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The Reaction Mechanism of Xanthine Oxidase: Evidence for Two-Electron Chemistry Rather Than Sequential One-Electron Steps

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The mechanism by which molybdenum enzymes such as xanthine oxidase carry out substrate hydroxylation has been the subject of continued interest,[1] and can now be understood in the context of the crystal structures for several enzymes.[2] The active sites of these enzymes have the basic structure $LMo^{VI}OS(OH)$, where L represents a pyranopterin cofactor common to all mononuclear molybdenum and tungsten enzymes;[1] this ligand binds to the metal via a side chain with an RC(SH)=C(SH)R′ structure. The coordination geometry is square-pyramidal, with Mo=O, Mo−OH, and pyranopterin in the equatorial plane and Mo=S in the apical position.

The available evidence favors a mechanism in which an active-site base (proposed to be a conserved glutamate residue[2b,c]) abstracts the proton from the Mo−OH group, initiating nucleophilic attack on substrate with concomitant hydride transfer from C-8 of substrate to the Mo=S. This yields an $LMo^{IV}O(SH)(OR)$ species, where OR represents product coordinated to the metal via the newly introduced hydroxyl group. This first observable intermediate in the reaction subsequently breaks down by electron transfer to other redox-active centers in the enzyme and displacement of product by hydroxide from solvent.[3b,c] A base-catalyzed mechanism is consistent with the observations that: (1) the reaction requires neutral substrate rather, than the monoanion, and involves an active-site base with a pK_a of 6.6;[3a] (2) the Mo−OH is the proximal donor of the oxygen atom incorporated into product,[3d−f] being regenerated by hydroxide from solvent at the completion of the reaction; (3) the C-8 proton of substrate is transiently transferred to the molybdenum center in the course of the reaction;[3g] and (4) the EPR-active $LMo^{V}OS(OR)$ species is formed by oxidation of an $LMo^{IV}O(SH)(OR)$ precursor.[3b] ENDOR studies of the paramagnetic $LMo^{V}OS(OR)$ species[4a] as well as the crystal structure of the *Rhodobacter capsulatus* protein in complex with the product analogue and inhibitor alloxanthine[2d] are consistent with simple, end-on coordination of product to the metal in both the Mo^{V} and Mo^{IV} oxidation states. Also, computational studies of the reaction of an $LMo^{VI}OS(OH)$ active-site model with formamide (a slow enzyme substrate) indicate negative charge accumulation on the hydrogen being transferred in the transition state, specifically connoting hydride transfer per se.[4b]

Nevertheless, it has recently been suggested that the reaction proceeds instead via two discrete one-electron steps (rather than the two-electron chemistry implied by a mechanism involving nucleophilic attack and hydride transfer).[5] Direct one-electron transfer from substrate to molybdenum yields a $Mo^{V}\cdots S^{\bullet+}$ species that subsequently breaks down by a second one-electron step (with uptake of oxygen) to give the $LMo^{IV}O(SH)(OR)$ intermediate. This first step is presumably very unfavorable thermodynamically and must be significantly slower than the second to account for the failure to detect a $Mo^{V}\cdots S^{\bullet+}$ radical pair at even the shortest time scales examined under a wide range of experimental conditions; it must thus represent the rate-limiting step in the formation of the $LMo^{IV}O(SH)(OR)$ intermediate. Given the short distance (≤3 Å) over which even such a very unfavorable initial electron transfer would take place, the rate constant for the initial forward (thermodynamically unfavorable) electron-transfer step in the equilibrium $Mo^{VI}\cdots S \leftrightarrow Mo^{V}\cdots S^{\bullet+}$ could still be fast enough to support turnover, which proceeds with a k_{cat} of $\sim 17\ s^{-1}$ under optimal conditions.[6a] Indeed, given the radical-based chemistry seen in the reaction mechanisms of other enzymes,[6b] a reaction mechanism involving two discrete one-electron steps cannot be excluded a priori. Direct experimental evidence for or against either one- or two-electron chemistry is thus highly desirable.

Table 1. Reduction Potentials ($S^{\bullet+}/S$) and Kinetic Parameters of Purines Used (for Experimental Details, See Supporting Information)

compound	$\Delta E^{\circ\prime}$ (V)	k_{red} (s^{-1})	k_{red}/K_d ($M^{-1}\,s^{-1}$)
xanthine	1.08	7.0	1.3×10^7
1-methylxanthine	1.07	13.9	3.1×10^6
7-methylxanthine	1.27	ND	2.0×10^3
1,7-dimethylxanthine	1.26	0.070	56
guanine	1.09	0.0001	1.7
2,6-diaminopurine	1.10	0.001	17
2-amino-6-chloropurine	1.10	0.013	100
2-OH-6-methylpurine	1.15	0.133	1.2×10^5
2-thioxanthine	1.05	3.0	ND
6-thioxanthine	1.03	1.4	1.5×10^6

If the reaction proceeds via sequential one-electron steps, then the effectiveness of a given purine as substrate should correlate inversely with the reduction potential for the one-electron $S^{\bullet+}/S$ couple for the heterocycle, since from the above the first one-electron oxidation step must be rate-limiting for overall reduction of the molybdenum center. Taking advantage of the well-established broad substrate specificity of xanthine oxidase[6c] we have determined the reduction potentials for a set of substituted purines and have also examined the kinetics of enzyme reduction by these potential substrates to determine whether a correlation exists that would support a mechanism involving individual one-electron steps.

Tables 1 and S1 give the reduction potentials (at pH 7.0) for the 10 purine derivatives examined in the present study, as determined by pulse radiolysis.[7] Purines were selected from those available commercially on the basis of substitutions about the purine ring that were expected to give the largest range in reduction potential (on the basis of inductive effect) with minimal potential steric conflicts.[7] Consideration was also limited to those substrates substituted at both positions 2 and 6 so that the enzyme can only hydroxylate at the C-8 position. The purines, ranging from effective substrates such as xanthine and 1-methylxanthine to ineffective ones

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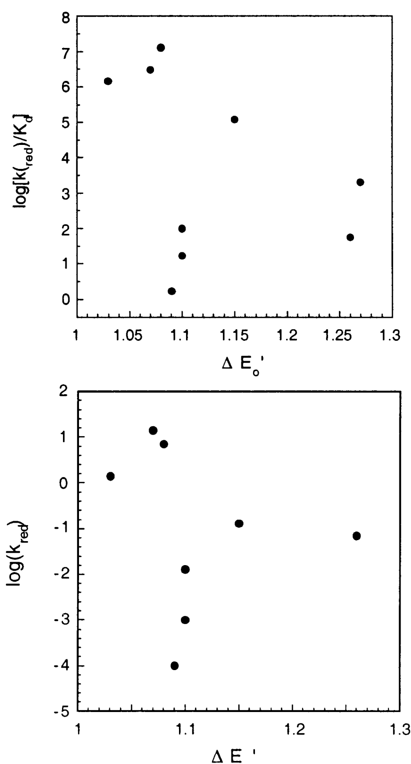

Figure 1. Plots of log[k_{red}/K_d] vs $\Delta E^{\circ\prime}$ (left) and log[k_{red}/K_d] vs $\Delta E^{\circ\prime}$ (left). Reduction potentials and kinetic parameters were obtained as described in Tables S1 and S2, respectively, in Supporting Information.

such as guanine and theophylline (1,3-dimethylxanthine), are found to have reduction potentials from +1.29 to +1.03 V (vs NHE), covering a range of 260 mV. For a reaction proceeding by rate-limiting one-electron chemistry, the Nernst equation predicts a range of rate constants covering 4 orders of magnitude.

Tables 1 and S2 gives the results of rapid kinetic studies of enzyme with each of the purine derivatives. The parameters to be considered are k_{red}, the limiting first-order rate constant for breakdown of the E·S complex in the high [substrate] regime, and k_{red}/K_d, the effective second-order rate constant for reaction of free enzyme and free substrate in the low [substrate] regime.[4c] Both terms follow the reaction through the first (functionally) irreversible step of the reaction[8]—here, formation of the $LMo^{IV}O(SH)(OR)$ species. In several cases, the rate of reaction with enzyme was so slow as to prevent an accurate determination of rate constant; in these cases only an upper limit on the rate of reaction could be estimated from the observed extent of reduction of enzyme in, for example, an overnight anaerobic incubation of enzyme with substrate.

Figure 1 shows plots of both log(k_{red}) and log(k_{red}/K_d) versus the one-electron reduction potential for each purine substrate from Table S1. It is evident that neither plot exhibits the linear relationship expected for a reaction involving a rate-limiting single-electron-transfer step within the E···S complex to give $Mo^{V}\cdots S^{\bullet+}$. While the two purines with reduction potentials above +1.25 V vs NHE are indeed poor substrates (as expected for a sequential one-electron mechanism), and while each of the three best substrates have among the lowest potentials of those examined, three of the least effective substrates also possess low reduction potentials. Over the potential range 1.00−1.10 V vs NHE (which includes the physiological substrate xanthine), it is clear that no linear relationship exists between reduction potential and the reaction kinetics, with effectiveness as substrate essentially random over more than 5 orders of magnitude in rate.

The present results are inconsistent with a mechanism involving individual one-electron steps and indicate that factors other than the one-electron reduction potential of substrate are important in determining catalytic effectiveness. One such consideration that is reflected in the present data, in which substrates methylated at N3 are found to be poor ones, is substrate tautomerization in the course of the reaction, with a proton transferred from N3 to N9.[3h] Methylation at N3 prevents this tautomerization, which is readily rationalized in the context of a reaction initiated by nucleophilic attack, but not one initiated by a Marcus-like one-electron-transfer event.[5] Another factor is the need for neutral substrate rather than the monoanionic form,[3a] which is difficult to rationalize for a rate-limiting Marcus-like process, but readily understood if the reaction is initiated by nucleophilic attack on substrate. We conclude that xanthine oxidase most likely operates via two-electron chemistry, with formation of the initially appearing $LMo^{IV}O(SH)(OR)$ intermediate proceeding in a single two-electron process involving nucleophilic attack on substrate followed by hydride transfer to the molybdenum center.

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Supporting Information Available: Two tables consisting of the reduction potentials and kinetic parameters determined in the present work (PDF). This material is available free of charge at http://pubs.acs.org.

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