Published in final edited form as:

Chem Biodivers. 2012 September ; 9(9): 1756–1760. doi:10.1002/cbdv.201200073.

C12073. Correlating C-H Bond Cleavage with Molybdenum Reduction in Xanthine Oxidase

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Xanthine oxidoreductase (XO) is a member of the xanthine oxidase family (molybdenum hydroxylases) of pyranopterin molybdenum enzymes.^[1] The importance of this enzyme with respect to human health is underscored by the fact that XO catalyzes the oxidative hydroxylation of xanthine to uric acid, the latter of which is responsible for gouty arthritis and its subsequent complications. The connection to human health is further exemplified by the fact that both XO and the structurally related aldehyde oxidase (AO) have also been implicated in pro-drug activation and drug metabolism.^[2] In addition to xanthine oxidation, XO also catalyzes the oxidative hydroxylation of a diverse array of other chemical compounds that include aldehydes, purines, and related heterocyclic organic compounds. The mechanism of XO mediated hydroxylations is quite different from their monooxygenase counterparts in that the oxygen atom inserted into a substrate C-H bond derives from metal-activated water and not dioxygen.^[1b] An additional difference between XO and monooxygenase enzymes is the generation, and not consumption, of reducing equivalents by XO in the reductive half reaction.^[1c] The oxidized active site of XO possesses a 5-coordinate square pyramidal coordination geometry (Figure 1)^[1b, 3] with a unique electronic structure that stems from the presence a terminal sulfido ligand in the equatorial plane oriented *cis* to a terminal axial oxo donor.^[4] A number of computational studies exploring the reaction coordinate of XO have been performed that converge upon a mechanism of substrate hydroxylation which is initiated by nucleophilic attack of metal-activated water on a substrate C-H carbon atom.^[1a, 1b, 3a, 5] This results in the formation a tetrahedral intermediate and subsequent transition state that breaks down by formal hydride transfer to the terminal sulfido ligand with a concomitant reduction of the molybdenum center to the Mo(VI) oxidation level. Additional support for this mechanism is provided by spectroscopic and structural studies on enzyme-product complexes that clearly indicate the presence of a Mo-O_{eq}-C_{product} linkage (O_{eq} = equatorial oxygen donor that derives from H₂O/OH⁻).^[3a, 6] Although this C_{substrate}-H bond-scission step has been described as a hydride transfer, a very recent natural bond orbital (NBO) analysis of the reaction coordinate clearly indicates that substrate C-H bond breaking results from nearly equivalent Mo=S $\pi \rightarrow$ C-H σ^* (H⁺ transfer) and C-H $\sigma \rightarrow$ Mo=S π^* (H⁻ transfer) donor $\rightarrow$ acceptor interactions along the reaction coordinate.^[5e] Additionally, this NBO study also showed that a key O_{eq} $\rightarrow$ (Mo + C_{substrate}) donor $\rightarrow$ acceptor interaction dominantly contributes to the stabilization of the transition state. This O_{eq} $\rightarrow$ (Mo + C_{substrate}) interaction is important since it reduces electronic repulsions that contribute to the energy of the transition state. Furthermore, the O_{eq} $\rightarrow$ (Mo + C_{substrate}) interaction contributes to Mo reduction and results in the formation of the product C=O bond.

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SUPPORTING INFORMATION PARAGRAPH

Details of the calculations are available in the SI.

The admixture of $\text{Mo}=\text{S } \pi \rightarrow \text{C-H } \sigma^*$ and $\text{C-H } \sigma \rightarrow \text{Mo}=\text{S } \pi^*$ donor $\rightarrow$ acceptor interactions facilitates C-H bond activation and cleavage by transferring electron density to the C-H antibonding orbital while simultaneously removing electron density from the C-H bonding orbital. The combination of forward and back charge donation processes acting in concert to activate the substrate C-H bond strongly suggests that the C-H hydrogen that is transferred to the terminal sulfido at the transition state is approximately charge neutral.^[5e] As a result, we calculated the Mulliken charge for aldehyde, amide, pterin, and purine substrates prior to C-H bond cleavage and at the transition state, and the results are presented in Figure 2. Here it is clearly observed that the C-H hydrogen is transferred to the terminal sulfido ligand as a near charge neutral species. These results are in complete agreement with earlier studies using xanthine and acetaldehyde as reducing substrates.^[5a, 5e] Thus, XO family enzymes are able to avoid a large buildup of charge along the reaction coordinate for C-H bond activation by a unique mechanism that utilizes a combination of $\text{Mo}=\text{S } \pi \rightarrow \text{C-H } \sigma^*$ and $\text{C-H } \sigma \rightarrow \text{Mo}=\text{S } \pi^*$ donor $\rightarrow$ acceptor interactions.^[5e] We note that the mechanisms proposed for monooxygenase enzymes can also facilitate charge neutral H-transfer using a radical mechanism that is initiated by H-atom abstraction.^[7] That a charge neutral H is observed along the reaction coordinate for all XO substrate types that we have probed provides strong evidence for the $\text{Mo}=\text{S } \pi \rightarrow \text{C-H } \sigma^*$ and $\text{C-H } \sigma \rightarrow \text{Mo}=\text{S } \pi^*$ donor $\rightarrow$ acceptor mechanism of substrate C-H bond activation,^[5e] and this mechanism is relatively independent of the nature of the substrate.

The NBO derived $\text{O}_{\text{eq}} \rightarrow (\text{Mo} + \text{C}_{\text{substrate}})$ donor $\rightarrow$ acceptor interaction that figures prominently in transition state stabilization with acetaldehyde as substrate^[5e] suggests a strong relationship should exist between the degree of C-H bond breaking and amount of molybdenum reduction at the transition state. Furthermore, this should extend to the wide variety of substrates that can be hydroxylated by XO. We have investigated this relationship as a function of substrate and the results are depicted in Figure 3. Here it is observed that the data points can be easily fit to an exponential rise function. The correlation is remarkable since it indicates that the exponential decrease in C-H bond overlap that accompanies bond scission directly correlates with the degree of Mo(VI) reduction in a predictable manner. Furthermore, the data points cluster in three regions. The first (region A of Figure 3) implies an early transition state with respect to C-H bond breaking and corresponds to substrates that *are not* protonated in step b of Figure 1, while the second region (region B of Figure 3) corresponds to substrates that *are* protonated in step b. Finally, the third region (region C of Figure 3) corresponds to the substrate formate, which is bound directly to the Mo center. In this mechanism, formate oxidation does not proceed via a tetrahedral intermediate, but by direct H-transfer to the terminal sulfido in a mechanism has been discussed previously.^[1b]

In summary, this work has increased our understanding of substrate C-H bond activation in XO and related enzymes of the XO family. It has been determined that the C-H hydrogen for all XO substrates studied is transferred to the terminal sulfido at the transition state with near neutral charge, consistent with a recent NBO analysis of the C-H hydrogen charge for acetaldehyde along the reaction coordinate.^[5e] This agrees well with a mechanism by which $\text{Mo}=\text{S } \pi \rightarrow \text{C-H } \sigma^*$ and $\text{C-H } \sigma \rightarrow \text{Mo}=\text{S } \pi^*$ donor $\rightarrow$ acceptor interactions conspire to activate the substrate C-H bond for catalysis. Finally, it has been shown that there is a direct relationship between C-H bond scission and Mo reduction along the reaction coordinate for all XO substrates studied, with Mo reduction being a continuous and exponential function of C-H bond breaking along the reaction coordinate.

Acknowledgments

M.L.K. acknowledges the National Institutes of Health grant GM 057378 for financial assistance.

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Figure 1. Proposed reaction mechanism for XO with purines: (a) oxidized active site, (b) tetrahedral intermediate, (c) transition state, and (d) reduced Mo(IV) site with product formation and binding of H₂O. Note that the C=O bond of the keto product depicted in Figure 1 can tautomerize to form the enol product.

Figure 2.

The Mulliken charge on the C-H hydrogen transferred to the terminal sulfido ligand at the transition state plotted as a function of XO substrates (XA = xanthine, 6MPC8 = C8 carbon of 6-methylpurine, AL = allopurinol, HMP = 2-hydroxy-6-methylpurine, PUC8 = C8 carbon of purine, FM = formamide, 6MPC2 = C2 carbon of 6-methylpurine, FA = formaldehyde, AC = acetaldehyde, LU = lumazine, HY = hypoxanthine, PUC2 = C2 carbon of purine, PUC6 = C6 carbon of purine).

Figure 3.

Percent Mo reduction as a function of the substrate C-H bond distance at the transition state for XO substrates. Region A includes non-protonated substrates: In order of increasing % reduction: AL, 6MPC8, PUC8, HY, FA, LU, 6MPC2, HMP, PUC6, PUC2, AC, XA, FM. Region B includes protonated substrates: XA, FA, AC, FT, FM. Region C is FT that is directly bound to the oxidized Mo(VI) center prior to C-H scission. Percent reduction is defined as the percent Mo dx^2-y^2 character in the redox orbital divided by the percent Mo dx^2-y^2 character in the redox orbital of the fully reduced enzyme-product complex (100% reduction) with violoapterin bound at the active site. For comparative purposes, the point at 0% Mo reduction is the C-H bond distance for benzene (1.101 Å). Data have been fit to an exponential rise function with an R value of 0.97.