Azide inhibition of urate oxidase

Laure Gabison,^a Nathalie
Colloc'h^b and Thierry Prange^{a*}^aFaculty of Pharmacy, UMR 8015 CNRS
Laboratoire de Cristallographie et RMN
Biologiques, 4 Avenue de l'Observatoire, 75006
Paris, France, and ^bISTCT, UMR 6301–CNRS–
Université de Caen–Normandie Université–
CEA, Centre Cyseron, Boulevard Becquerel,
14074 Caen CEDEX, FranceCorrespondence e-mail:
thierry.prange@univ-paris5.fr

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The inhibition of urate oxidase (UOX) by azide was investigated by X-ray diffraction techniques and compared with cyanide inhibition. Two well characterized sites for reagents are present in the enzyme: the dioxygen site and the substrate-binding site. To examine the selectivity of these sites towards azide inhibition, several crystallization conditions were developed. UOX was co-crystallized with azide (N₃) in the presence or absence of either uric acid (UA, the natural substrate) or 8-azaxanthine (8AZA, a competitive inhibitor). In a second set of experiments, previously grown orthorhombic crystals of the UOX–UA or UOX–8AZA complexes were soaked in sodium azide solutions. In a third set of experiments, orthorhombic crystals of UOX with the exchangeable ligand 8-nitroxanthine (8NXN) were soaked in a solution containing uric acid and azide simultaneously (competitive soaking). In all assays, the soaking periods were either short (a few hours) or long (one or two months). These different experimental conditions showed that one or other of the sites, or the two sites together, could be inhibited. This also demonstrated that azide not only competes with dioxygen as cyanide does but also competes with the substrate for its enzymatic site. A model in agreement with experimental data would be an azide in equilibrium between two sites, kinetically in favour of the dioxygen site and thermodynamically in favour of the substrate-binding site.

1. Introduction

Urate oxidase (uricase; EC 1.7.3.3; UOX) from *Aspergillus flavus* catalyzes the oxidation of uric acid (UA) in the presence of molecular oxygen to 5-hydroxyisourate (Fig. 1*a*). UOX is a key enzyme in the degradation cascade of purine. It follows xanthine oxidase (Enroth *et al.*, 2000) and precedes hydroxyisourate hydrolase (Sarma *et al.*, 1999) and allantoinase (Cooper & Lawther, 1973). The gene for UOX is present but is not expressed in the genome of higher apes and humans. This leads to a high level of uric acid in the blood, which is considered to be an evolutionary benefit in terms of longevity and protection against cancer (Ames *et al.*, 1981; Wu *et al.*, 1992).

UOX belongs to the class of cofactorless oxidases (Fetzner & Steiner, 2010) and is functional as a tetramer with an overall size of 60 × 60 Å, enclosing a tunnel 50 Å long and 12 Å in diameter (Colloc'h *et al.*, 1997). The four chains (or subunits) are assembled according to an internal 222 symmetry, aligned on the symmetries of the *I*222 space group. There is one independent monomer in the asymmetric unit. Two distinct sites were characterized in UOX, one for the substrate (UA) and the other for dioxygen; both are located at the interface of two subunits. In all structures of UOX, the substrate (uric acid or UA) or competitive inhibitors (here 8-azaxanthine or 8AZA) are similarly positioned by molecular tweezers built by the side chains of Arg176 and Gln228 as depicted in Fig. 1(*b*). This specific arrangement is dedicated to the cyclic NH–CO–NH–CO– motif included in a six-membered ring. Thus, UOX can accommodate a large number of other flat molecules derived either from purines (xanthine, 8-nitroxanthine, 8-thiouric acid, guanine *etc.*) and from other monocyclic substrates such as oxonic acid, uracil, allantoin and synthetic

pyrimidines (Sedor & Sander, 1977; Retailleau *et al.*, 2004; Gabison *et al.*, 2006). On the other hand, with its two N atoms (N1 and N3) bearing methyl groups, caffeine cannot be caught by the tweezers and is not recognized, whilst 9-methyl uric acid is still a good inhibitor (Hurst *et al.*, 1985; Retailleau *et al.*, 2004). Close to the active site resides the dioxygen site through which all reagents pass: a water molecule and dioxygen as incoming reagents and hydrogen peroxide as the leaving product. This location displays important base and oxidative properties; it is delimited by the side chains of the two Thr57* and Asn254 residues, where * indicates a residue from another subunit (Colloc'h *et al.*, 1997; Gabison *et al.*, 2011). These two residues characterized by genetic engineering were designated as a catalytic dyad (Imhoff *et al.*, 2003), then a triad (Gabison *et al.*, 2006) and finally a tetrad by the inclusion of two further conserved residues, Lys10* on one side and His256 on the other. The flexibility of these tweezers has been reported (Gabison *et al.*, 2010); they allow the binding of a variety of 'small' ions/molecules such as, in addition to the above-mentioned water, dioxygen or hydrogen peroxide, other ions such as chloride, cyanide and azide, as demonstrated in the present study.

In contrast to xanthine oxidase, which is the preceding enzyme in the cascade, UOX does not have a cofactor or a metallic cation able to activate a radical for direct reaction with dioxygen, and the true mechanism has been the subject of numerous studies (Bentley & Neuberger, 1952; Modrić *et al.*, 1992; Kahn *et al.*, 1997; Imhoff *et al.*, 2003; Altarsha *et al.*, 2009; Gabison *et al.*, 2011). To gain more information about the mechanism, we investigated the specific inhibition of the dioxygen site (the peroxo hole).

Figure 1
(a) The catalyzed reaction leading to 5-hydroxyisourate (5-HIU). In solution, 5-HIU spontaneously evolves to ($\pm$)-allantoin with a lifetime of about 20 min. *In vivo*, 5-HIU is rapidly degraded to (+)-allantoin by another enzymatic system. (b) A schematic view of the substrate-binding site. In red, the minimal consensus for a chemical compound to be a 'good' ligand of UOX. The binding site at the interface of two subunits A and B is built by residues from two chains: A176 and A227–228 from the first subunit and B57–58 from the second. In addition (not shown), Phe159 from subunit A closely stacks with the six-membered ring of uric acid.

Azide (used as NaN_3 , called N3 in the following) and cyanide anions have been known for a long time to strongly inhibit oxygenases, oxidoreductases, cytochromes in the respiration chain and other biological systems (Stannard & Horecker, 1948; Wilson & Chance, 1967; Bennett *et al.*, 1996; Fei *et al.*, 2000). In that sense, a number of azide/cyanide-containing structures deposited with the Protein Data Bank have relevance to their inhibitory effects. In the case of the cofactorless UOX, cyanide and hydroxylamine have been shown to have a substantial inhibitory effect (Conley & Priest, 1980); azide inhibition of UOX has also been reported but with only limited data (Keilin & Hartree, 1936; Laboureur & Langlois, 1968). The present study aims at unravelling some mechanistic aspects of azide inhibition at the molecular level by analyzing UOX when co-crystallized and/or soaked with substrate or inhibitors in the presence of sodium azide.

2. Materials and methods

2.1. Crystallization

UOX from *Aspergillus flavus* was a gift from Sanofi-Aventis Co. The initial UOX concentration was estimated from OD measurements as $\sim 11 \text{ mg ml}^{-1}$. Before crystallization, the solution was exchanged against Tris acetate buffer (0.05 M, pH 8–8.5) and concentrated to 20 mg ml^{-1} . In some experiments chloride from the standard Tris–HCl buffer was found to compete with dioxygen, a fact that has previously been reported (Roeser *et al.*, 2007; Colloc'h *et al.*, 2008) and as observed in PDB entries 3cku (Colloc'h *et al.*, 2008) or 3p9o (L. Gabison, N. Colloc'h, M. El Hajji, B. Castro, M. Chiadmi & T. Prangé, unpublished work). We thus prepared, and preferentially employed, acetate-buffered Tris instead. Uric acid (UA), xanthine (XAN), 8-azaxanthine (8AZA) and sodium azide (N3) were purchased from Fluka Co. 8-Nitroxanthine (8NXN) was prepared by the direct nitration of xanthine according to Mosselhi & Pfeleiderer (1993).

Crystals of UOX complexes were obtained at 291 K following a previously reported protocol (Retailleau *et al.*, 2004) that was modified slightly because sodium azide increases the protein solubility. Two solutions, A and B, were prepared. Solution A was a protein solution at 20 mg ml^{-1} in 0.05 M Tris buffer. Solution B was in Tris buffer (the same as in solution A) plus 0.3 M N3 and was then adjusted to 18% PEG 8000 by volume by adding solid PEG 8000. For cryocooling, the crystals were soaked a few minutes in a mixture of 30% methyl-2,4-pentanediol (MPD) and 70% of the crystallization medium.

Two different techniques were employed: co-crystallization and soaking.

2.1.1. Co-crystallization. In co-crystallization experiments, solution B was saturated with either 8AZA or UA or used as such. The batch method was used throughout, *i.e.* mixing equal amounts of 15 μl of the two solutions A and B in wells of 100 μl . Crystals usually appeared within 1–2 d as small rods and grew up to 1 mm in length in favourable cases.

2.1.2. Soaking experiments. In soaking experiments, crystals of UOX in complex with its natural substrate (UA) or 8AZA were first prepared (Retailleau *et al.*, 2004). UOX–8AZA crystals are known to be very stable and can easily be stored for a long time at 291 K, but in the case of the UOX–UA complex, a rapid degradation occurs *in situ* and the resulting crystals contain allantoin, the end product of the reaction (Gabison *et al.*, 2006) after a few days. To overcome this problem in aerobic conditions, we first prepared the deep red orthorhombic crystals of the 8NXN complex according to Gabison *et al.*

Table 1

Data-collection and refinement statistics for UOX–UA and UOX–8AZA in the presence of sodium azide.

For all data, the space group is orthorhombic *I*222. Values in parentheses are for the highest resolution bin. Wavelengths are 0.964, 0.938 and 0.976 Å at the ESRF and 0.8856 Å at SOLEIL.

Conditions	Co-crystallized		Sequential soaking		
	With N3	With UA + N3	UOX–8AZA in N3	UOX–UA in N3	'Aged' crystals
Structure	Fig. 2(<i>a</i>)	Fig. 2(<i>b</i>)	Fig. 3(<i>a</i>)	Fig. 3(<i>b</i>)	Fig. 4
Ligands					
Substrate-binding site	N3	N3	8AZA	UA	N3
Dioxygen site	H ₂ O	H ₂ O	N3	N3	N3
Unit-cell parameters					
<i>a</i> (Å)	78.77	79.52	78.73	78.58	79.18
<i>b</i> (Å)	94.85	95.24	95.18	95.15	94.92
<i>c</i> (Å)	104.34	104.58	104.51	104.82	104.53
<i>V</i> (Å ³)	779559	792052	783066	783761	785623
Resolution (Å)	15–1.30 (1.32–1.30)	47.6–1.05 (1.12–1.05)	26.7–1.7 (1.79–1.70)	15.5–1.15 (1.28–1.15)	22.2–1.30 (1.34–1.30)
No. of unique reflections	89456 (14789)	172500 (24081)	43286 (6279)	110651 (8735)	97678 (8236)
Multiplicity	4.3 (4.8)	4.4 (3.2)	4.5 (4.5)	4.8 (2.5)	4.7 (4.2)
<i>R</i> _{merge} (%)	7.7 (22.3)	6.8 (32.5)	4.6 (18.0)	6.2 (27.2)	7.2 (21.7)
Completeness (%)	93.3 (95.3)	98.2 (54.8)	99.7 (99.4)	94.0 (34.2)	98.8 (99.0)
Mean <i>I</i> /σ(<i>I</i>)	22.7 (3.2)	17.9 (2.0)	25.0 (4.4)	18.2 (2.1)	16.1 (1.9)
Refinement					
No. of atoms in the model					
Protein	2362	2362	2362	2362	2362
Ligand			11 [8AZA]	12 [UA]	
Solvent					
H ₂ O	242	308	334	302	239
Na ⁺	1	1	1	1	1
Azide (N ₃ [−])	6	6	6	6	9
Resolution range (Å)	15–1.30	47–1.07	10–1.70	11–1.16	20–1.30
No. of <i>F</i> used	89456	172500	43286	110037	9 678
No. of observed <i>F</i> with <i>F</i> > 4σ(<i>F</i>)	82780	148909	43178	65992	66686
No. of parameters/No. of restraints	10515/9792	19325/23883	10995/9911	18181/21705	10481/9811
<i>R</i> _{work} , all <i>F</i> data (%)	18.8	18.5	18.9	18.4	21.1
<i>R</i> _{work} observed <i>F</i> data (%)	18.4	16.9	18.4	17.8	17.6
<i>R</i> _{free} [No. of <i>F</i> s] (%)			21.1 [2727]	21.6 [4830]	
<i>wR</i> ² on <i>F</i> ² (%)	47.7	47.9	48.8	47.6	44.4
Mean standard deviations from ideality					
1–2 distances					
No.	2429	2425	2464	2459	2427
Target/value (Å)	0.034/0.030	0.038/0.029	0.034/0.030	0.015/0.026	0.034/0.030
1–3 distances					
No.	3 291	3 286	3 339	3332	3 288
Target/value (Å)	0.082/0.076	0.082/0.076	0.082/0.073	0.030/0.070	0.082/0.075
Planes					
No.	785	785	800	793	785
Target/value (Å)	0.43/0.030	0.43/0.406	0.43/0.025	0.43/0.401	0.43/0.029
Chiral zero volumes					
No.	380	379	381	382	379
Target/value (Å ³)	0.10/0.060	0.10/0.075	0.10/0.038	0.08/0.046	0.10/0.069
Chiral nonzero volumes					
No.	368	368	368	368	369
Target/value (Å ³)	0.10/0.071	0.10/0.092	0.10/0.049	0.08/0.057	0.10/0.073
Mean isotropic ⟨ <i>B</i> ⟩ factors					
Main-chain atoms	12.4	13.4	13.2	18.1	12.8
Side-chain atoms	16.5	17.0	16.7	22.6	19.7
Ligand			9.8	20.6	
Water molecules	23.3	26.7	26.2	31.4	26.9
Azide	19.6	20.2	18.7	16.2	27.1
Na ⁺	14.8	14.5	8.8	17.4	14.1
PDB code	4oqc	4poc	3p9f	4pr8	4puv

al. (2010). These crystals are also very stable and can be stored at 277 K for several months, but the 8NXN ligand is particularly mobile. In the presence of an excess of UA (or 8AZA), ligand exchange proceeds readily and the bleaching of the red UOX–8NXN crystals is a convenient way to monitor the reaction. Once colourless (exchange completed), the UOX–UA crystals are rapidly transferred for soaking. In anaerobic conditions (*i.e.* under an argon atmosphere), this exchange procedure is not necessary; the crystals of UOX–UA are then as stable as those of UOX–8AZA.

2.1.3. Sequential soaking. The UOX–UA or UOX–8AZA crystals were transferred into a solution containing excess N3 corresponding

to a final concentration of 0.3 *M*. After a few hours, the crystals were harvested, cryocooled in liquid nitrogen and stored. A second batch of crystals was harvested after a period of one or two months. The two batches of crystals were called 'fresh' and 'aged' crystals.

2.1.4. Competitive soaking. The red UOX–8NXN crystals were soaked in solution *B* containing the UA and N3 reagents together. The exchange of 8NXN to UA was much slower in the presence of N3 and took several days as indicated by the colour evolution. Several crystals were also harvested at different times: the first batch immediately after equilibration ('fresh' crystals) and the second batch after one or two months ('aged' crystals). All crystals were cryocooled in

liquid nitrogen and stored at 100 K. At this stage, it was evident that N3 was also able to perturb the substrate-binding site in addition to the dioxygen site.

2.2. Data recording

Data were collected on beamlines BM30 (Quantum Q315r detector), BM14 (MAR 225 CCD detector), ID14-4 (Quantum Q315r detector) and ID23-1 (Pilatus 6M detector) at the ESRF, Grenoble, France and on beamline PX1 (Pilatus 6M detector) at SOLEIL, Gif-sur-Yvette, France.

The same protocol was used for all CCD detectors: 1° rotation per frame over a complete ω range of 120°. On pixel detectors, 1200 frames of 0.1° each were recorded over the same ω range of 120°. In both cases, the crystal-to-detector distance was set according to the maximum resolution determined on a first test frame. Low-

temperature recordings were adopted throughout, especially with UA-exchanged crystals, to avoid any risk of enzymatic activity within the crystal lattice.

All data sets were processed using either *ipmosflm* (Battye *et al.*, 2011) or *XDS* (Kabsch, 2010), merged according to the *CCP4* package of programs (Winn *et al.*, 2011) and then formatted as *hkl F²* files suitable for input into the *SHELX* suite of program (Sheldrick, 2008).

Several different cooled states and snapshots of the reaction were obtained with azide either in the substrate-binding site or in the oxygen site, as well as with azide in both sites. They are all described below and the corresponding data and statistics are reported in Table 1.

2.3. Refinement

All refinements started from the previously refined model of UOX–xanthine at atomic resolution (PDB entry 3l8w; Gabison *et al.*, 2010) by first removing ligands, ions and water molecules. After a preliminary round of rigid-body refinement, atoms were refined with individual isotropic thermal factors using *SHELXL* (Sheldrick, 2008). Constraints on distances, angles, planes and chiral volumes were applied throughout all refinements. Disordered side chains were modelled as far as possible with two static orientations. Water molecules, ions and ligands were progressively introduced and refined, according to σ_A and $|F_o - F_c|$ maps. Finally, in the case of high-resolution data (better than 1.3 Å) H atoms were imposed at their calculated positions with isotropic thermal factors riding on that of the bonded atom. In the case of atomic resolution structures (better than 1.2 Å) individual but constrained anisotropic thermal parameters were introduced. Refinement parameters and statistics are also reported in Table 1. Figs. 2–4 were prepared using *PyMOL* (<http://www.pymol.org>).

3. Results

3.1. Co-crystallization of UOX or UOX complexes with azide

3.1.1. Co-crystallization of UOX with azide alone. It was previously established that the high resolution diffracting orthorhombic crystals of UOX can only be obtained if a specific ligand is present in the substrate-binding site (Retailleau *et al.*, 2005). Azide alone was however equally able to induce large orthorhombic crystals, in contrast to cyanide. The structure of a ‘fresh’ crystal clearly showed that azide was present in the substrate-binding site (Fig. 2a) connected through a number of hydrogen bonds mediated by three structural water molecules with low *B* factors, but not in the dioxygen site as was expected. In this site, a water molecule (W1) with loose interactions towards the N^{δ2} atom of Asn254 was observed. Clearly, this experiment shows better selectivity of azide towards the substrate-binding site than the dioxygen site.

3.1.2. Co-crystallization of UOX with azide and uric acid. Large orthorhombic crystals were also obtained, some of which diffracted to near-atomic resolution (1.05 Å), when solution *B* contained both UA and N3. The substrate-binding site did not contain UA but instead contained azide, as in the crystals obtained previously with azide alone (Fig. 2b).

3.1.3. Co-crystallization of UOX with azide and 8-azaxanthine. When crystallization of UOX was performed with solution *B* containing 8AZA (instead of UA) and azide, the resulting crystals now contained 8AZA in the substrate-binding site and azide in the dioxygen site (the peroxo hole) as shown in Fig. 3(a).

Figure 2

Co-crystallizations. (a) Contents of the substrate active site when UOX is crystallized in the presence of 0.3 M sodium azide. The dioxygen site (peroxo hole; Thr57* and Asn254) contains a water molecule, while an azide is bound in the substrate-binding site, filling the cavity with two structural water molecules (W2 and W3). A third water (W4) completes the hydrogen bonding but with weaker interactions. (b) The same result from competitive co-crystallizations of UOX with UA (saturated) and 0.3 M sodium azide. Omit maps are at the 3σ level in both cases.

3.2. Soaking of UOX complexes (UOX–UA and UOX–8AZA)

These experiments were performed with the aim of obtaining some snapshots of the ongoing azide inhibition. In the first case UOX–UA crystals were prepared by ligand exchange prior to use. Under the soaking conditions the degradation of UA probably still proceeds, but slowly with a pseudo-zero kinetic order because the limiting step is the slow diffusion of UA within the crystal. This ensures a near-isotropic repartition of UA throughout the crystal lattice.

Different results were obtained depending on the way that reagents were added or mixed.

3.2.1. Sequential soaking in N3: 8-azaxanthine complex. Crystals of the UOX–8AZA complex incubated for a few hours in solution *B* containing 0.3 M N3 gave the stable ternary complex UOX–8AZA–N3. The structure is exactly the same as when co-crystallization was performed, with an active-site content as depicted in Fig. 3(a).

3.2.2. Sequential soaking in N3: uric acid complex. As mentioned earlier, the UOX–UA crystals were obtained by ligand exchange; UOX–8NXN crystals were first incubated in buffered solution *B* in

the presence of UA. The colour turned from deep red to pale yellow and then to colourless as a consequence of the ligand exchange. After a period of ~20 min, N3 was then added followed by incubation for a further ~1 h. The crystals were quenched in liquid nitrogen. The resulting ternary structure UOX–UA–N3 was obtained (Fig. 3b).

Fig. 3 clearly shows that 8AZA and UA are indeed in the substrate-binding site and azide is in the dioxygen site.

3.2.3. Competitive soaking of UOX–8NXN in UA plus N3. Soaking of UOX–8NXN was now performed with solution *B* containing both UA and azide. Exchange in the presence of azide proceeded very slowly, taking place over several days to a week rather than in 1 h. In the case of crystals picked out after this period (the ‘fresh’ structure), the azide occupies the substrate-binding site, exactly as in co-crystallization experiments obtained with azide alone or with azide in competition with UA, as depicted in Fig. 2.

3.3. ‘Aged’ crystals

When crystals of UOX co-crystallized with N3 alone were left for one or two months for complete equilibration, two azide anions were found to fill the two sites, one in the substrate-binding site, again with three water molecules as a complementary binding system mimicking UA, and a second azide located in the dioxygen site (Fig. 4). The same results were obtained by a long-term competitive soaking of exchangeable UOX–8NXN crystals in a solution containing UA and azide for one or two months. Here again the water molecule sitting in the dioxygen site was slowly but completely replaced by a second azide above the first located in the substrate-binding site.

On the other hand, when crystals of the ternary UOX–8AZA–N3 or UOX–UA–N3 complexes were soaked in solution *B* for one or two months, there was no modification of the ligand binding: 8AZA or UA still remained bound in the enzymatic site with an azide in the dioxygen site.

3.4. A subsidiary binding site of azide

In all structures, whatever the conditions that were used (co-crystallization or soaking), a subsidiary azide was always observed in the tunnel delimited by the subunits involving residues 4–7 from one subunit and residues 290*–292* from another, far from the enzymatic sites. This structural azide is loosely bound on one side to the N^ε atom of Arg7 (distance of ~3 Å) but is more tightly solvated by two water molecules on both sides as a hydrogen-bond relay to the O^γ atom of Thr291 and the N^ω atom of Arg7. The role of this location remains undetermined but is likely to have no particular enzymatic function.

4. Discussion

The substrate-binding and dioxygen sites, although close to each other, display different dynamics: the substrate-binding site is rather rigid with little deformation, as observed in all UOX structures solved to date, while the dioxygen site (or peroxo hole) has a greater flexibility. The Thr57*/Asn254 pair of residues forming the molecular tweezers adapts its gap to the size of the reagent, usually a water in most of the crystal structures (*e.g.* W1 in Figs. 2a and 2b) and an azide in the others (Figs. 3 and 4). However, the two sites share some degree of cooperativity. When the substrate-binding site is empty, Thr57* and Asn254 are flipped away as observed in the monoclinic ligand-free structure (PDB entry 1r56; Retailleau *et al.*, 2004) and do not build functional tweezers. Among all anions observed in the dioxygen site, azide is probably one of the bulkiest, leading to a *gauche* orientation, and increases the opening to ~5.9–6.05 Å (Thr57* O^{γ1}–Asn254 N^{δ2} distance). This distance is equivalent when

Figure 3
Soaking of the UOX–UA and UOX–8AZA complexes with N3. (a) 8AZA, (b) UA. The substrate is locked in the active site whilst azide fills the dioxygen site. No change was observed when analyzing ‘aged’ crystals (harvested after one or two months).

a cyanide is present but becomes shorter when smaller molecules such as water are present, in which case the distance reduces to ~ 5.5 Å.

Inhibition of UOX by cyanide was first mentioned a long time ago (Conley & Priest, 1980). The X-ray structure of the UOX–UA–CN[−] complex showed that cyanide interacts exclusively with the dioxygen site (Colloc'h *et al.*, 2008; Gabison *et al.*, 2008). Here, investigations of UOX–azide complexes show that azide does bind to the enzyme but in a different manner to cyanide. In this respect questions arise as to how the different crystallization assays influence azide inhibition and how the mixing of reagents together can explain the different results obtained. Three main points can be drawn up and are summarized as follows:

(i) Azide has a preference for the substrate-binding site over the dioxygen site (Fig. 2).

(ii) When the substrate-binding site is initially occupied, as in preformed UOX–8AZA or UOX–UA complexes, the dioxygen site is the immediate target ('fresh' crystals; Fig. 3). This remains stable if the crystals are further soaked in azide solutions, even after one or several months ('aged' crystals) with no exchange between 8AZA (or UA) and azide in spite of a large excess of the latter. However, in the case of the labile UOX–8NXN crystals, the 8NXN ligand is slowly exchanged for azide.

(iii) When the substrate-binding site is empty and when the soaking periods are increased to one or several months, the azide progressively saturates the two sites, leading to a di-azide complex (Fig. 4).

To explain these observations, two remarks may be made. The first concerns the size and binding energy of the hydrated azide anion compared with cyanide, while the second is connected to the concentrations used in our crystallization assays.

In all the structures, azide in the substrate-binding site is always strongly hydrated at both ends. With a triplet of structural waters, it mimics the substrate in size, polarity and bonds. The situation is different in the dioxygen site, where its size imposes an increase of the distance between the tweezers Thr57*–Asn254 and hence introduces a destabilization effect. It is interesting to note that azide is usually found to be slightly bent (165 – 171°) in the dioxygen site and quasi-linear (175 – 180°) in the substrate-binding site, an indication of a more constrained state in the first case than in the second. From an energetic point of view, it seems reasonable to conclude that azide would

be more stable in the substrate-binding site rather than in the dioxygen site. Conversely, cyanide is shorter and similar in size to dioxygen; it would perfectly fit the dioxygen site and not the too wide substrate-binding site in the vicinity (Gabison *et al.*, 2008). This would explain its better selectivity for the dioxygen site compared with azide. However, this did not explain why, when the substrate-binding site is occupied by UA or 8AZA, azide binds so easily and rapidly in the dioxygen site without displacing the substrate. In fact, there is no contradiction here; azide did follow the normal pathway *via* the dioxygen site as all the reagents do but stops there in the case of an occupied substrate-binding site or continues to move if it is empty. Another relevant point concerns the competitive soaking experiments, which show that azide is the winner in substrate-binding-site access when competing with UA, but not in the case of 8AZA; we believe this is the result of the large difference in the concentrations used: 0.3 M for azide and less than 1 mM for UA because of its low solubility. Le Chatelier's principle clearly favours the first (N3) at the expense of the second (UA). With regard to the more soluble 8AZA (~ 10 mM), the competition is reversed in favour of 8AZA.

The best model accounting for azide (N3) inhibition would be the following three-stage equilibrium:

Here, *a* and *b* are the dioxygen and the substrate-binding sites, respectively.

In the steady-state approximation, assuming $k_2 \gg k_1$ and $k_3 \ll k_2$, only E_b^{aN3} (Fig. 2) and E_{bN3}^{aN3} (Fig. 4) can be isolated after the long-standing assays of crystallization. The intermediate E_{bN3}^a would be isolated only if the three-stage equilibrium (shown above) is limited to the first equation, *i.e.* if site *b* is occupied and unavailable. This is the case in Fig. 3.

5. Conclusion

All our experimental conditions show that azide has a high affinity for the substrate-binding site, in addition to the dioxygen site, in contrast to cyanide, which only has a strong affinity towards the dioxygen site. A plausible model that could explain all of the results described here would be a primary filling of the dioxygen site by azide that results in a structure of a high conformational energy owing to its bulkiness. This would be followed by a migration to the substrate-binding site located in the vicinity, where azide would remain at a lower energy. In the case of a substrate (or an inhibitor) already present in the active site, no displacement would be possible owing to the better affinity for the site towards its substrate. This can also be viewed for the free enzyme according to the scheme above as an azide in equilibrium between two sites with the dioxygen site kinetically favoured but with the substrate-binding site thermodynamically favoured.

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Figure 4
'Aged' crystals from co-crystallizations: the evolution of the contents of the active sites in the case of the UOX–N3 crystals depicted in Fig. 2. Two azide anions fill both the dioxygen and the substrate-binding sites.

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