraction study of Uox represents one of the highest resolution neutron datasets thus far.

Refinement of the neutron structures is under way and clearly shows enhanced visibility for hydrogen isotope positions of the protein, solvent (as full D_2O) and substrate analogues. The direct determination of

the protonation states of the residues and the orientation of the water molecules within the active site is crucial for a more complete understanding of the enzymatic mechanism of this protein drug. In addition, this study represents a significant advance in our work on Uox. To date, the present neutron diffraction data obtained with the different Uox complexes are the only existing neutron structural data concerning this enzyme. The supplementary information derived from an ensemble of neutron structures of Uox will further improve our understanding of the catalytic mechanism of this therapeutically important enzyme.

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