

# Crystal Structures of the Active and Alloxanthine Inhibited Forms of Xanthine Dehydrogenase from *Rhodobacter capsulatus*

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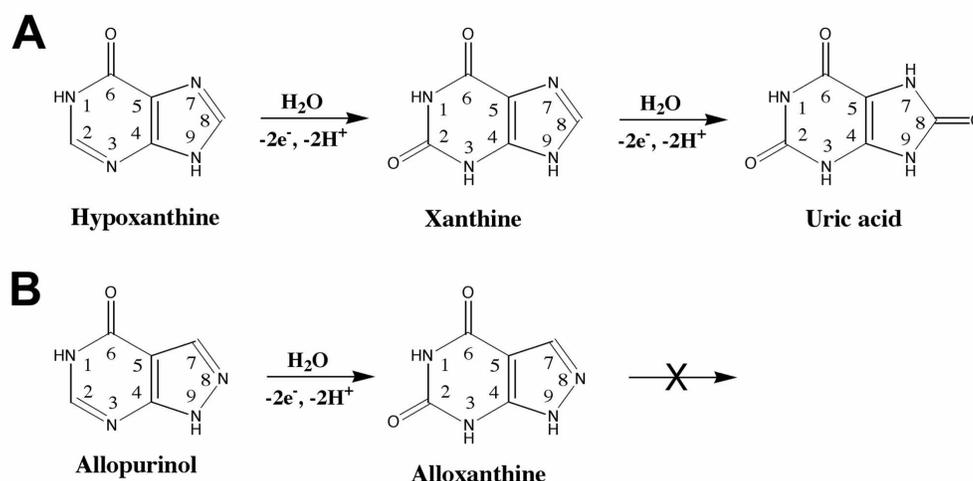
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Xanthine dehydrogenase/oxidase (XDH/XO) is a complex metallo-flavoprotein that catalyzes the oxidative hydroxylation of purines, pyrimidines, pterins, and aldehyde substrates (Figure 1) [1]. Upon substrate oxidation by the molybdopterin center, electrons are transferred across the enzyme via two [2Fe-2S] clusters to the FAD cofactor where either NAD<sup>+</sup> (XDH) or molecular oxygen (XO) is reduced. The enzyme is synthesized as a dehydrogenase, but those from mammalian sources can undergo a dehydrogenase to oxidase conversion whereby they lose the ability to use NAD<sup>+</sup> as electron acceptor. A well-characterized prokaryotic XDH with similar activity to the eukaryotic counterparts is the enzyme isolated from the phototrophic purple bacterium *Rhodobacter capsulatus* [3]. *R. capsulatus* XDH (RcXDH) is a cytoplasmic enzyme with an (ab)<sub>2</sub>

heterotetrameric structure and a molecular mass of 275 kDa. Although very similar to eukaryotic XDH enzymes, RcXDH is isolated with high reactivity towards NAD<sup>+</sup> and low reactivity towards oxygen and does not undergo conversion to the oxidase form. The cofactors were identified to be located on two different polypeptides with the iron sulfur clusters and the FAD bound to the XDHA subunit, and the molybdenum cofactor (Moco) bound to the XDHB subunit. In contrast, all three cofactors are bound within a single subunit in eukaryotes. The amino acid sequence of RcXDH has a high degree of similarity to eukaryotic xanthine dehydrogenases/oxidases. Furthermore, analysis of the Moco in RcXDH revealed the presence of molybdopterin (MPT), as found for all eukaryotic Moco containing enzymes. This is in contrast to most bacterial molybdoenzymes



**Figure 1.** Reactions catalyzed by Xanthine dehydrogenase. (A) Hypoxanthine is converted to xanthine and then to uric acid in the oxidative half-reaction while the Mo center is reduced from the +VI to the +IV state in each reaction. The reductive half-reaction to reestablish the Mo(VI) state is not shown. (B) Inhibition by allopurinol. Allopurinol is converted to alloxanthine, which is not further oxidized by XDH, but binds tightly at the active site of XDH when in the Mo(IV) state. The atom-numbering scheme is indicated.

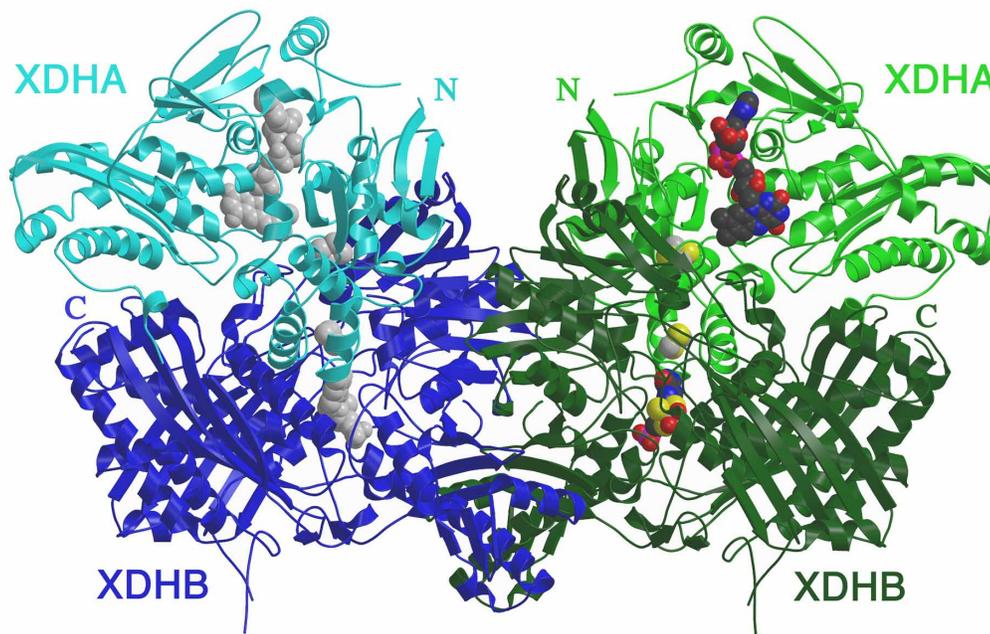
in which the terminal phosphate of MPT is covalently attached to an additional nucleotide such as GMP or CMP to generate different dinucleotide forms of the cofactor.

The crystal structure of active *R. capsulatus* XDH was solved by molecular replacement using data obtained from NSLS beamlines X26C and X25 (Figure 2) [4] and was refined to 2.7 Å resolution. Despite differences in subunit composition, the overall structure of RcXDH and the bovine XDH (bXDH) are surprisingly similar, but differ in important details. This includes the regions necessary for XDH to XO conversion in bXDH, a process not observed in RcXDH. Interestingly, sequence stretches allowing this conversion are strictly conserved in eukaryotes, as are sequence stretches in prokaryotes that appear to inhibit this conformational change in RcXDH. It has been proposed that in mammals, the presence of XO in milk has a beneficial bactericidal effect. Taking into account that the chicken enzyme is also not converted to the XO-form, it is possible that the ability of XDH to be converted to XO has emerged through divergent evolution in mammals, but not in other organisms.

In humans, urate, the ionized form of uric acid that predominates at physiological pH, is the final product of the XDH-catalyzed purine degradation and is ex-

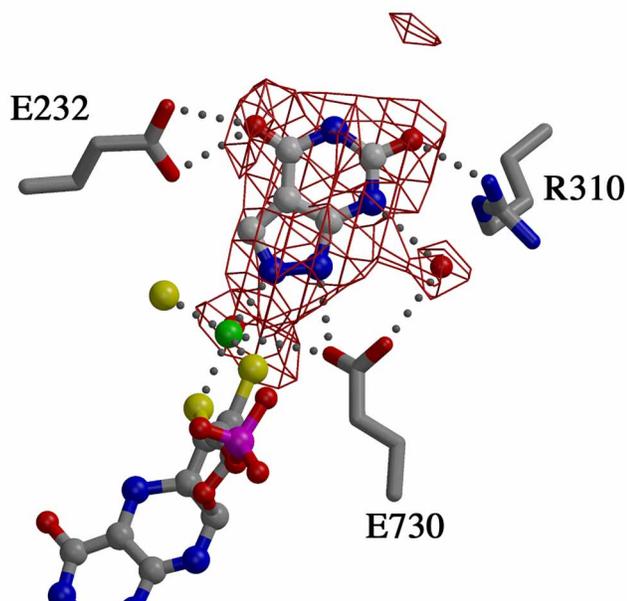
creted in the urine. High circulating levels of uric acid (hyperuricemia) can have severe effects due to the low solubility of sodium urate. The XDH-inhibitor allopurinol (1*H*-pyrazolo[3,4-*d*] pyrimidin-4-ol; Figure 1) is a very common, orally administered drug used to treat hyperuricemia associated with chronic gout (gouty arthritis), cancer chemotherapy, or hyperplastic or radiation therapy. It has also been used to aid in post ischemic reperfusion injury caused partly by the formation of oxygen-derived free radicals from XO. XDH (or XO), in the Mo(VI) state, oxidizes allopurinol to alloxanthine (1*H*-pyrazolo [3,4-*d*] pyrimidine-4,6-diol; also known as oxypurinol). The now reduced enzyme in the Mo(IV) state forms a tight, inactive complex with alloxanthine, thereby blocking the substrate-binding site.

RcXDH was crystallized in the presence of allopurinol. The inhibited crystal structure was solved to 3.0 Å by difference Fourier methods using data collected at NSLS beamline X4A. Difference density was observed at the active site, but the exact chemical nature of the ligand could not be determined unambiguously by crystallographic data alone. To verify that RcXDH turns over allopurinol under crystallization conditions and remains bound to alloxanthine, excess allopurinol was removed by gel filtration, the protein was denatured by heat, and the extract analyzed by reversed



**Figure 2.** Structure of RcXDH. RcXDH forms an  $(\alpha\beta)_2$  heterotetramer. The XDHA subunits are drawn in light green and light blue and the XDHB subunits in dark green and dark blue. The N- and C-termini of each subunit are labeled in their respective colors with N and C. The [2Fe-2S] and FAD cofactors of XDHA and the Moco of XDHB are shown as space-filling models. In one heterodimer the cofactors are color coded with the Fe in gray and the Mo in green. The latter is hidden by the dithiolene sulfurs in this view.

phase HPLC. The results clearly demonstrate that alloxanthine is bound to RcXDH during the inactivation process. Notably, alloxanthine coordinates directly to the molybdenum ion of the molybdenum cofactor, displacing the hydroxo-ligand (Figure 3). This structure provides the first crystallographic insight into the mechanism of inhibition by alloxanthine.



**Figure 3.** Inhibition of the Moco active site.  $F_o - F_c$  difference electron density contoured at  $4\sigma$  (red mesh) obtained from crystals of allopurinol-incubated RcXDH shows RcXDH inhibited by alloxanthine. The density was calculated before introducing the inhibitor into the crystallographic model to avoid model bias and was averaged over all four active sites present in the a.s.u. to increase the signal to noise ratio. Residues interacting with the inhibitor are shown in all-bond representation and are indicated by numbers. The molybdenum and its sulfo and oxo ligand are shown as green, yellow and red (partially hidden in this view) spheres, respectively.

Because of the high structural similarity of RcXDH and bXDH, the bacterial enzyme is a good model system for studying the mechanism of drug action. Allopurinol has been widely used for the clinical control of uric acid production in conditions of hyperuricemia. However, allopurinol toxicity and a severe life-threatening toxicity syndrome have been reported in some patients, leading to eosinophilia, vasculitis, rash, hepatitis, and progressive renal failure [5]. Although new as well as previously known compounds have recently been discovered to inhibit XDH/XO activity, a clinically effective inhibitor has not been developed to treat hyperuricemia since allopurinol was introduced for patient use in 1963. The crystal structure of RcXDH with alloxanthine bound advances our understanding of XDH/XO inhibition by allopurinol, revealing its direct coordination to the Mo ion of the Moco. The high structural and sequence similarity between RcXDH and its mammalian counterpart bXDH, especially in their respective active sites, will allow these data to aid in the design of new, more effective clinical inhibitors.

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