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Crystal Structures of Xanthine Oxidoreductase in Action: Mechanism of Catalysis and Inhibition by Drug Candidates for Clinical Therapy

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Xanthine oxidoreductase (XOR), a molybdenum containing metallo-flavoprotein, catalyzes the two steps of reaction prior to formation of uric acid, *i.e.*, conversion of hypoxanthine to xanthine and of xanthine to uric acid. As over-production of uric acid causes gout disease, it is a proven target of drug for clinical therapy of it. In addition, as XOR (as the xanthine oxidase form) produces reactive oxygen species (both superoxide anion and hydrogen peroxide), it has been implicated to link to many pathological events, suggesting a possible important target of drug for oxygen-stress linked diseases, too. We have determined the crystal structures of XOR during catalysis of substrates and inhibition by various inhibitors. Our main goal is to understand the mechanism of molybdenum dependent hydroxylation and its inhibition by drug candidates. This provides important information essential for clinical application.

The oxidative hydroxylation of xanthine to uric acid takes place at the molybdenum center ($LMo^{VI}O(=S)(OH)$) of the enzyme, that center becomes reduced and protonated to $LMo^{IV}O(SH)(OH)$ in the course of the reductive half-reaction of the catalytic sequence [1]. Electrons thus obtained are passed on to the FAD site, via the two iron-sulfur centers, prior to removal by either NAD^+ or O_2 (depending on whether the enzyme is in the dehydrogenase or oxidase form) in the oxidative half-reaction of the catalytic sequence [1]. Two contradictory models have been proposed for the binding mode of substrates and activation mechanism of substrates during catalysis in the active site pocket of the molybdenum centers of XOR [1]. In an effort to demonstrate the binding model, we determined the crystal structure of the urate complex reduced bovine milk enzyme at 2.1Å (Fig. 2, *right*). Bovine XOR crystals were soaked with 250μM uric acid in a large excess of NADH and titanium citrate under strictly anaerobic conditions in order to reduce cofactors. The crystal was cryo-cooled in liquid nitrogen and data were collected at BL17A and NW12A. In the urate bound form of reduced XOR structure, a covalent linkage between molybdenum and C8 of urate via an oxygen atom was observed, indicating that the structure represents an intermediate of hydroxylation of xanthine as the substrate. This provides unequivocal model for the binding mode of xanthine. We have proposed activation mechanism of substrate by amino acid residues in the active site of the molybdenum center of XOR [1, 2].

We have determined crystal structures of XOR bound with various inhibitors. The structures reveal that inhibitors are categorized into three types. Allopurinol, which has been widely used for gout for more than 40 years, is hydroxylated to oxipurinol by the enzyme and the product (oxipurinol) forms a covalent linkage to the molybdenum atom (OH was replaced by N atom of oxipurinol) as $Mo^{IV}O(SH)(N)$ (Fig.1, *upper left*). Thus it is a type of mechanism-based inhibitor [3]. In contrast, febuxostat (TEI-6720, Teijin Pharma Limited) is a structure-based inhibitor with extreme high affinity for XOR [4]. Crystallographic study has revealed that febuxostat makes multiple interactions between the active site structure of the enzyme protein without direct interaction to the Mo atom (Fig.1, lower). After successful clinical trials, the US Food and Drug Administration approved the drug in 2009. Subsequently, it was approved also in EU (2009) and Japan (2011). Another inhibitor, FYX-051, has recently been developed and is found to be a hybrid type of inhibitor based on both mechanism and protein structure [5]. The inhibitor not only forms a covalent linkage to Mo atom via oxygen in the hydroxylation reaction intermediate (Fig.1, *upper right*), but also interacts with amino acid residues of the solvent channel.

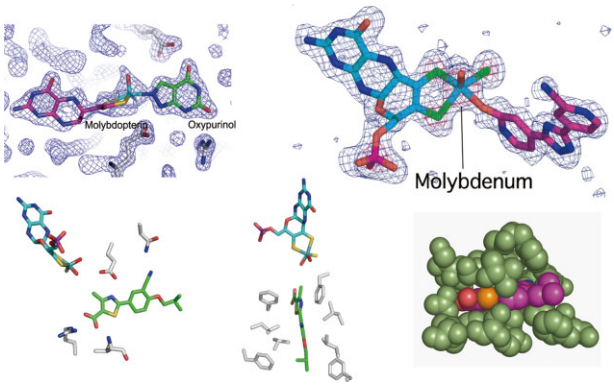

Fig.1 Complex structures of bovine XOR and inhibitors. *Upper left*; oxipurinol bound form. *Upper right*; FYX-051 bound form. *Lower left*; stick model presentation of febuxostat bound form. *Lower right*; van der Waals Presentation of febuxostat and interacting residues.

We further investigated the inhibitor-enzyme interaction and the *in vivo* hypouricemic effect of FYX-051. We trapped and observed a Mo-N linkage via CN-group of tri-OH-FYX-051, a further hydroxylated product by XOR itself, in the crystal structure (3AM9) at 2.2Å resolution correlated with the prominent spectral perturbation due to charge transfer interaction (Fig. 2, *left*) [6]. FYX-051 is now undergoing a clinical trial (phase II) in Japan.

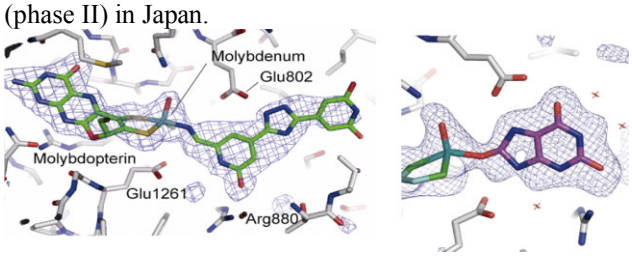

Fig.2 *Left*; Complex structures of bovine XOR and tri-hydroxylated FYX-051 (3AM9). *Right*; Reaction intermediated structure of XOR bound with xanthine (3AMZ).

References

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