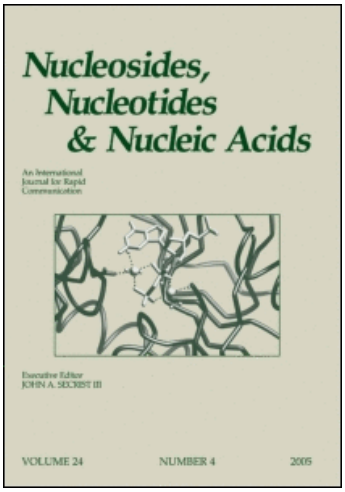


Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

Mechanism of Inhibition of Xanthine Oxidoreductase by Allopurinol: Crystal Structure of Reduced Bovine Milk Xanthine Oxidoreductase Bound with Oxipurinol

Ken Okamoto[a]; Bryan T. Eger[b]; Tomoko Nishino[a]; Emil F. Pai[b]; Takeshi Nishino[a]
[a] Department of Biochemistry and Molecular Biology, Nippon Medical School, Tokyo, Japan [b] Departments of Biochemistry, Medical Biophysics, and Molecular Genetics, University of Toronto and Division of Cancer Genomics & Proteomics, Ontario Cancer and Institute/University Health Network, Tornoto, Ontario, Canada

To cite this Article Okamoto, Ken , Eger, Bryan T. , Nishino, Tomoko , Pai, Emil F. and Nishino, Takeshi(2008) 'Mechanism of Inhibition of Xanthine Oxidoreductase by Allopurinol: Crystal Structure of Reduced Bovine Milk Xanthine Oxidoreductase Bound with Oxipurinol', Nucleosides, Nucleotides and Nucleic Acids, 27: 6, 888 — 893
To link to this Article: DOI: 10.1080/15257770802146577
URL: http://dx.doi.org/10.1080/15257770802146577

Nucleosides, Nucleotides, and Nucleic Acids, 27:888–893, 2008

ISSN: 1525-7770 print / 1532-2335 online
DOI: 10.1080/15257770802146577

Taylor & Francis
Taylor & Francis Group

MECHANISM OF INHIBITION OF XANTHINE OXIDOREDUCTASE BY ALLOPURINOL: CRYSTAL STRUCTURE OF REDUCED BOVINE MILK XANTHINE OXIDOREDUCTASE BOUND WITH OXIPURINOL

Ken Okamoto,[1] Bryan T. Eger,[2] Tomoko Nishino,[1] Emil F. Pai,[2] and Takeshi Nishino[1]

[1]Department of Biochemistry and Molecular Biology, Nippon Medical School, Tokyo, Japan
[2]Departments of Biochemistry, Medical Biophysics, and Molecular Genetics, University of Toronto and Division of Cancer Genomics & Proteomics, Ontario Cancer and Institute/University Health Network, Tornoto, Ontario, Canada

□ *Inhibitors of xanthine oxidoreductase block conversion of xanthine to uric acid and are therefore potentially useful for treatment of hyperuricemia or gout. We determined the crystal structure of reduced bovine milk xanthine oxidoreductase complexed with oxipurinol at 2.0 Å resolution. Clear electron density was observed between the N2 nitrogen of oxipurinol and the molybdenum atom of the molybdopterin cofactor, indicating that oxipurinol coordinated directly to molybdenum. Oxipurinol forms hydrogen bonds with glutamate802, arginine880, and glutamate1261, which have previously been shown to be essential for the enzyme reaction. We discuss possible differences in the hypouricemic effect of inhibitors, including allopurinol and newly developed inhibitors, based on their mode of binding in the crystal structures.*

Keywords Xanthine oxidase; xanthine dehydrogenase; xanthine oxidoreductase; gout; hyperuricemia; allopurinol; febuxostat

INTRODUCTION

Xanthine oxidoreductase (XOR) catalyzes the last two reactions of uric acid formation in human purine catabolism. It oxidizes hypoxanthine to xanthine and xanthine to uric acid at the molybdopterin center with concomitant reduction of NAD^+ to NADH at the FAD cofactor.[1] XOR is a target of drugs for treatment of gout and hyperuricemia; that is, inhibitors of XOR block formation of uric acid. Allopurinol, an analogue of hypoxanthine, was introduced by Elion et al. 30 years ago as a potent inhibitor of XOR and has been widely prescribed for hyperuricemia and gout.[2] Allopurinol is further hydroxylated by XOR at the 6-position (2-position based on

Address correspondence to Takeshi Nishino, Department of Biochemistry and Molecular Biology, Nippon Medical School, 1-1-5, Sendagi, Bunkyo-ku, Tokyo 113-8602, Japan. E-mail: nishino@nms.ac.jp

purine numbering) to give oxipurinol, the actual potent inhibitor of XOR. Oxipurinol has been supposed to bind tightly to the reduced molybdenum ion of the enzyme (Mo^{4+}), but it is simply a competitive inhibitor of oxidized XOR.[3] Thus, allopurinol is designated as a mechanism-based inhibitor.

Recently, three potent inhibitors of XOR (febuxostat, Y-700 and FYX-051) have been developed. We have solved the crystal structures of the complexes of these inhibitors with bovine milk XOR and defined their inhibition mechanisms.[4–6] In these crystal structures, all the inhibitors bind in a water channel near the molybdenum atom of the molybdopterin cofactor, and therefore inhibit the enzyme activity by obstructing the substrate binding. In this paper, we report the crystal structure of the complex of mammalian XOR with oxipurinol and discuss the possible differences in the in vivo effects of all four inhibitors based on the mechanism of inhibition.

EXPERIMENTAL PROCEDURES

Fully active bovine XOR was obtained by the method of Nishino et al.[7] with minor modifications. The enzyme was reduced with dithionite and mixed with oxipurinol under anerobic conditions, applied to a Sephadex G-25 column and then a folate affinity column. Enzyme solution eluted from the affinity column was concentrated to 30 mg/ml without liberating bound oxipurinol, and crystallized using the batch method as described by Eger et al.[8] Briefly, 10 μl of the solution was mixed with 10 μl of 50 mM potassium phosphate buffer (pH 6.5) containing 4.5–7.5% polyethylene glycol 4,000, 30% glycerol (wt/vol), 0.2 mM EDTA, 5 mM DTT, and 2 mM allopurinol. The batch solutions were set up on siliconized glass plates, and kept in the dark at 20°C. Crystals grew after 5 days; they belonged to the same C2 space group as the previously reported bovine XOR crystals. The crystals were collected with a nylon loop, shock-frozen, and stored in liquid nitrogen. A complete 2.0 Å diffraction data set was collected at Spring-8 (Harima Garden City, Hyogo, Japan), beamline BL40B2 at $\lambda = 1.000$ Å. Data were processed with the program package HKL2000.[9] The structure was solved by molecular replacement using the program EPMR[10]with salicylate-bound XOR (Protein Data Bank ID: 1FO4) as a search model. The molecular model was built by using the program O.[11] Refinement was done following standard protocols of the program CNS, Version 1.0.[12] Statistical values will be included in the PDB file of the structure (PDB; 3 BDJ).

RESULTS AND DISCUSSION

Crystal Structure of Oxipurinol-Reduced XOR

The inhibition of XOR with allopurinol was first suggested to be competitive,[13] but subsequently it was shown that the reduced

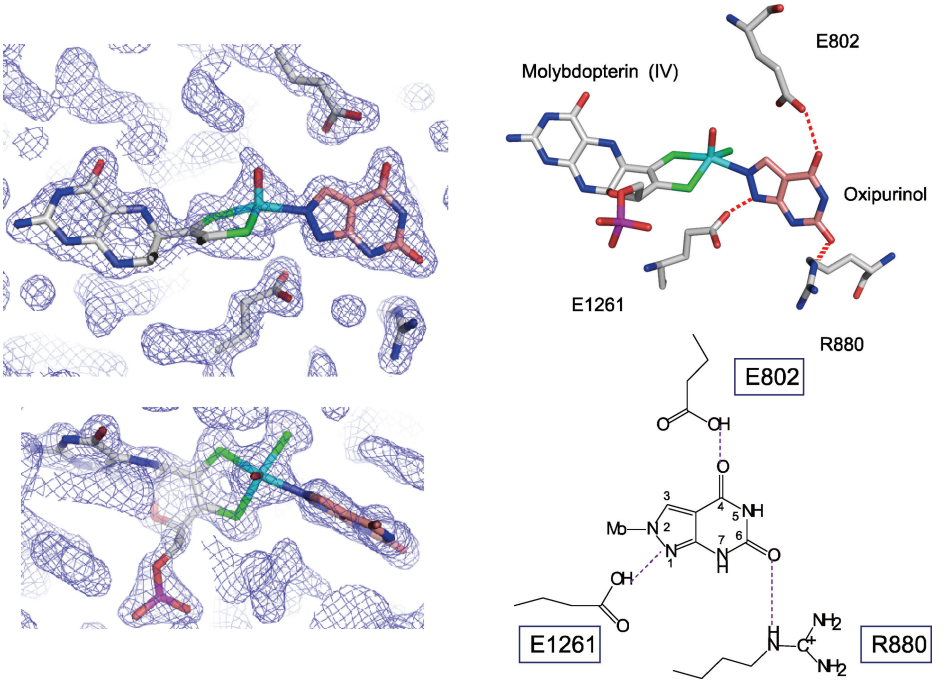


FIGURE 1 Left top and left bottom: Two views of the 2Fo–Fc electron density map contoured at a 1.5 σ cutoff in the active site of the enzyme with the covalent inhibitor bound. Right top: Interaction of oxipurinol and surrounding residues. Oxipurinol (orange), molybdopterin, and catalytically important amino acid residues (CPK-atom colored) are illustrated as stick models. Hydrogen bonds are shown with red broken lines. Right bottom: Schematic representation of the molybdenum-oxipurinol complex. Hydrogen bonds are indicated by broken lines. Residue E802 is presented in its protonated form, consistent with the previously reported structures.[4–6]

molybdenum interacted more tightly with oxipurinol, the hydroxylation product of allopurinol generated by XOR itself.[3] The crystal structure of bacterial XOR mixed with excess allopurinol has been determined at 3Å resolution.[14] In that report, the authors suggested that the bound complex was not allopurinol, but oxipurinol. Here, we discuss the complex of bovine XOR and the oxipurinol inhibitor in more detail, based on a determination of the crystal structure of the complex of pre-reduced fully active bovine XOR and oxipurinol at 2.0 Å resolution.

In this structure, strong electron density was observed connecting oxipurinol and the molybdenum atom (Figure 1, left). The N2 nitrogen replaces an equatorial hydroxyl ligand of the molybdenum atom, coordinating directly to the metal atom at a distance of 2.3 Å with an 180° angle between Mo-ion, N2-atom and the pyrazole ring (Figure 1, left bottom). We conclude that the oxipurinol nitrogen atom replaced the water-exchangeable OH ligand of the molybdenum atom, because the other accessible equatorial position[6] is occupied by a sulfur atom, located at a distance of about 2.4 Å from the central metal ion, a bond length appropriate for a reduced

sulfide group (-SH). Moreover, oxipurinol forms hydrogen bonds with glutamate802 via its 4-position (=O; corresponding to the 6-position of xanthine), arginine880 via its 6-position (=O; corresponding to the 2-position of xanthine) and glutamate1261 via N1. These interactions are essential for the catalytic reaction[15] (Figure 1, right), as shown by an analysis of mutant human enzymes.

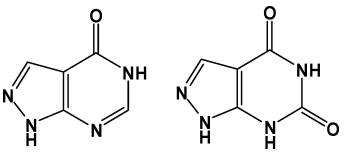
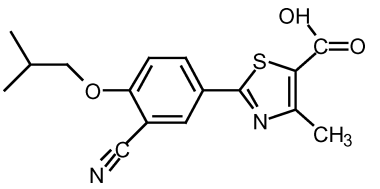
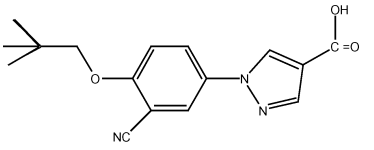
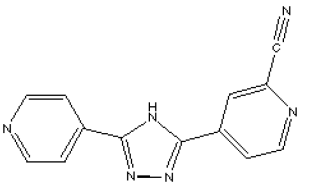
Mutants E803 and R881 (corresponding to E802 and R880 in bovine XOR) exhibit very low, although still significant, hydroxylation activity toward allopurinol, but not inhibition by oxipurinol.[15] Hydrogen bonds between oxipurinol and these amino acids seem to be necessary for the formation of a tight binding complex between the Mo atom and the inhibitor.

Differences in Binding Mode and Mechanism of Inhibition Among Various Inhibitors and Possible Differences of in vivo Effects

We previously determined the crystal structures of xanthine oxidoreductase complexed with the potent inhibitors oxipurinol, febuxostat, Y-700 and FYX-051 (Table 1).[4–6] It was found that each inhibitor has a different binding mode, that is, structure-based or/and mechanism-based. Such variation of the inhibition mechanism seems likely to affect the hypouricemic activity of the inhibitors in vivo.

Febuxostat (TEI-6720, TMX-67) is a potent, nonpurine inhibitor of XOR, and we have reported the mechanism of inhibition based on the crystal structure of the inhibitor-enzyme complex. This inhibitor is currently under clinical evaluation for the treatment of hyperuricemia and gout.[16] Steady-state kinetics measurements revealed mixed-type inhibition with Ki and Ki′ values of $1.2 \pm 0.05 \times 10^{-10}$ M and $9 \pm 0.05 \times 10^{-10}$ M, respectively.[4] In the crystal structure, febuxostat does not form a covalent bond with the molybdenum, in contrast to what has been described above for the oxipurinol-reduced XOR complex. Rather, febuxostat fills the substrate channel of XOR and has numerous interactions with the enzyme protein, including hydrogen bonds, salt bridges, hydrophobic interactions, π-π interactions between aromatic rings, and van der Waals interactions. The potent inhibition of XOR by febuxostat is based on these multiple interactions and the inhibitory mechanism is designated as structure-based. The structural basis of enzyme inhibition by Y-700 is very similar to that of febuxostat, though the potency of Y-700 to inhibit XOR is slightly weaker than that of febuxostat.[5] The difference in the steric bulk of the heterocyclic rings is a plausible explanation for the difference in potency. The thiazole moiety of febuxostat more completely fills the binding pocket. FYX-051, another tight inhibitor, is a slow substrate of XOR and forms a stable reaction intermediate with the enzyme.[6] FYX-051 binds covalently to the molybdenum atom and also has various interactions with amino acid residues. As FYX-051 has features of both mechanism-based inhibition and structure-based

TABLE 1 Chemical structures and inhibition potency of XOR inhibitors

Allopurinol and Oxipurinol		$t_{1/2}$ = 300 minutes, 25°C[3]
Febuxostat (new drug)		Ki = 0.12 nM Ki′ = 0.9 nM[4]
Y-700 (new drug)	 Y-700	Ki = 0.6 nM Ki′ = 3.2 nM[5]
FYX-051 (new drug)		$t_{1/2}$ = 22 hours, 25°C (K. Matsumoto, personal communication, August 1, 2007)

Middle column: chemical structures of the inhibitors. Right column: Ki and Ki′ values are shown for febuxostat and Y-700. Due to the inhibition mechanisms of allopurinol and FYX-051, the Ki and Ki′ values do not properly reflect their inhibition potency. Instead of these values, the half lifes of the inhibitor-enzyme complexes are shown in the table.

inhibition, it is expected to inhibit the enzyme strongly even in the absence of a covalent bond, whereas oxipurinol exhibits only weak inhibition of the enzyme without the covalent bond.

Here, we describe the in detail the features of the clinically relevant interactions between XOR and allopurinol based on the crystal structure of the pre-reduced XOR-oxipurinol complex. Although the inhibition is tight, the oxipurinol-inhibited enzyme is reactivated in a time-dependent manner ($t_{1/2}$ = 300 minutes at 25°C) by spontaneous reoxidation of the molybdenum cofactor.[3] From the clinical point of view, the result is that the enzyme-oxipurinol complex is cleaved spontaneously, accompanied with loss of strong inhibition in vivo. Consequently, patients have to take allopurinol frequently. However, new-generation inhibitors form stable, long-lasting complexes, even with the oxidized enzyme. The complexes may remain stable during the lifetime of the enzyme, so a longer-lasting hypouricemic

effect can be expected. The new inhibitors, because they have different inhibition mechanisms from allopurinol, should be effective alternatives to allopurinol for the treatment of gout and hyperuricemia.

REFERENCES

1. Hille, R.; Nishino, T. Flavoprotein structure and mechanism. 4. Xanthine oxidase and xanthine dehydrogenase. *FASEB J.* **1995**, 9, 995–1003.
2. Elion, G.B. The purine path to chemotherapy, *Science* **1989**, 244, 41–47.
3. Massey, V.; Komai, H.; Palmer, G.; Elion, G.B. On the mechanism of inactivation of xanthine oxidase by allopurinol and other pyrazolo[3,4-D]pyrimidines. *J. Biol. Chem.* **1970**, 245, 2837–2844.
4. Okamoto, K.; Eger, B.T.; Nishino, T.; Kondo, S.; Pai, E.F.; Nishino, T. An extremely potent inhibitor of xanthine oxidoreductase: crystal structure of the enzyme-inhibitor complex and mechanism of inhibition. *J. Biol. Chem.* **2003**, 278, 1848–1855.
5. Fukunari, A.; Okamoto, K.; Nishino, T.; Eger, B.T.; Pai, E.F.; et al. Y-700 [1-[3-Cyano-4-(2,2-dimethylpropoxy)phenyl]-1H-pyrazole-4-carboxylic acid]: a potent xanthine oxidoreductase inhibitor with hepatic excretion. *J. Pharmacol. Exp. Ther.* **2004**, 311, 519–528.
6. Okamoto, K.; Matsumoto, K.; Hille, R.; Eger, B.T.; Pai, E.F.; Nishino, T. The crystal structure of xanthine oxidoreductase during catalysis: implications for reaction mechanism and enzyme inhibition. *Proc. Natl. Acad. Sci. USA* **2004**, 101, 7931–7936.
7. Nishino, T.; Nishino, T.; Tsushima, K. Purification of highly active milk xanthine oxidase by affinity chromatography on Sepharose 4B/folate gel. *FEBS Lett.* **1981**, 31, 369–372.
8. Eger, B.T.; Okamoto, K.; Enroth, C.; Sato, M.; Nishino, et al. Purification, crystallization and preliminary x-ray diffraction studies of xanthine dehydrogenase and xanthine oxidase isolated from bovine milk. *Acta Crystallogr. Sect. D Biol. Crystallogr.* **2000**, 56, 1656–1658.
9. Otwinowski, Z.; Minor, W. Processing of x-ray diffraction data collected in oscillation mode. In *Methods in Enzymology, Volume 276: Macromolecular Crystallography, Part A*, eds. C.W. Carter, Jr., R.M. Sweet, Academic Press, New York, 1997, p. 307–326.
10. Kissinger, C.R.; Gehlhaar, D.K.; Fogel, D.B. Rapid automated molecular replacement by evolutionary search. *Acta Crystallogr. D Biol. Crystallogr.* **1999**, 55, 484–491.
11. Kleywegt, G.J.; Jones, T.A. Efficient rebuilding of protein structures. *Acta Crystallogr. D Biol. Crystallogr.* **1996**, 52, 829–832.
12. Brunger, A.T.; Adams, P.D.; Clore, G.M.; Delano, W.L.; Gros, P.; et al. Crystallography & NMR system: A new software suite for macromolecular structure determination. *Acta Crystallogr. D Biol. Crystallogr.,* **1998**, 54, 905–921.
13. Elion, G.B. Enzymatic and metabolic studies with allopurinol. *Ann. Rheum. Dis.* **1966**, 25, 608–614.
14. Truglio, J.J.; Theis, K.; Leimkühler, S.; Rappa, R.; Rajagopalan, K.V.; Kisker, C. Crystal structures of the active and alloxanthine-inhibited forms of xanthine dehydrogenase from *Rhodobacter capsulatus*. *Structure* **2005**, 10, 115–125.
15. Yamaguchi, Y.; Matsumura, T.; Ichida, K.; Okamoto, K.; Nishino, T. Human xanthine oxidase changes its substrate specificity to aldehyde oxidase type upon mutation of amino acid residues in the active site: roles of active site residues in binding and activation of purine substrate. *J. Biochem.* (Tokyo) **2007**, 141, 513–524.
16. Becker, M.A.; Schumacher, H.R.Jr.; Wortmann, R.L.; MacDonald, P.A.; Eustace, D.; et al. Febuxostat compared with allopurinol in patients with hyperuricemia and gout. *N. Engl. J. Med.* **2005**, 353, 2450–2461.