

# Xanthine Dehydrogenase Active Site: Chiral Switching and Substrate Coordination

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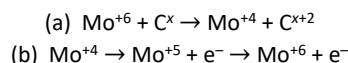
**Abstract:** Analysis of electronic, structural and mechanistic parameters of the enzyme-substrate reaction of xanthine oxidase, a member of the xanthine dehydrogenase class of mono-molybdopterin oxidoreductive enzymes, shows that the molybdenum center in the enzyme active site acts as a reversible chiral switch. The metal center cycles from the (*S*)-absolute configuration, *SPY-5-42-A*, in the fully oxidized state, Mo(VI), to the (*R*)-absolute configuration, *SPY-5-43-C*, for the fully reduced metal center, Mo(IV). This process is complemented by induction of chirality at the substrate carbon center (*pro-S<sub>C</sub>* → *S<sub>C</sub>*) and is involved in the control of coordination and, likely, protonation of imino-centers of conjugated heterocyclic substrates in the enzyme active site.

**Keywords:** xanthine dehydrogenase, molybdopterin cofactor, oxidoreductive catalysis, first-principles electronic structure calculation, Mo-center chirality switching, enzyme-substrate coordination control.

## INTRODUCTION

**X**ANTHINE dehydrogenase, XDH, [EC 1.17.1.4],<sup>[1]</sup> is the central member of the class of molybdopterin-containing oxidoreductive metalloenzymes. Expressed in variety of tissues across the phylogenetic tree of organisms, the enzyme catalyses 2-electron oxidation of a range of C-substrates.<sup>[2]</sup> Each of the C2-symmetry related 1333 amino-acid peptide subunits, XDH\_HUMAN,<sup>[3]</sup> in the enzyme homodimer houses four cofactors: (i) a phosphomolybdopterin unit, MPT, Moco – the active site, (ii, iii) two ferredoxin-type iron-sulfur units, [2Fe-2S] and (iv) a flavin adenine dinucleotide unit, FAD.<sup>[2,4]</sup> In the course of a XDH-catalyzed oxidative reaction the Mo(VI) center in the enzyme active site undergoes a 2-electron-equivalent reduction to Mo(IV) and, in the reverse half-cycle, two 1-electron re-oxidations to the initial Mo(VI) state.<sup>[5,6]</sup> The redox reactions for the two half-cycles are given in Scheme 1.

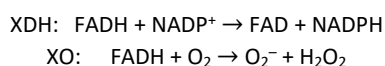
With hypoxanthine and xanthine, the principal substrates in the rate-controlling terminal steps of the oxidative degradation of purine metabolites in mammals, the formal oxidation state of the target carbon atom, “*x*” in the Scheme 1a, is +2. Additionally, C-substrates with



**Scheme 1.** (a) In the first half-cycle of the oxidoreductive reaction molybdenum center accepts 2 electrons from substrate and (b) in the second half-reaction molybdenum undergoes stepwise re-oxidation, forming in the process an experimentally detected Mo(V) species. (Note: The hydroxyl group transferred in the first half-cycle from enzyme to the substrate C-center does not change oxidation state and is omitted for clarity.)

oxidation states  $x = -1$ ,<sup>[7]</sup> and  $x = +1$ <sup>[8]</sup> are known to be on the enzyme substrate menu.

The two-electron equivalents passed from substrate to the Mo-center active site are then relayed to FADH and then to the terminal electron acceptor. In XDH this is nicotinamide adenine dinucleotide, NAD<sup>+</sup>.<sup>[9,10]</sup> With mammalian enzyme xanthine oxidase, XO, EC 1.17.3.2,<sup>[11]</sup> a member of the same class of enzymes derived from XDH either by reversible reduction/oxidation of a pair of Cys residues<sup>[12]</sup> or irreversible transformation by partial



**Scheme 2.** In XDH the reduced flavin adenine dinucleotide cofactor, FADH, the terminal cofactor in the protein electron transfer relay line, passes two electrons to an oxidized nicotinamide adenine dinucleotide, as the terminal electron acceptor, while in XO the terminal two-electron acceptor is dioxygen which then forms dioxygen radical anion and dioxygen dianion (hydrogen peroxide).

proteolysis<sup>[13,14,15,16]</sup> dioxygen rather than the NAD<sup>+</sup>/NADH redox pair serves as terminal electron acceptor,<sup>[6]</sup> as illustrated by Scheme 2.

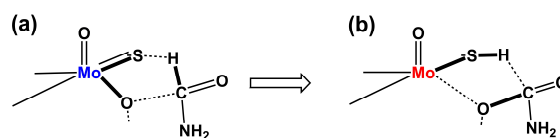
A two-point mutation in the XDH\_HUMAN gene,<sup>[14]</sup> hXDH,<sup>[17–19]</sup> degrades the enzyme performance and results in the phenotypic xanthinuria-1 pathology (XAN1) while a co-deficiency of XDH and Aldehyde Oxidoreductase, AOXA\_HUMAN,<sup>[20]</sup> another enzyme in the same class, or a genetically-induced deficiency in the Mo-pterin cofactor itself, causes xanthinuria type II (XAN2), a genetic deficiency resulting in a wide range, from asymptomatic to relatively mild, conditions. The deficiency of the molybdopterin cofactor in the related, sulfite oxidase, class of enzymes is a cause of imminent infant mortality.<sup>[21,22]</sup>

In the reactions catalyzed by the XO form of the enzyme [EC 1.17.3.2], generation of O<sub>2</sub><sup>-</sup> and H<sub>2</sub>O<sub>2</sub> species introduces yet another set of biological variables, relating this class of enzymes with oxidative injury as well as the formation of the NO· hormone; this is apparently an aspect attracting progressively more attention and scrutiny.<sup>[23]</sup>

The relative stability of this class of proteins, their ubiquity and importance in humans, plants and bacteria<sup>[24–26]</sup> have attracted considerable research interest, starting with Franz Schardinger in as early as 1902<sup>[27]</sup> (hence the long-standing name “Schardinger enzyme” for what was allegedly the XO form of the enzyme) or even earlier. The large opus of kinetic, molecular biological, UV/VIS, scattering and resonance Raman, ENDOR, ESEEM and EPR spectroscopic, and XAFS and x-ray structural, studies accrued over the past century has brought about significant advances in our understanding of the XDH class of enzymes. Here, I recognize and highlight another intrinsic property – the absolute configuration of the enzyme metal center – and introduce it as a novel and more precise descriptor of the enzyme structural and catalytic properties.

## RESULTS AND DISCUSSION

Electronic structure calculations of enzyme models have brought a focused integration of the experimental evidence and provided a plausible picture of chemical-mechanistic



**Scheme 3.** The topology of the enzyme-formamide substrate transition state: The enzyme Mo(VI) and the substrate C-center in +2 formal oxidation state (a) undergo a metathetic exchange which yields the enzyme Mo(IV) form and the substrate C-center in +4 oxidation state (b).

parameters not amenable to experiment. Begun in part in 1997 and 1998,<sup>[28–30]</sup> first-principles electronic structure calculation of the reaction transition state were subsequently carried out in a more systematic way,<sup>[31]</sup> starting from 46 completely random pair-coordination of enedithiolate model of molybdopterin center, LMo<sup>+6</sup>OS(OH) center (L = enedithiolate: –S–CH=CH–S–) and formamide (a bona fide enzyme substrate).<sup>[7]</sup> A first-order saddle point on the Mo-enedithiolate : formamide potential energy surface [PES] suggests a synchronous metathetic-type exchange between the enzyme Mo-center and the substrate C-center, without formation of an organometallic bond,<sup>[31]</sup> Scheme 3. The result was obtained using the Møller/Plesset’s method for perturbative correction of electron correlation (MP4//MP2<sup>[32]</sup> – *i.e.* fourth order energy correction using the structure optimized using the second MP order), with the double-zeta Los Alamos basis set LanLDZ,<sup>[33,34]</sup> as implemented within the Gaussian computer program.<sup>[35]</sup>

In the calculations using the MP4//MP2 formalism for electron correlation correction the electron density on the substrate H-center shows a significant increase with the reaction progress, thus providing a strong support for the reaction model based on substrate-enzyme hydride transfer and a 2-electron reduction of the Mo center. It should be noted that this has not been the case in the calculations reported to use Density Functional Theory, DFT, methods, even for the DFT calculations using identical reaction model.<sup>[36]</sup> Subsequent calculations of the same reaction scenario<sup>[37]</sup> have branched out along approximately two principal directions. One direction followed an extension of the size of the Mo-enedithiolate : formamide model, from the initial 17-atom reaction pair,<sup>[28–31]</sup> to a 39 atom model with the inclusion of xanthine substrate,<sup>[38]</sup> and to a 23357-atom cluster comprising, in addition to the enzyme-substrate reacting pair, solvent molecules treated by molecular mechanics, MM, methods.<sup>[39]</sup> The other direction was focused principally on the use of more refined and also more varied theoretical formalism, for example the CCSD(T) formalism<sup>[40,41]</sup> and a number of DFT methods. While expanding the theater of possible atomic

exchange scenarios in the reactions catalyzed by this class of enzymes the results of these calculations have largely supported the geometry – if not the energy and the electron density distribution - of the transition state model presented in Scheme 3.

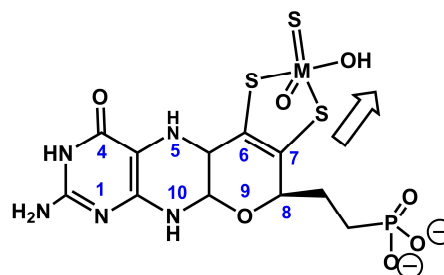
I deem a note on nomenclature to be appropriate at this point. Firstly, in virtually all mechanistic, structural and computational studies on the XDH class of enzymes the thioxo ligand, =S, has been consistently named “sulfide” thus confusing the ligand inventory in the oxidized, Mo(VI), with that of the reduced, Mo(IV), forms of the cofactor. Secondly, the hydron particle passed from a C-substrate to the =S ligand of the Mo-pterin center in the enzyme active site has been consistently named “proton” and the transfer has been designated as protonation. This is doubly wrong as, firstly, it conflates a Brønsted-acid-type reaction with a hydride transfer or hydridation and, secondly, it completely contradicts the currently accepted reaction mechanistic model for this class of enzymes. This is an unfortunate practice of promoting erroneous nomenclature and causing unnecessary confusion.

This geometry of a XDH : substrate transition state is posited to comprise a nearly planar pentagon subtended by the molybdenum center and its thioxo and hydroxyl ligands on the enzyme side and the carbon-hydride pair on the substrate side. The suggested scission of the Mo–O(H) and the formation of the S–H bonds on the enzyme side, and the scission of the C–H and the formation of the C–O(H) bonds on the substrate side, lead to a completion of the substrate oxidation and a separation of the reaction product.

A brief inspection of the reaction geometry and pair-coordination, and the posited change in the character and position of the Mo ligands prompts a question about the chirality of the enzyme active site. Aside from a reference on the absolute configuration of the C<sup>(2)</sup>-center in the C6-side-chain of a de-metalized 2-aminopteridine precursor / oxidation product<sup>[42]</sup> there have been no reported studies of the absolute configuration of the metal center in molybdopterin enzymes. Nor, seemingly, in other natural metalloenzymes.

A note of caution is needed at this point regarding the “correct” structure of the molybdopterin center as it has been reported for the XDH class of oxidoreductases. Firstly, for the correct ranking of molybdenum ligands we need to consider the complete molybdo-enedithiolato-pyranopterin center Figure 1, as the Mo-enedithiolate model of the enzyme active site used successfully in the energy profile calculations, Scheme 1, does not provide sufficient structural information.

This point requires additional scrutiny as the position of the thioxo ligand, Mo = S, in the enzyme molybdopterin cofactor has not been well defined in a number of protein

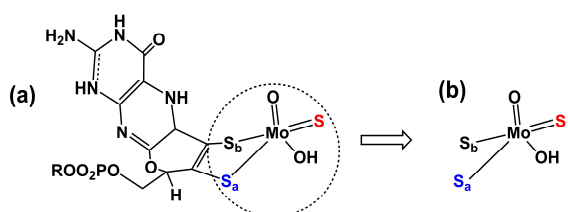


**Figure 1.** Mo(VI) molybdopterin monophosphate, MPT, Moco: {[{(5aR,8R,9aR)-2-amino-4-oxo-6,7-di(sulfanyl-κS)-1,5,5a,8,9a,10-hexahydro-4H-pyrano[3,2-g]pteridin-8-yl)methyl dihydrogenato(2-) phosphate}(oxo-thiohydroxy)molybdate, C<sub>10</sub>H<sub>10</sub>N<sub>5</sub>O<sub>7</sub>PS<sub>3</sub>Mo [CAS RN: 9002-17-9 (dioxo form)], viewed “from above”, that is, along the O=Mo line, as indicated by the enclosed arrow.

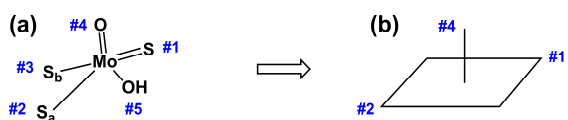
crystal x-ray diffraction experiments; this was either because the x-ray diffraction was collected from a sample of de-sulfo enzyme form<sup>[43]</sup> or the experimental electron densities on the oxo and sulfoxo ligands were not sufficiently discernible.<sup>[44]</sup> One consequence of this uncertainty is that the molecular models used in numerous electronic structure studies of Mo-enedithiolate or Mo-enedithiolate : substrate reported since 1996 are either rotamers-enantiomers<sup>[45]</sup> or enantiomers of the correct structure;<sup>[28–31,36,46,47]</sup> in at least one report the correct structure is given in the article but enantiomers are provided in the supplementary material.<sup>[36]</sup> The recent reports on the crystal structure of XOR with an inhibitor<sup>[48]</sup> and the crystal structure of quinoline 2-oxidoreductase, Q<sub>OR</sub>,<sup>[49]</sup> have brought this important issue to closure. Here, I use a consensus of the molybdenum center structures reported for the bovine milk inhibitor-bound xanthine dehydrogenase, PDB ID codes 3UNA<sup>[16]</sup> and 1FO4,<sup>[48]</sup> xanthine oxidase, PDB ID code 1FIQ,<sup>[13]</sup> and the bacterial quinoline 2-oxidoreductase, PDB ID code 1T3Q.<sup>[49]</sup> The structure of this molybdopterin cofactor is defined by a pyramidal Mo-enedithiolate ring, cata-condensed with pyranopterine; the four-ring cofactor is twisted to assume M-helicity [M = minus]. The molybdenum oxo ligand, Mo=O, is apical and the hydroxyl, Mo-OH, and thioxo, Mo=S, ligands are equatorial. The methylphospho, –CH<sub>2</sub>–OPO<sub>3</sub>R, substituent at the position C2' of the pyrano ring is *cis*- to the Mo=O apical ligand; the structure of this cofactor is given in Step #1 below.

When determining the absolute configuration of the metal center in the Mo-pterin active site of the XDH class of enzymes, I note that the original CIP rule provides no guidance, stating specifically: “However, since we know of no molecule whose chirality depends on that of a configurationally stable, chiral, quinqueliant atom, we shall for the present refrain from detailing such a

procedure".<sup>[50]</sup> So I resorted to the early works delineating an extension of the CIP rules<sup>[51,52]</sup> and the current rules for the structure and nomenclature of inorganic complexes, given in the so-called "Red Book 2005",<sup>[53]</sup> referred to as "Recommendations" in this text. Given the possible broader didactic benefits I present the procedure applied here in full detail, Step #1 through Step #6.



**Step #1.** Abstraction of a full monophosphomolybdopterin, MPT, (a) to a 5-coordinated molybdenum center (b) with clearly designated dithiolate sulfur centers,  $S_a$  and  $S_b$ .



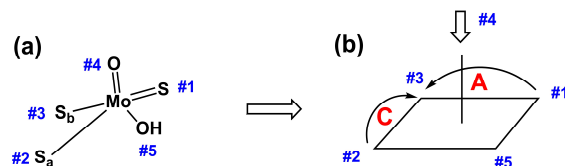
**Step #2.** In the geometrical (a) and topological (b) representation of the Mo-center the ligands are ranked and numbered according to the CIP rule:<sup>[50]</sup> the  $S_a$  sulfide, being closer to the pyrano oxygen (3<sup>rd</sup> tier, see Step #1), has a higher rank than  $S_b$  sulfide and is assigned higher priority while the  $S_b$  sulfide, closer to the pyrazine nitrogen (3<sup>rd</sup> tier), is assigned lower priority.

**Step #3:** The *geometry* of the complex is determined to be 5-coordinate square pyramidal, SPY-5, according to the Table IR-9.3 of Recommendations.

**Step #4:** According to the Rule IR-9.3.3.5 of Recommendations, the *configuration index* of the Mo(VI) center is determined to be 42. The first digit, 4, is the priority ranking of the axial ligand (=O, #4) pointing to the metal center (along the axis of observation) and the second digit, 2, is the priority ranking of the ligand ( $S_b$ , #2) which is *trans*- to the highest priority ligand, the thioxo group (=S, #1). So the designation of the *geometry* and the *configuration index* of the  $O=Mo^V(=S)S_aS_b(OH)$  complex is SPY-5-42.<sup>[53,54]</sup>

**Step #5:** According to Recommendations (IR-9.3.4.6), *The C/A convention for square pyramidal centres*, the clockwise and anticlockwise sequences of priority numbers are compared and the structure is assigned the symbol C or A according to whether the clockwise (C) or anticlockwise (A) sequence is lower at the first point of difference. The point of difference in the Mo-pterin center is ligand #3, the

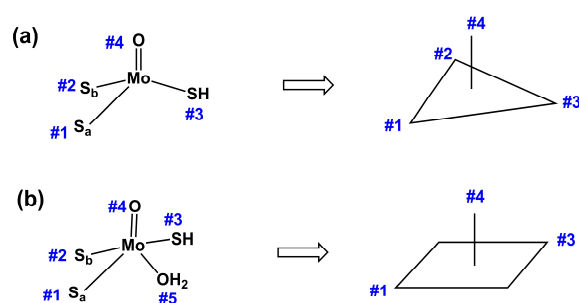
lower ranking dithiolate sulfur; see also Scheme 4a. The path from #1 to #3 is anti-clockwise (counter-clockwise), A, and the path from #2 to #3 is clockwise, C. Since  $1 + 3 = 4$  is a lower numbered sequence than  $2 + 3 = 5$ , the prevalent configuration is A. So the Mo-center has left-handed *absolute configuration* and its full designation is SPY-5-42-A.



**Step #5.** The Mo(VI)-pterin center geometrical abstraction (a) and the absolute configuration of its topological representation (b), SPY-5-42-A.

The same absolute configuration for the Mo(VI) center in XDH has been derived using a different methodology.<sup>[51,52]</sup>

**Step #6:** The preceding Mo-pterin coordination is that of the oxidized, reaction-ready Mo(VI) center in the active site of xanthine and aldehyde oxidoreductive enzymes, XDH, AOX, XO, XOR. Following the metathetic exchange with substrate, Scheme 3, the Mo(VI)-center loses the O(H) ligand to the substrate to become a two-electron-reduced Mo(IV) tetrahedral complex,<sup>[31,41]</sup> (a), or, as invoked in early mechanistic considerations and suggested by some x-ray structural studies<sup>[43]</sup> a 5-coordinated aqua-Mo(IV) complex (b):



**Step #6.** The enzyme Mo-center and its topological representation, immediately following separation of reaction product (a), and the same center, Mo(IV), after acquisition of a water molecule (which is thermodynamically preferred to hydroxyl group) and its topological representation (b).

The tetrahedral Mo(IV) complex is of *R*-absolute configuration and the 5-coordinated aqua complex could be shown to possess the SPY-5-43-C absolute configuration. Both centers are right-handed. As the full oxidoreductive

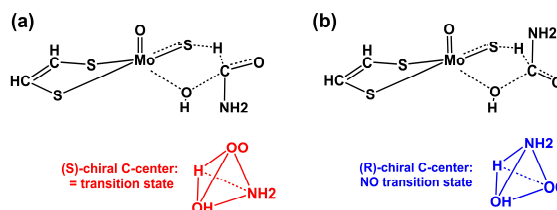
cycle involves both the Mo(VI) and Mo(IV) states of the enzyme active site I postulate the following:

The molybdenum center of the Mo-pterin complex in the active site of the xanthine dehydrogenase enzyme acts in the course of a complete oxidoreductive cycle as a reversible chiral switch.

And how does this relate to the spatial distribution of the atomic centers in the enzyme substrates and products? Given the type of chemical transformation of the substrate carbonyl carbon and the transient topology of the enzyme-substrate complex one could argue that the relative orientation of substrate may not be of particular importance to the reaction outcome. With formamide as substrate the transition state topology, Scheme 3, would allow for a possibility of two enzyme – substrate coordination: one in which the apical Mo=O fragment in the enzyme center and the C-N fragment in the substrate are *anti*-, inducing (*S*)-chirality in the substrate carbon center, Figure 2a, and another, where the same two molecular fragments are *syn*-, giving rise to an (*R*) – transition state configuration of the substrate carbon atom, Figure 2b. In the following, I will call these two coordination *anti*-transition state, *anti*-TS, and *syn*-transition state, *syn*-TS, respectively.

Only the interacting geometries defined by the *anti*-TS coordination have led to a true transition state in the course of the electronic structure calculations of the Mo-enedithiolate : formamide molecular pair, using the MP2/LanL2DZ formalism.<sup>[31]</sup> Simulated reaction trajectories starting with the *syn*-TS type input geometries, Figure 2b, do not result in a transition state but typically drift toward a complete separation of a chemically unchanged substrate and the enzyme cofactor. This may at first seem surprising given a possibility for the intermolecular Mo=O ... HNH hydrogen bonding interaction that could potentially facilitate formation of the transition state. With this idea in mind, a number of *syn*-TS input geometries were created with the (enzyme)=O ... H-(substrate) intermolecular distance ranging from 220 pm (weakly interacting) to 160 pm (medium-to-strongly interacting). However, none of these coordination resulted in a transition state.

Further analysis indicated that this may be not a result of a numerical coincidence only. In the course of spatial and electronic changes leading to a transition state the following changes occur in the formamide substrate molecule: (i) a significant twisting of the H-C=O plane (~ 42°) with respect to the HNH moiety, (ii) a partial loss of the carbonyl sp<sup>2</sup> character, and (iii) a slight elongation of the C-N bond (from 132 pm to 138 pm). As demonstrated by NMR spectroscopic studies of peptides,<sup>[55,56]</sup> a donating hydrogen-bonding interaction of an amido hydrogen induces the opposite effect - an increase in the C-N bonding character. Hydrogen-bonding interaction between the enzyme Mo=O and the substrate H-N, as anticipated for



**Figure 2** The absolute configuration of the substrate C-center (not shown in the tetrahedral representation) in (a) the *anti*-TS coordination, and the absolute configuration of the C-center in (b) the *syn*-TS coordination (note that in the tetrahedral representation carbonyl oxygen, =O, is depicted as OO).

the *syn*-TS coordination, Figure 2b, would therefore be disruptive to the electronic structure changes formamide is undergoing in the course of oxidative hydroxylation. On the enzyme cofactor side, neither would a hydrogen-bonding interaction accepted by the Mo(VI)-coordinated oxo group, the so-called “spectator” oxygen,<sup>[57,58]</sup> be expected to promote the transition state formation as the resulting partial loss of the Mo=O oxo character would have led to a decreased nucleophilicity of the Mo-coordinated *cis*-hydroxyl group, as indicated by the electron density distribution in our calculations.<sup>[31,46]</sup> It should be clear that, these arguments notwithstanding, the results of our transition state calculations could be of no more significance than a fortuitous outcome of a numerical simulation executed within a relatively narrow window of atomic and geometric parameters and within a specific theoretical formalism. A more careful analysis, however, does indicate a possible emergence of a pattern.

Translated to simple chemical terms the results of our calculations suggest an accumulation of the negative charge on the hydrogen atom and an increase in the C-H bond polarization in formamide. As pointed early on, the transfer of hydride from substrate to the Mo(VI)=S fragment of the enzyme cofactor is the principal reaction coordinate and constitutes the formal transfer of two reducing equivalents from substrate to the enzyme. Calculations with oxo-congeners of the enzyme molybdopterin center,<sup>[46,39,41]</sup> on the one hand, and the formamide thioxo-congener substrate, on the other hand [Ilich & Hille, unpublished calculations], show that absent any of the changes associated with the substrate carbon atom re-hybridization the oxidoreductive reaction will not take place in either case. It should also be noted that in formamide – a small, simple and relatively flexible molecule<sup>[59]</sup> – the electronic structure changes leading to formation of transition state are centered on the target carbon atom. In case of larger, conjugated heterocyclic, substrates and inhibitors (purines, quinoline, allopurine, and pteridine and pyridine derivatives) the analogous

electronic structure changes will depend also on the participation of the atoms adjacent to the target carbon center.

Protonation, even partial, of the nitrogen vicinal to the target carbon will facilitate the electronic structure changes leading to  $sp^2 - sp^3$  rehybridization in, for example, the quinoline C2 and the hypoxanthine C2 centers (see Figures 3 and 4 for atom labeling). This imposes another condition on the formation of the transition state: a requirement for a properly positioned proton donor within the enzyme active site. The quinoline 2-oxidoreductase : quinoline pair seems particularly well suited to illustrate this point. The target atom, C2 in the quinoline ring, can be coordinated in the enzyme active site so that the C=N bond is either *syn*- or *anti*- to the molybdopterin Mo=O fragment, Figure 3a & 3b.

Only the *anti*-coordination, Figure 3a, will lead to a formation of the transition state and the oxidative hydroxylation of the ring at C2, as suggested by analysis presented here and confirmed by a high-resolution x-ray diffraction study of the  $Q_{OR}$  : quinoline complex.<sup>[49]</sup>

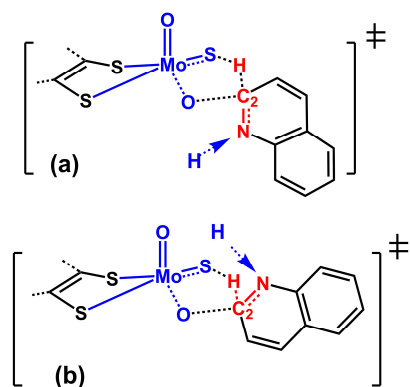
Quinoline is in spite of its size a relatively simple molecule that provides a good demonstration for the principle of *anti*-TS orientation of the substrate imino fragment and the Mo=O group in the enzyme active site. In larger and more complicated substrates other factors, like the size of the substrate molecule, its effective volume, the presence of multiple intranuclear nitrogen centers and multiple extranuclear carbonyl groups could be envisaged as dominant secondary factors in the formation of the transition state. I illustrate this aspect in the case of hypoxanthine, the penultimate product of the metabolic degradation of nucleic acids in mammals and the principal substrate for the XO form of this class of enzymes. The product of the XO-catalyzed oxidative hydroxylation of hypoxanthine (or purine-6-one) is xanthine (or purine-2,6-

dione) and the enzyme-substrate coordination pattern I have identified in this class of enzymes leads to two possible transition state geometries, Figure 4a & 4b.

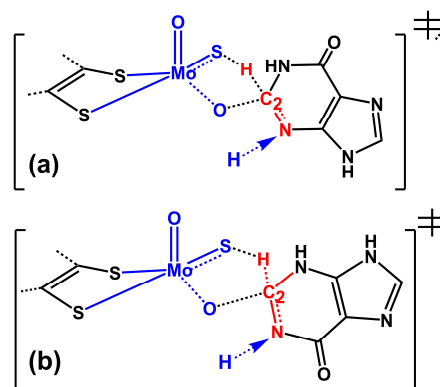
While no direct experimental evidence for a transition state is available, the recent x-ray diffraction studies of the XO protein crystals treated with hypoxanthine suggest the enzyme : substrate coordination given in Figure 4b as the likely pre-transition state reaction geometry.<sup>[60,61]</sup> I proffer three interpretations of this structure: (i) the *anti*-TS coordination condition for the oxomolybdenum : imino fragments is not valid in the case of purine substrates, (ii) the x-ray diffraction data for the protein : substrate co-crystal do not directly relate to the enzyme : substrate transition state, and (iii) the substrate is selected for a certain predominant proto-tautomeric form in the reaction course.

I posit that the first assumption is likely not valid given the evidence I have presented. The second assumption – while of potential relevance in numerous other studies – is in principle impossible to evaluate. This leaves us with the third assumption. Heterocyclic aromatic compounds like purines and pteridines (the latter are left out from the present consideration) are known, by both experiment and numerical simulations,<sup>[62,63,64]</sup> to exist in multiple proto-tautomeric forms which easily exchange under the effects of immediate environment. If the x-ray evidence for the two proto-tautomers of dry and wet guanine, respectively,<sup>[65,66]</sup> is any guidance I conclude that the transition state depicted in Figure 4b forms as a result of the *anti*-TS coordination requirement accommodated with a specific hypoxanthine proto-tautomeric form selected by the reaction conditions within the XO active site.

It is interesting to note another thread common to the structures presented in Figures 3 and 4 and also shared with the Mo-enedithiolate : formamide transition state,



**Figure 3** Two a priori possible coordination of the quinoline substrate in the  $Q_{OR}$  active site, (a) *anti*-TS, and (b) *syn*-TS. Note the proton (blue) to be supplied by the active site environment.



**Figure 4** The two postulated geometries of the XO : hypoxanthine transition state, (a) and (b), that are both subject to the condition that the substrate N=C and the enzyme Mo=O fragments assume *anti*-orientation.

Figure 2b: in all transition states the substrate carbon atom undergoing oxidative hydroxylation has the (*S*)-absolute configuration. It thus seems that structural and configurational analysis of the Mo-enedithiolate : formamide model, supported by the crystal structures of the Q<sub>OR</sub> : quinoline substrate complex and the XOR : pyridine-derivative inhibitor complex, suggests a model suitable for both the coordination of substrate and a location of a Brønsted-acid site in the catalytic center of xanthine oxidoreductase.

## SUMMARY

Analysis of the electronic and molecular structure changes predicted for a model reaction of xanthine oxidoreductase with substrate reveals new reaction mechanistic parameters. Firstly, the molybdenum center in the enzyme active site acts as a chiral switch, changing the absolute configuration from (*S*)- in the fully oxidized state, Mo(VI), to the (*R*)-absolute configuration in the fully reduced state, Mo(IV). Secondly, this process is complemented by induction of chirality in the transition state in the reaction with a pro-chiral substrate formamide (*pro-S<sub>C</sub>* → *S<sub>C</sub>*). When extended to the conjugated heterocyclic substrates (e.g. purine, pteridine and quinoline), this observation suggests that protonation of the substrate imino nitrogen vicinal to the oxidized carbon center is a prerequisite for the sp<sup>2</sup> – sp<sup>3</sup> re-hybridization of the carbon center and formation of transition state. The absolute configurations of the enzyme molybdenum center and the substrate carbon center further require that the substrate imino nitrogen be positioned *anti*- to the Mo=O ligand. This in turn requires a proton transfer from the protein and imposes coordination constraints on heterocyclic substrates.

The relevance of the approach presented here is potentially twofold: (i) it provides a basis for novel applications of signed optical spectroscopies in studies of kinetic and mechanistic properties of this class of enzymes (and this class of cofactors), and (ii) it recognizes the absolute configuration of the enzyme active site as a novel parameter suitable for classification of the structure and catalytic activity in molybdopterin oxidoreductive enzymes or possibly in metalloenzymes in general.

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## REFERENCES

- [1] BRENDA (BRAunschweig ENzyme DATA base) EC (Enzyme Commission number) 1.17.1.4; the enzyme was previously classified as EC 1.1.1.204.
- [2] R. Hille, J. Hall, P. Basu, *Chem. Rev.* **2014**, *114*, 3963.
- [3] UniProtKB [*Nucleic Acids Res.* **2015**, *43*, D204-D212; <http://www.uniprot.org/uniprot/?query=XDH%2C+h uman&sort=score>, accessed Nov/2016, entry P47989.
- [4] R. Hille, *Chem. Rev.* **1996**, *96*, 2757.
- [5] T.D. Tullius, D. NM. Kurtz, Jr., S. D. Conradson, K. O. Hodgson, *J. Am. Chem. Soc.* **1979**, *101*, 2776.
- [6] J. S. Olson, D. P. Ballou, G. Palmer, V. Massey, *J. Biol. Chem.* **1974**, *249*, 4363.
- [7] F. Morpeth, R. C. Bray, *Biochemistry* **1984**, *23*, 1332.
- [8] C. H. C. H. Shackleton, M. S. Neres, B. A. Hughes, P. M. Stewart, C. E. Kater, *Steroids* **2008**, *73*, 652.
- [9] E. Della Corte, F. Stirpe, F., *Biochemical J.* **1972**, *126*, 739.
- [10] T. Nishino, T. Nishino, L. M. Schopfer, V. Massey, *J. Biol. Chem.* **1989**, *264*, 2518.
- [11] BRENDA EC 1.17.3.2, previously classified as EC 1.1.3.22.
- [12] T. Nishino, K. Okamoto, Y. Kawaguchi, H. Hori, T. Matsumura, B. T. Eger, E. F. Pai, T. Nishino, *J. Biol. Chem.* **2005**, *280*, 24888.
- [13] C. Enroth, B. T. Eger, K. Okamoto, T. Nohino, T. Nishino, E. F. Pai, *Proc. Natl. Acad. Sci. USA* **2000**, *97*, 10723.
- [14] T. Nishino, K. Okamoto, Y. Kawaguchi, T. Matsumura, B. T. Eger, E. F. Pai, T. Nishino, *Febs J.* **2015**, *282*, 3075.
- [15] RCSB PDB [Research Collaboratory for Structural Bioinformatics - Protein Data Bank] identification numbers: 4YRW, 4YSW, 4YTY.
- [16] H. Ishikita, B. T. Eger, K. Okamoto, T. Nishino, E. F. Pai, *J. Am. Chem. Soc.* **2012**, *134*, 999; PDB ID 3UNA.
- [17] K. Ichida, Y. Amaya, N. Kamatani, T. Nishino, T. Hosoya, O. Sakai, *J. Clin. Invest.* **1997**, *99*, 2391.
- [18] HGNC (HUGO Gene Nomenclature Committee) entry 12805.
- [19] K. Ichida, Y. Amaya, K. Noda, S. Minoshima, T. Hosoya, O. Sakai, N. Shimizu, T. Nishino, *Gene* **1993**, *133*, 279.
- [20] UniProtKB [*Nucleic Acids Res.* **2015**, *43*, D204-D212] entry Q06278.
- [21] OMIM (Online Mendelian Inheritance in Man) entry 252150 ICD+ MOLYBDENUM COFACTOR DEFICIENCY, COMPLEMENTATION GROUP A; MOCODA.

- [22] J. L. Johnson, W. R. Waud, K. V. Rajagopalan, M. Duran, F. A. Beemer, S. K. Wadman, *Proc. Natl. Acad. Sci. USA* **1980**, *77*, 3715.
- [23] N. Cantu-Medellin, E. E. Kelley, *Redox Biol.* **2013**, *1*, 353.
- [24] E. I. Stiefel, *The Biogeochemistry of Molybdenum and Tungsten: Their Roles in Biological Processes*, in *Metal Ions in Biological Systems, Vol. 39* (Eds. A. Siegel, H. Siegel), Marcel Dekker, New York, NY, USA, **2002**, pp. 1–30.
- [25] R. Hille, T. Nishino, F. Bittner, *Coord. Chem. Rev.* **2011**, *255*, 1179.
- [26] D. J. D. Nicholas, *Nature* **1957**, *179*, 800.
- [27] F. Schardinger, *Z. Unters. Nahr.-Genussm. (Eur. Food Res. Technol. since 1926)* **1902**, *5*, 1113.
- [28] M. R. Bray, R. J. Deeth, *J. C. S. Dalton Trans.* **1997**, 1267.
- [29] A. A. Voityuk, K. Albert, M. J. Romao, R. Huber, N. Rösch, *J. Am. Chem. Soc.* **1997**, *119*, 3159.
- [30] A. A. Voityuk, K. Albert, M. J. Romao, R. Huber, N. Rösch, *Inorg. Chem.* **1998**, *37*, 176.
- [31] P. Ilich, R. Hille, *J. Phys. Chem. B* **1999**, *103*, 5406.
- [32] C. Møller, M. S. Plesset, *Phys. Rev.* **1934**, *46*, 618.
- [33] W. R. Wadt, P. J. Hay, *J. Chem. Phys.* **1985**, *82*, 299.
- [34] T. H. Dunning, Jr.; P. J. Hay, In *Modern Theoretical Chemistry*; (Ed. E. H. Schaefer, III), Plenum, New York, **1976**, pp 1–28.
- [35] Gaussian 09, Revision D.01, M. J. Frisch, G. W. Trucks, H. B. Schlegel, G. E. Scuseria, M. A. Robb, J. R. Cheeseman, G. Scalmani, V. Barone, B. Mennucci, G. A. Petersson, H. Nakatsuji, M. Caricato, X. Li, H. P. Hratchian, A. F. Izmaylov, J. Bloino, G. Zheng, J. L. Sonnenberg, M. Hada, M. Ehara, K. Toyota, R. Fukuda, J. Hasegawa, M. Ishida, T. Nakajima, Y. Honda, O. Kitao, H. Nakai, T. Vreven, J. A. Montgomery, Jr., J. E. Peralta, F. Ogliaro, M. Bearpark, J. J. Heyd, E. Brothers, K. N. Kudin, V. N. Staroverov, T. Keith, R. Kobayashi, J. Normand, K. Raghavachari, A. Rendell, J. C. Burant, S. S. Iyengar, J. Tomasi, M. Cossi, N. Rega, J. M. Millam, M. Klene, J. E. Knox, J. B. Cross, V. Bakken, C. Adamo, J. Jaramillo, R. Gomperts, R. E. Stratmann, O. Yazyev, A. J. Austin, R. Cammi, C. Pomelli, J. W. Ochterski, R. L. Martin, K. Morokuma, V. G. Zakrzewski, G. A. Voth, P. Salvador, J. J. Dannenberg, S. Dapprich, A. D. Daniels, O. Farkas, J. B. Foresman, J. V. Ortiz, J. Cioslowski, D. J. Fox, Gaussian, Inc., Wallingford CT, **2013**.
- [36] J. F. Alfaro, J. P. Jones, *J. Org. Chem.* **2008**, *73*, 9469.
- [37] M. Hofmann, *J. Mol. Struct. THEOCHEM* **2006**, *773*, 59.
- [38] T. Amano, N. Ochi, H. Sato, S. Sakaki, *J. Am. Chem. Soc.* **2007**, *129*, 8131.
- [39] S. Metz, W. Thiel, *J. Am. Chem. Soc.* **2009**, *131*, 14885.
- [40] L. Hu, H. Chen, *J. Chem. Theory. Comput.* **2015**, *11*, 4601.
- [41] J. Li & U. Ryde, *Inorg. Chem.* **2014**, *53*, 11913, and references therein.
- [42] E. C. Taylor, P. S. Ray, I. S. Darwish, J. L. Johnson, K. V. Rajagopalan, *J. Am. Chem. Soc.* **1989**, *111*, 7664.
- [43] R. Huber, P. Hof, R. O. Duarte, J. J. G. Moura, I. Moura, M.-Y. Liu, J. LeGall, R. Hille, M. Archer, M. J. Romao, *Proc. Natl. Acad. Sci. USA* **1996**, *93*, 8846.
- [44] J. J. Truglio, K. Theis, S. Leimkühler, R. Rappa, K. V. Rajagopalan, C. Kisker, *Struct.* **2002**, *10*, 115.
- [45] M. J. Romao, N. Rösch, R. Huber, *J. Bioinorg. Chem.* **1997**, *2*, 782.
- [46] P. Ilich, R. Hille, *J. Am. Chem. Soc.* **2002**, *124*, 6796.
- [47] X.-H. Zhang & Y.-D. Wu, *Inorg. Chem.* **2005**, *44*, 1466.
- [48] K. Okamoto, K. Matsumoto, R. Hille, B. T. Eger, E. F. Pai, T. Nishino, *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 7931.
- [49] I. Bonin, B. M. Martins, V. Purvanov, S. Fetzner, R. Huber, H. Dobbek, *Struct.* **2004**, *12*, 1425.
- [50] R. S. Cahn, C. Ingold, V. Prelog, *Angew. Chem. Int. Ed.* **1966**, *5*, 385.
- [51] M. F. Brown, B. R. Cook, T. E. Sloan, *Inorg. Chem.* **1975**, *14*, 1273.
- [52] J. C. Martin, T. M. Balthazor, *J. Am. Chem. Soc.* **1977**, *99*, 152.
- [53] N. G. Connelly, T. Damhus, R. M. Hartshorn, A. T. Hutton, "Nomenclature of Inorganic Chemistry, IUPAC Recommendation 2005", *Internat. Union Pure Appl. Chem.*, Section IR-9.3., p. 179.
- [54] The depiction of the "Mo-coordination sphere" used here,  $O=Mo^{VI}(=S)_2S_2(OH)$ , is preferred as a more precise and more informative way of describing the configuration and more informative way of describing the configuration of the Mo-complex (an SPY-5 geometry with O=Mo axis) with clearly and intuitively expressed characters (sulfido vs. thioxo) and the CIP-derived ranking of the equatorial ligands (#1, #2, #3, #5). The usual way of representing the Mo-coordination sphere as  $LMo^{VI}OS(OH)$ , L=enedithiolate, does not discern between the two enedithiolate sulfides and is also lacking the configuration, rank, and ligand ordering information.
- [55] N. Juranic, P. Ilich, S. Macura, *J. Am. Chem. Soc.* **1995**, *117*, 405.
- [56] P. Ilich, N. Juranic, *Chem. Phys. Phys. Chem.* **2003**, *4*, 1358.
- [57] A. K. Rappe, W. A. Goddard, III., *Nature*, **1980**, *285*, 311.
- [58] A. K. Rappe, W. A. Goddard, III., *J. Am. Chem. Soc.* **1982**, *104*, 448.



- [59] K. B. Wiberg, P. R. Rablen, *J. Am. Chem. Soc.* **1995**, *117*, 2201.
- [60] J. M. Pauff, H. Cao, R. Hille, *J. Biol. Chem.* **2009**, *284*, 8760.
- [61] U. Dietzel, J. Kuper, J. A. Doebbler, A. Schulte, J. J. Truglio, S. Leimkühler, C. Kisker, *J. Biol. Chem.* **2009**, *284*, 8768.
- [62] P. Ilich, R. Hille, *Inorg. Chim. Acta* **1997**, *263*, 87.
- [63] J. H. Kim, J. A. Odotola, J. Popham, S. von Laven, *J. Inorg. Biochem.* **2001**, *84*, 145.
- [64] S. T. Madaraiga, J. G. Contreras, *J. Chilean Chem. Soc.* **2003**, *48*, 129.
- [65] K. Guille, W. Clegg, *Acta Cryst.* **2006**, *C62*, o515, and references therein.
- [66] U. Thewalt, C. Bugg, R. E. Marsh, *Acta Cryst.* **1971**, *B27*, 2358.