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Computational exploration of reactive fragment for mechanism-based inhibition of xanthine oxidase

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ABSTRACT

The xanthine oxidase (XO) family is a group of molybdenum-containing enzymes that catalyze the transfer of an oxygen atom from water to a substrate, such as xanthine or aldehyde. The proposed mechanism for the reductive half-reaction of xanthine oxidase involves nucleophilic addition of Mo-bound hydroxide to the substrate and hydride transfer from the substrate to sulfido group (Mo=S) at the molybdenum cofactor (Moco) reaction center. A DFT study of a mechanism involving the near-by glutamic acid (Glu) residue for the oxidation of several model substrates found that the direct involvement of the Glu residue side chain significantly increases the reactivity of the cofactor. The reaction is stepwise with a hydride transfer as the rate determining step. On the basis of mechanistic studies, a QM model was established to search the potential reactive fragments for mechanism-based inhibitors. Two important parameters were designed to evaluate each fragment: the activation enthalpy ($\Delta H^{\neq}$) which estimates how effectively an inhibitor competes with xanthine to react with the Moco site, and the enthalpy associated with release of the product (ΔH) which estimates how easily the product in product complex (**PRO**) could be replaced by a water molecule. The computational findings add to our understanding of the catalytic mechanism of xanthine oxidase, and also assist the screening of mechanism-based inhibitors for this important drug target.

1. Introduction

The xanthine oxidase family is a group of molybdenum-containing enzymes that catalyze the transfer of an oxygen atom from water to a substrate, such as xanthine or aldehyde [1–4]. In the metabolism of purines in the human body, xanthine oxidase catalyzes the oxidative hydroxylation of hypoxanthine to xanthine and of xanthine to uric acid [1–4]. A high expression of xanthine oxidase is associated with overproduction of uric acid which leads to gout [5,6], hyperuricemia [7], cancer [8], and other diseases [9–12]. Xanthine oxidase is a validated target of drugs or inhibitors for therapeutic treatment of gout and hyperuricemia. Numerous inhibitors, which show considerable promise in the treatment of these conditions, have been developed [13–15].

Three inhibitors, allopurinol, febuxostat and topiroxostat (Japan in 2013), have been clinically approved as *anti*-hyperuricemic/gout agents. Among them, the recently approved topiroxostat (Fig. 1(a)) is cost effective and well tolerated in patients, reduces serum uric acid levels in hemodialysis patients with or without chronic kidney disease or gout [16–20].

Topiroxostat is metabolized mainly by glucuronidation and oxidation in the liver and there is no need for dose reduction when treating patients with mild-to-severe renal impairment [21]. A kinetics study showed that topiroxostat initially behaves as a competitive-type inhibitor with $K_i = 5.7 \times 10^{-9}$ M [22]. The mechanism of reaction and enzyme inhibition was further revealed by the crystal structure of a key intermediate in the hydroxylation reaction of XO with topiroxostat [23]. In the crystal structure (PDB: 1V97), topiroxostat forms a covalent bond with molybdenum through oxygen, along with various interactions at the binding site [23]. Topiroxostat is a hybrid-type inhibitor exhibiting both structure- and mechanism-based inhibition of XO. Therefore,

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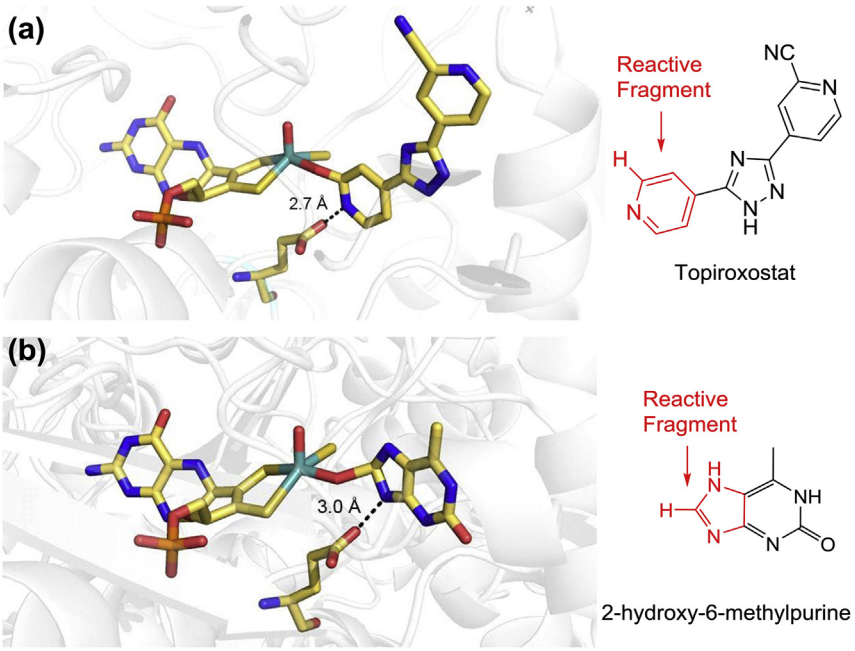

Fig. 1. The crystal structures of the XO Moco center involving (a) topiroxostat (1V97) and (b) 2-hydroxy-6-methylpurine (2-HMP) (3B9J).

understanding the reaction mechanism of this enzyme is a key to the development of mechanism-based inhibitors [13]. The crystal structures of several enzymes of the family are available and provide much information for mechanistic study [23–27]. Huber and his co-workers proposed the structure-based mechanism on the basis of the crystal structures of aldehyde oxidoreductase from *Desulfovibrio gigas* (Mop), including an alcohol-bound form [25]. Additional crystal structures involving 2-hydroxy-6-methylpurine (2-HMP) reveal more details of the inhibition mechanism (Fig. 1 (b)) [28]. The proposed mechanism involves nucleophilic addition of the Mo-bound hydroxide to the substrate associated with hydride transfer from the substrate to the sulfido group (Mo=S) at molybdenum cofactor reaction center [25].

In the xanthine oxidase family, a glutamic acid residue is universally conserved at the environment of the Moco site, [3]. As depicted in Fig. 2, the glutamic acid residues conserved in different enzymes, such as xanthine dehydrogenase (XDH), aldehyde oxidase 3 (mAOX3), and quinoline 2-oxidoreductase (Qor) show a high similarity. The oxygen atom O1, which is at the bottom of the entry pocket approaching the Moco, is believed to be the catalytically labile oxygen [31,32]. The distances between oxygen atoms O1 and the O2 of the Glu carboxylate are 2.9 Å, 4.0 Å and 3.1 Å in three enzymes, suggesting the presence of hydrogen bonds. In addition to the interaction between the Glu residue and the Moco, interaction of the same Glu residue with the substrates or inhibitors (Fig. 1), thought to involve a H-bond, was also reported [23]. It indicated that the Glu could form an H-bond not only with the Moco but also with the substrates or inhibitors. Since the Glu residue side chain is likely to be deprotonated at pH = 7, it can act only as a proton-acceptor. Therefore, the proton is presumed to originate from the substrate. Leimkühler and her co-workers demonstrated the indispensable role of this Glu in the catalysis by site-directed mutagenesis studies of xanthine dehydrogenase from *R. capsulatus* [33]. When Glu730 was mutated in E730A, E730Q, E730R, or E730D mutants, the reaction rate was decreased by a factor of at least 10^7 in the kinetic studies [33], implying that the glutamic acid residue stabilizes the transition state by at least 10 kcal/mol in energy [34]. This "conserved" glutamic acid residue was supposed to deprotonate the Mo-H_2O moiety [23,25,35], and orient the substrates [23].

Comprehensive quantum mechanical/molecular mechanical (QM/MM) studies conducted by Thiel and co-workers [36,37], revealed a multistep mechanism, in which the hydride transfer is the rate-determining step. Several side chains of nearby residues including Glu802, Arg880, and Glu1261, were considered in the QM region. Glu1261 was found to play important roles in the reaction, such as deprotonation/protonation of the substrate and guiding the approach of the substrate to the Mo cofactor. A simplified model of the reductive half reaction mediated by the Moco with the assistance of Glu1261 is depicted in Scheme 1. Upon the coordination of water to Mo(VI), one of the hydrogen atoms is abstracted by the Glu residue to form the stable oxidized Moco precursor. A hydrogen bond is formed between the carbonyl of the Glu residue and the OH group of Moco. The carboxyl group of the Glu residue may either release the proton to or form a hydrogen bond with a nearby base, which could be a water molecule found in the active site of xanthine oxidases [25]. The *p*Ka of the glutamic acid at the active site (6.5) [32,35] is higher than the intrinsic *p*Ka value (4.3) of a glutamic acid residue, indicating that the neutral carboxylic acid is stabilized in the enzyme circumstance. Even at pH = 7, the proton of the glutamic acid is supposed to be around the active site as a counter-ion, bound to a nearby base. This proton is also required for product release following protonation of N7 of the xanthine. The presence of the proton is supported by existence of an H-bond between Glu and the oxidized product [23]. When the xanthine substrate approaches the active site, it is protonated and complexes with the Glu anion by an N7–H-O hydrogen bond. This complex formation is followed by nucleophilic attack at C8 of xanthine by the OH group of the Moco. Meanwhile, the Glu abstracts the proton from the Moco-OH, facilitating the reaction by enhancing the nucleophilicity of the hydroxyl group. The C = X (X = O, or N) bond of the substrate is activated by the protonation on X as well. The next step is hydride transfer from the C8 of the xanthine to the sulfido group of the Moco, and in this process the Mo(VI) is reduced to Mo(IV) [38]. An alternative pathway requires the nucleophilic attack and the hydride transfer to be concerted. The product