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Studies on the Mechanism of Aldehyde Oxidase and Xanthine Oxidase

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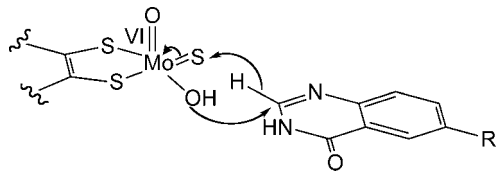
Received May 15, 2008



DFT calculations support a concerted mechanism for xanthine oxidase and aldehyde oxidase hydride displacement from the sp^2 carbon of 6-substituted 4-quinazolinones. The variations in transition state structure show that C−O bond formation is nearly complete in the transition state and the transition state changes are anti-Hammond with the C−H and C−O bond lengths being more product-like for the faster reactions. The C−O bond length in the transition state is around 90% formed. However, the C−H bond is only about 80% broken. This leads to a very tetrahedral transition state with an O−C−N angle of 109°. Thus, while the mechanism is concerted, the antibonding orbital of the C−H bond that is broken is not directly attacked by the nucleophile and instead hydride displacement occurs after almost complete tetrahedral transition state formation. In support of this the *C*═N bond is lengthened in the transition state indicating that attack on the electrophilic carbon occurs by addition to the *C*═N bond with negative charge increasing on the nitrogen. Differences in experimental reaction rates are accurately reproduced by these calculations and tend to support this mechanism.

The mechanism(s) of molybdoenzymes such as aldehyde oxidase and xanthine oxidase are of interest since these enzymes are more frequently being linked to major metabolic pathways of drugs[1−4] and, in the case of xanthine oxidase, serve an important physiological function.[5] Recent studies by our laboratory have shown that the regioselectivity of aldehyde oxidase, but not xanthine oxidase can be predicted by relatively simple DFT calculations.[6] A better understanding of the overall mechanism should enhance our ability to control the metabolic properties of potential drug molecules metabolized by these enzymes.

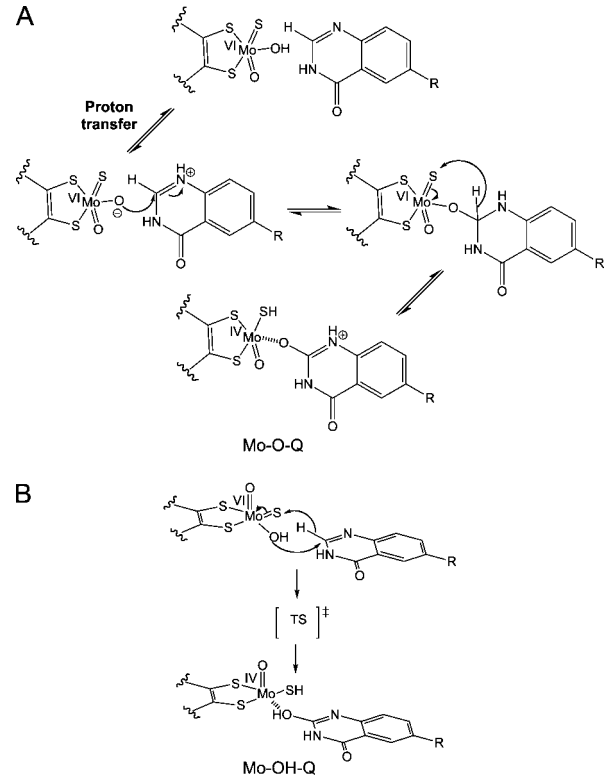


FIGURE 1. Reaction mechanisms for xanthine oxidase and aldehyde oxidase. (A) Step-wise. (B) Concerted.

Both xanthine oxidase and aldehyde oxidase enzymes contain a molybdenum pyranopterin cofactor (MoCo) and oxidize electron deficient substrates via a nucleophilic oxidation. The MoCo for these enzymes contains three sulfurs and two oxygens. The catalytically labile oxygen, which comes from water,[7,8] and the sulfur atom that accepts the hydride are in equatorial positions of the square-pyramidal molybdenum coordination complex.[9,10] The mechanism of the reaction could proceed either through a stepwise mechanism with a tetrahedral intermediate (Figure 1a) or a concerted mechanism as shown in Figure 1b.[7]

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10.1021/jo801053u CCC: $40.75 © 2008 American Chemical Society
Published on Web 11/11/2008

Experimental evidence supports a concerted mechanism.[11] Skibo and co-workers used substituted quinazolinones as shown in Figure 1 as electronic probes and found electron withdrawing groups increase reaction rates, suggesting a negative charge build-up in the transition state, and that nucleophilic addition was rate determining. In addition, large deuterium isotope effects of up to 5.2 indicate that hydride transfer is also rate determining, consistent with a concerted mechanism. Without any other experimental data it is possible to conclude that the electronic effects are associated with an equilibrium addition to form a tetrahedral intermediate, followed by rate-limiting hydride transfer. However, kinetic isotope effects for the series indicate that the more electron withdrawing groups, which would form the most stable tetrahedral intermediate, have the smallest isotope effect (R = CH_3 > Cl > NO_2). The Melander−Westheimer Postulate predicts this order would only result from an endothermic reaction coordinate for hydride transfer, meaning that the tetrahedral intermediate is more stable than the product of hydride transfer, the Mo(IV)−OH−Q intermediate. Experimental results indicate that the intermediate (Mo(IV)−OH−Q) is stable at least for reactions with 2-hydroxy-6-methylpurine[12] and 2,6-dihydroxypteridine[13] which is not consistent with an endothermic reaction coordinate. UV−visible spectroscopy indicates that the Mo(IV)−OH−Q intermediate is rapidly formed, and then is slowly oxidized to the EPR active Mo(V)−OH−Q intermediate.[13,14] An alternative explanation is another partially rate-limiting step masks the kinetic isotope effect for the electron withdrawing groups more than the electron donating groups. While this might explain the changes in isotope effects for a concerted mechanism a stepwise mechanism would lead to slow hydride transfer for electron withdrawing groups which is not consistent with the observed trend in isotope effects.

Orbital considerations would appear to argue against a concerted displacement of a hydride from a sp^2 carbon. A more chemically plausible stepwise mechanism with initial nucleophilic attack to create a tetrahedral intermediate followed by hydride transfer would be consistent with the substituent effects, but cannot explain the large deuterium isotope effect or the trend in isotope effects for different substituents.

Computational studies on the concerted mechanism using formamide as a substrate and Mo−ditholene as a model for the complete MoCo provided evidence that the concerted mechanism was possible, but gave an activation enthalpy of 78 kcal/mol using a UMP2 level of theory and the LanL2DZ basis set.[15] This high barrier appears to support the orbital argument that the concerted pathway is a high energy pathway. However, the high barrier to reaction could be a computational artifact. More recent calculations using B3LYP,[16,17] and CCSD[17] indicate that the barrier is much lower at around 26 kcal/mol for the concerted reaction from infinitely separated reactants. (A higher barrier of ca. 40kcal/mol was reported from a preassociation complex that is not on the reaction pathway. This preassociation complex is unlikely to form in the enzyme active-site.) Studies were also done on stepwise pathways. None of the stepwise mechanisms gave barriers significantly lower than the concerted pathway, and the predicted rate-limiting step is a proton transfer from the Mo−OH to formamide, or coordination of the carbonyl oxygen with the Mo. Both mechanisms can be excluded because they would not result in a kinetic isotope effect when the substrate has a deuterium. However, no experimental kinetic isotope effects have been measured for formamide, and it is possible that this substrate would not show an isotope effect. Since the required experimental data is not available for formamide we performed density functional calculations on selected 6-substituted 4-quinazolinones described in the experimental work of Skibo.[11] This is the first report of theoretical calculations that can be directly compared to experiment.

Calculations were performed using a DFT method at a B3LYP level of theory with a modified cc-aug-pVDZ basis set[18] on hydrogen, carbon, nitrogen and sulfur, and cc-aug-pVDZ basis set with an effective core potential on molybdenum (see Supporting Information for basis set). Reactant and product geometries were optimized and all stationary points confirmed by frequency calculations. Transition state structures were verified using frequency calculations to have only one negative eigenvalue and to smoothly decompose to reactants and products for the formamide and quinazolinone (R = H) transition states. A tetrahedral intermediate was not observed in either case.

As a means of comparing our results to previous calculations, we determined the transition state energy and geometry for the reaction between Mo-ditholene and formamide. We found an activation enthalpy of 31.69 kcal/mol, much lower than the barrier (78 kcal/mol) reported by Hille and co-workers and similar to those reported by Amano et. al,[17] and Zhang and Wu.[16] Transition state geometries varied from the MP2 calculations as well. Hille reported C−O and S−H bond length of 1.74 and 1.54 Å, respectively. Our calculations yielded bond lengths for C−O of 1.64 and 1.55 Å, respectively. The energetics and structures are similar for the DFT calculation with the smaller LanL2DZ basis set on Mo, consistent with previous findings that the difference in barrier height and geometry is a result of the method and not the basis set.[16,17]

To compare our results with the experimental difference in rates reported by Skibo and co-workers, we examined activation enthalpies for the reaction between Mo-ditholene and 6-substituted 4-quinazolinones. The geometries of the transition states and the activation enthalpies for the B3LYP functional are in Table 1. When zero point energy corrections were applied the activation energy for all substituents decreased 2.0 kcal/mol for R = H, CH_3, CL, NH_2, 2.1 kcal/mol for R = CH_3, and 1.9 kcal/mol for R = NO_2, and CF_3. Activation enthalpies decrease with electron withdrawing substituents suggesting a negative charge build up in the transition state consistent with the substituent effects data obtained by Skibo and are given in Table 2. To test for

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TABLE 1. Transition State Bond Lengths and Activation Enthalpies for Substituted Quinazolinones[a]

R	C−H	S−H	C−O	Mo−O	$\Delta H^{\ddagger}$
NH_2	1.391	1.599	1.496	2.179	33.4 (31.2)
OCH_3	1.394	1.596	1.493	2.184	32.5 (30.4)
CH_3	1.397	1.594	1.492	2.180	31.9 (29.6)
H	1.400	1.592	1.490	2.181	31.1 (28.9)
Cl	1.413	1.583	1.480	2.187	28.6 (26.5)
CF_3	1.421	1.577	1.474	2.191	27.0 (ND)
NO_2	1.445	1.562	1.459	2.202	22.1 (19.3)

[a] Bond lengths reported in Å and activation enthalpies reported in kcal/mol. Values in parentheses are the energies determined with the TPSS functional.

TABLE 2. Experimental and Theoretical Differences in Activation Energies for 6-Substituted 4-Quinazolinones

R	$\Delta H^{\ddagger}_{Exp}$[a]	$\Delta H^{\ddagger}_{Theo}$[b]
NH_2	2.4	1.5
OCH_3	−0.1	0.6
CH_3	0	0
H	0.9	−0.7
Cl	−2.2	−3.2
CF_3	ND	−4.9
NO_2	−2.8	−9.8

[a] Difference in energies calculated relative to the 6-methyl-4-quinazolinone for reported k_{cat}/K_m in ref 11. [b] Difference between theoretical enthalpies of activation relative to 6-methyl-4-quinazolinone.

dependence on the functional, we used the nonempirical TPSS functional[19] and found very similar activation energies and trends (parentheses Table 1).

When Skibo plotted the log k_{cat}/K_M versus the pK_a of the parent amide a linear relationship was seen for the differences in rates for the amino to chloro substituents as shown in Table 2. The nitro substituted compound showed a similar rate to the chloro compound indicating that the more electron withdrawing nitro group had a change in either the kinetic or chemical mechanism. To directly compare the differences in rates associated with different substituents we calculated the difference in activation energies, determined from the k_{cat}/K_m values reported by Skibo, and compared those to differences we obtained for the theoretical activation enthalpies. The differences in experimental activation energies were determined from the ratio of rates at 310 K using the Arrhenius equation. These values are reported in Table 2, and show a good correlation between experiment and theory. Obviously since we assume all the compounds in the series react with the same chemical and kinetic mechanism our results for the nitro substituted compound does not correlate.

These calculations support the concerted mechanism shown in Figure 1b. The variations in transition state structure show that C−O bond formation is nearly complete in the transition state. The C−O bond length in the transition state is around 90% formed relative to the product geometries. However, the C−H bond is only about 80% broken relative to the reactant geometries. This leads to a very tetrahedral transition state with an O−C−N angle of 109° (Figure 2).

Thus, while the mechanism is concerted, the antibonding orbital of the C−H bond that is broken is not directly attacked

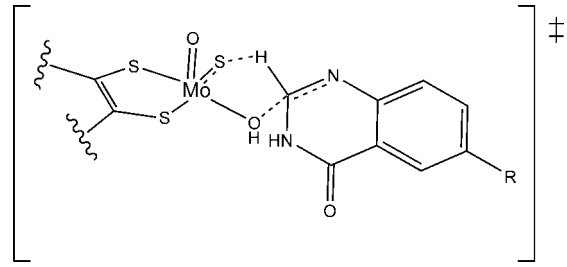

FIGURE 2. Transition state geometries for 6-substituted 4-quinazolinones.

by the nucleophile and instead hydride displacement occurs after almost complete tetrahedral transition state formation. In support of this, the *C*═N bond length is lengthened in the transition state indicating that attack on the electrophilic carbon occurs by addition to the *C*═N antibonding orbital with negative charge increasing on the nitrogen.

In the transition state the C−H and C−O bond lengths get more product-like for electron withdrawing groups, while the rates become faster. Normally, the Hammond postulate predicts that transition state structure will get more reactant-like with faster reaction rates.[20] However, this anti-Hammond effect is consistent with electron withdrawing groups enhancing the rate of tetrahedral transition state formation, while hindering hydride transfer. The C−O bond lengths must be shorter for the electron withdrawing groups in order to build-up sufficient electron density to support hydride transfer.

Though our calculated activation enthalpies for the reaction between MoCo and various substrates are reasonable, we hypothesize the energy barriers could be lowered further by enzyme catalysis. The crystal structure of xanthine oxidase suggests the possibility that an active site glutamate may function as a base to accept the Mo−OH−C proton as it becomes more acidic in the transition state.[5]

To explore the formation of a tetrahedral intermediate, and mechanism A in Figure 1, we transferred a proton from the nucleophilic oxygen to the nitrogen in the substrate ring for R= -Cl, -CH_3, -NH_2 (simply deprotonating the nucleophilic oxygen also gives a concerted mechanism). The proton transfer is extremely endothermic in the gas phase (>150 kcal/mol) since the overall charge on the MoCo is negative two and a positive charge is placed on the substrate. The resulting tetrahedral intermediate is exothermic relative to the substrate and cofactor prior to proton transfer by between −2.5 (-NH_2) and −7.7 kcal/mol (-Cl). This would lead to build-up of the tetrahedral intermediate for the substrates with electron withdrawing groups. The increased concentration of the tetrahedral intermediate for electron withdrawing groups would increase the rate of product formation consistent with experimental results. However the resulting products after hydride transfer from the carbon to the sulfur of the cofactor is exothermic by −0.75 (R = Cl) to −2.9 kcal/mol (R = NH_2) which is inconsistent with the experimental trend in kinetic isotope effects.

Our calculations for the reaction between MoCo and various substrates support previous experimental evidence that AO and XO oxidize substituted quinazolinone substrates via a concerted mechanism.[7,11] The tetrahedral characteristics

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of the transition state provide insight into our ability to predict the sites of metabolism for aldehyde oxidase based on the stability of the tetrahedral intermediate.[6] While the data is more consistent with a concerted mechanism, the data presented herein does not exclude a stepwise mechanism, and further experimental studies will be required to fully characterize this reaction.

Acknowledgment. This work was supported by GM84546, and ES009122. We also thank Professor Greg Crouch for thoughtful discussions and Professor Kirk Peterson for making the basis sets available to us.

Supporting Information Available: The cc-aug-pVDZ basis set used for calculations, charges, transition state cartoons, and tables of Cartesian coordinates for transition state geometries. This material is available free of charge via the Internet at http://pubs.acs.org.

JO801053U