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A Theoretical Study on the Mechanism of the Reductive Half-Reaction of Xanthine Oxidase

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On the basis of the crystal structure of an aldehyde oxidoreductase, Huber et al. proposed a catalytic mechanism for the reductive half-reaction of xanthine oxidase which involves nucleophilic addition of Mo-bound hydroxide (Moco **1**) to the substrate and hydride transfer from the substrate to sulfido group (Mo═S). Density functional theory calculations have been carried out for the oxidation of formaldehyde, acetaldehyde, formamide, and formamidine with Moco **2** to understand more detailed catalytic pathways. Our calculation results indicate that the anionic catalyst model acts as a nucleophile and is reactive for the oxidation of aldehyde substrates, which are reactive for nucleophilic addition. In these cases, a concerted mechanism is found to be more favorable than a stepwise mechanism. The concerted mechanism is further shown to be promoted by the presence of a nearby water molecule, in the active site, which serves as a Lewis acid for the nucleophilic addition of hydroxide. For less reactive formamide and formamidine (a model for xanthine) substrates, the calculated activation energies with the above mechanisms are high. These reactions also do not benefit from the presence of the water molecule. The results indicate that different catalyst forms might be responsible for the oxidation of different substrates, which could be regulated by the enzyme active site environment.

Introduction

The xanthine oxidase family[1] is a group of molybdenum-containing enzymes that catalyze the transfer of an oxygen atom from water to a substrate, such as xanthine or aldehyde. In the human body, the xanthine oxidase catalyzes the hydroxylation of xanthine to uric acid, the crystal deposition of which is believed to cause gout.[2] For this reason, the inhibition of this enzyme becomes a target of drug design,[2] and understanding the mechanism of this enzyme is the key in the design process. Although the first xanthine oxidase related enzyme was discovered about a century ago, its catalytic mechanism is still unclear to date. The mechanistic study on this family of enzymes was revived after the crystal structures of several enzymes became available.[3−5]

All enzymes in the xanthine oxidase family contain a structurally similar molybdopterin cofactor in the active site. In the oxidized state, the Mo(VI) center processes an oxo group, a sulfido group, a dithiolene side chain to a pterin,[6] and a water or hydroxide ligand (Scheme 1). Hindered by the challenge of incorporating all these groups on the molybdenum atom, synthetic modeling remains a difficult problem.[7]

Several catalytic mechanisms were proposed in the last two decades.[1,8−11] Among them, the most promising one is the structure-based mechanism proposed by Huber and co-

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(5) After the original submission of this Article, the first key intermediate crystal structure in the xanthine oxidoreductase catalysis process was reported by Okamoto et al. This involves the oxidation of a less reactive substrate, FYX-051. The intermediate is trapped in a Mo-bound form. This should provide useful information for more detailed analysis of the catalytic mechanism. See: Okamoto, K.; Matsumoto, K.; Hille, R.; Eger, B. T.; Pai, E. F.; Nishino, T. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 7931−7936.

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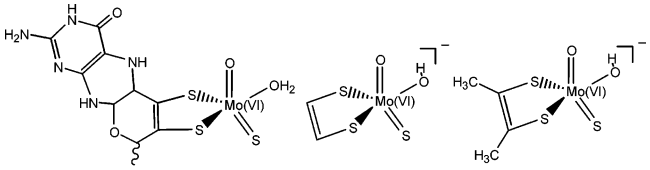
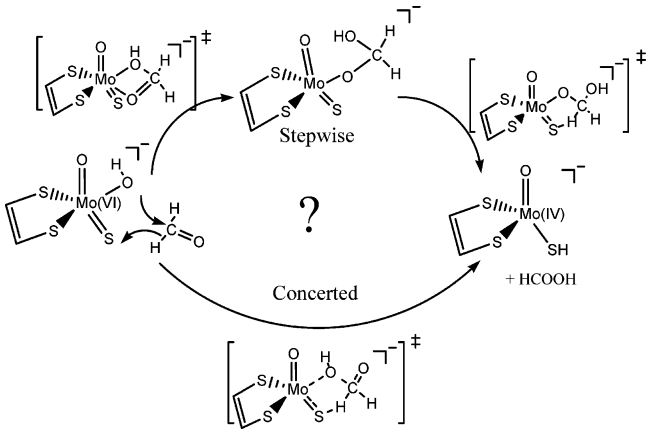
Scheme 1. Native Moco Active Site in the Xanthine Oxidase Family, Moco Model 1, and Model 2



Scheme 2. Proposed Mechanism Involving Hydroxide Nucleophilic Addition and Hydride Transfer



workers based on the crystal structures of aldehyde oxidoreductase from *Desulfovibrio gigas* (Mop), including an alcohol-bound form.[8] The reaction is supposed to proceed (1) by a reaction in which the initial molybdenum-bound water molecule is turned into OH^- by proton transfer to Glu-869 near the molybdenum active site and (2) by nucleophilic attack on the substrate by metal-coordinated OH^- and hydride transfer from the substrate to the metal sulfido group (Scheme 2).[8] An open question is whether these two processes are stepwise or concerted. Several theoretical studies have been carried out based on the above catalytic model.[12−14] Voityuk et al. suggested a stepwise pathway based on a model density functional study.[13b] By studying the reaction of the Moco (molybdenum cofactor) model **1** (Scheme 1) with formaldehyde, they found that the nucleophilic addition of the hydroxide on formaldehyde leads to a stable intermediate. This intermediate undergoes a hydride-transfer reaction with a low activation energy of about 7.7 kcal/mol. They also found that the release of the bound product formic acid costs about 4.8 kcal/mol. It should be noted that the transition structure for the nucleophilic addition of hydride on formaldehyde was not reported.[13b] It would be critical to know whether this step is the rate-determining step and how high the barrier is for the step. On the other hand, Ilich et al. proposed a concerted mechanism that involves simultaneous nucleophilic addition of hydroxide and hydride transfer (see Scheme 2). Using the MP2 method, they located the concerted transition structure for the reaction of Moco model **1** with formamide,[14] and reported an activation energy of 78 kcal/mol for the reaction. This barrier seems to be very high but might be due to the MP2 method, which might not treat the energetics properly. The studies of the two groups employed different calculation methods and different substrates. A simple comparison of their results is difficult.

A key difference between the above two mechanisms is the coordination of the metal center. In the stepwise mechanism, the carbonyl group of the substrate is activated by the coordination of the carbonyl oxygen with the metal center. On the other hand, in the concerted mechanism, the carbonyl group is not involved in coordination with the metal and, therefore, is available for acid (or Lewis acid) catalysis. In this connection, it has been found that there is a water molecule in the vicinity of the Mop active site.[3] It is hypothesized that the water molecule might assist the half-reaction.[8] Therefore, it is important to explore the function of the water molecule, which might also provide some help in distinguishing the above two mechanisms.

We report here on a theoretical study based on density functional theory calculations on the mechanism of the half-reaction of xanthine oxidase. Using Moco catalyst model **2**, we have explored the reaction potential energy surfaces of both the concerted and stepwise mechanisms so that a comparison can be made more systematically. The influence of a water molecule on the two mechanisms has been studied. Using a series of substrates, we attempted to address the reactivity of the enzyme model toward substrates with different properties, thus revealing insight into further possible mechanisms. We have also modeled the influence of the enzyme environment by using a continuum solvent model. Our results reveal useful information for the understanding of the reaction mechanism.

Computational Details

All calculations were carried out with the density functional method of B3LYP[15,16] using the Gaussian 03 program.[17] Geometries were optimized with the following basis sets: Mo, LanL2DZ with an f polarization function;[18] S, LanL2DZ with a d polarization function;[19] 6-31+G** for the rest of the elements. Vibration frequency calculation was performed on each structure with the same method. The optimized minima and the transition structures

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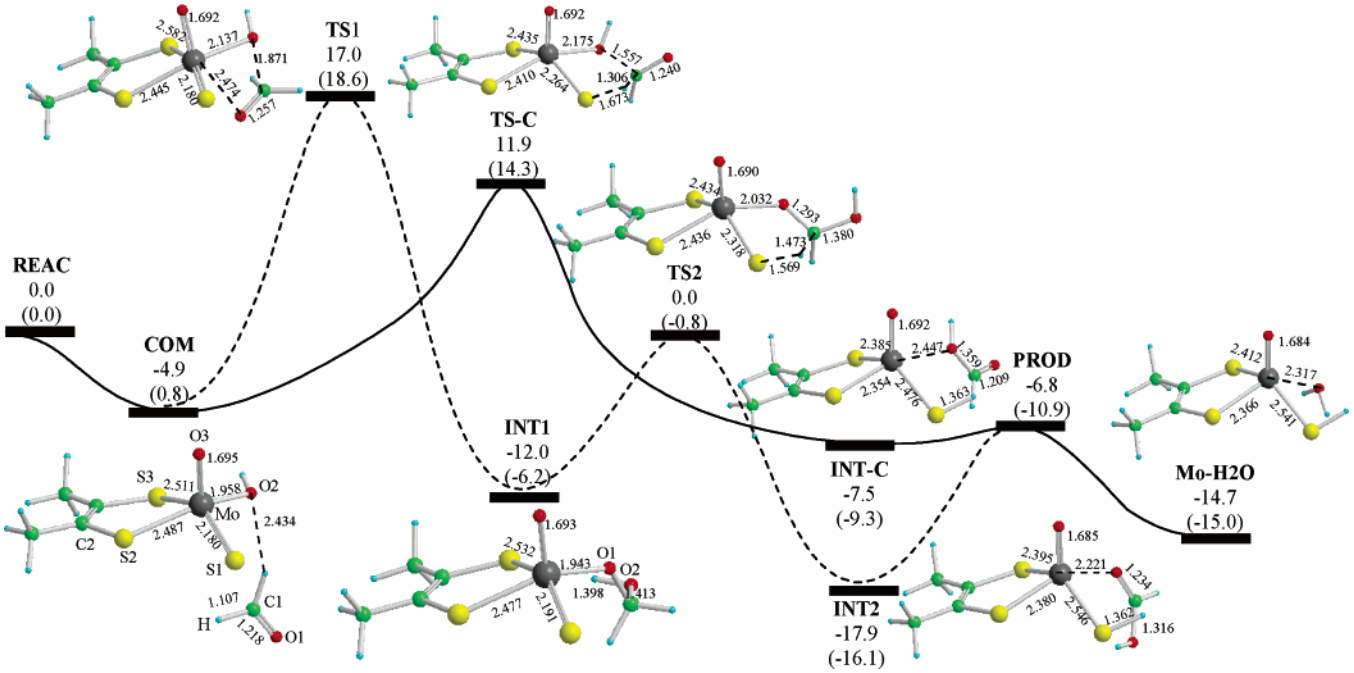

Figure 1. Calculated enthalpy profile for the reaction of Moco model **2** with formaldehyde. Enthalpies at 298 K are in kilocalories per mole. Single-point energies with the PCM solvent model calculations are shown in parentheses. Bond distances are in angstroms.

have been confirmed by harmonic vibration frequency calculations. The relative enthalpies reported have been corrected with zero-point energies (ZPEs). Single-point energy calculations with solvent effect were carried out with the polarized continuum model (PCM),[20] using a dielectric constant of 5.621.[21]

Our calculated Moco model $[LMo^{IV}(=O)(=S)(OH)]^-$ (L = dimethyl ene-1,2-dithiolate) (**2**; shown in Scheme 1) is similar to the model (**1**) that was employed in previous theoretical studies reported by other groups.[12−14] The only difference is that we use dimethyl ene-1,2-dithiolate instead of ene-1,2-dithiolate. Since the pyranopterin dithiolate plays an important role in the mononuclear molybdenum-containing enzymes,[6] we examined the simplest model ene-1,2-dithiolate model **1** and the dimethyl ene-1,2-dithiolate model **2** at first. Although the geometries of the two models are similar, the reaction enthalpies calculated by model **1** (−5.7 kcal/mol) and model **2** (−6.8 kcal/mol) differ by about 1.1 kcal/mol. Therefore, we adopted the dimethyl ene-1,2-dithiolate for its better presentation of the natural enzyme.

Table 1. Calculated Mulliken Atomic Charges of the Species Shown in Figure 1 in the Gas Phase

	COM	TS1	INT1	TS2	INT2	TS-C	INT-C
Mo	0.838	0.639	0.872	0.652	0.474	0.531	0.652
S1[a]	−0.343	−0.366	−0.346	−0.294	−0.387	−0.268	−0.377
S2[a]	−0.122	−0.085	−0.111	−0.148	−0.165	−0.149	−0.164
S3[a]	−0.200	−0.163	−0.208	−0.180	−0.193	−0.172	−0.223
O3[a]	−0.659	−0.612	−0.703	−0.714	−0.651	−0.640	−0.626
O2[a]	−0.819	−0.762	−0.544	−0.435	−0.370	−0.531	−0.493
O1[a]	−0.386	−0.366	−0.662	−0.547	−0.424	−0.501	−0.437
C1[a]	0.069	0.139	0.160	0.214	0.291	0.246	0.340
H[a]	0.120	0.090	0.104	0.027	0.092	0.031	0.069

[a] The atoms are labeled as in COM in Figure 1.

Results and Discussion

Comparison of the Stepwise Mechanism and the Concerted Mechanism. We first studied the Moco reductive half-reaction with the simplest substrate, formaldehyde. The calculated enthalpy profiles for the two mechanistic pathways are depicted in Figure 1, and the calculated Mulliken atomic charges are listed in Table 1. REAC and **PROD** represent the isolated reactants (Moco) and isolated products, respectively. The two pathways share a common starting complex (COM), in which the substrate and the Moco model complex via Mo−O2---H electrostatic interaction instead of metal carbonyl coordination. This is consistent with the crystallographic data that the substrate does not coordinate to the

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molybdenum center.[8] As a result, the geometrical parameters of the Moco in COM are similar to those of the isolated reactants (REAC). Compared with the oxo group (O3), it is believed that the hydroxide group (O2) is a better nucleophile for its higher negative charge (−0.819; see Table 1). That is consistent with recent experimental suggestion that the catalytically labile site should be the metal-coordinated hydroxide rather than the Mo═O group.[9,10,11] From the charge distribution on oxo (O3), and molybdenum center along the reaction paths (Table 1), one can say that the oxo oxygen (O3) might likely act as a "spectator" group[22] in this reaction.

For the stepwise pathway, the nucleophilic attack by the hydroxyl group on the carbonyl C1 is associated with the coordination of carbonyl O1 to the Mo center, leading to a hexacoordinated transition structure (TS1). The transition structure is a four-membered ring. The forming O---C and O---Mo bond lengths are 1.871 and 2.471 Å, respectively. It is considered to be a late transition structure. The calculated activation enthalpy with respect to COM is about 21.9 kcal/mol. This barrier is high compared to those of other metal-mediated nucleophilic addition reactions.[23] This indicates that the coordination of the carbonyl oxygen does not have a significant effect on promoting the nucleophilic addition. The other plausible nucleophilic attack transition structure without the Mo---O1 coordination could not be located. That might be due to the lack of activation on the carbonyl and the Mo---O2 bond. Since the reaction can be regarded as a [2 + 2] metathesis, the Mo---O1 bond formation and the Mo---O2 bond cleavage are simultaneous, and the oxidation state of Mo(VI) remains unchanged. The Mo---O1 bond length (1.943 Å) in the following intermediate (INT1) is close to the Mo---O2 bond length (1.958 Å) in COM, representing a molybdenum−oxygen single bond. This INT1 is somewhat overstable as an intermediate in the enzyme catalysis process.[24] The next step is the hydride transfer from the substrate to the sulfido group of Moco, in which the redox reaction occurs.[25] The transition structure involves considerable C−H bond (1.473 Å) breaking and H−S bond (1.569 Å) formation. The calculated activation enthalpy of this step is about 12.9 kcal/mol, which is much lower than that of the hydroxide nucleophilic addition step (TS1). Thus, the rate-determining step of the stepwise pathway should be the hydroxide nucleophilic attack rather than the hydride transfer. The redox reaction occurs in the hydride-transfer process, and the resulting oxidized substrate HCOOH coordinates to Mo(IV) with its carbonyl O1 (INT2). The dissociation of HCOOH requires an energy of about 11.1 kcal/mol, which is larger than the complexation energy of oxidized Moco with substrate. This conflicts with the experimental observation that E_{ox}·S binding is stronger than E_{red}·P binding.[11] In addition, the enthalpy of water binding to the reduced Moco is about −7.9 kcal/mol. This means that the replacement of the Mo-bound formic acid product by a molecule of water is endothermic. Therefore, this stepwise mechanism would display a product inhibition.

The redox reaction in the concerted pathway is a one-step reaction with a transition structure (TS-C) in which C1−H forms a five-membered ring with O2−Mo−S1 in a roughly coplanar manner. Compared with TS1, the longer Mo− O2 (2.175 Å) and the shorter O2−C1 (1.557 Å) distances in TS-C indicate that the nucleophilic attack of hydroxide is in a later stage. On the other hand, compared with TS2, C1−H (1.306 Å) is shorter and S1−H (1.673 Å) is longer, revealing that the hydride transfer is in an earlier stage. Therefore, the concerted mechanism can be considered as dominated by the hydroxide nucleophilic addition. The concerted pathway has a calculated barrier of about 16.8 kcal/mol,[26] which is lower than that of the stepwise pathway by about 5.1 kcal/mol, indicating that the concerted pathway is more favorable than the stepwise one. In the product complex INT-C, HCOOH coordinates to Mo by the hydroxyl oxygen, with a binding energy of 0.7 kcal/mol. Thus, this means that product dissociation and water coordination to the reduced Moco in the concerted pathway are much easier. Since the hydride transfer is involved in the rate-determining step of the concerted pathway but not in that of the stepwise one, a kinetic isotope effect experiment should be able to distinguish these two pathways.

The enzyme environment can have a significant effect on the overall reaction pathway. While modeling of the real environment of the enzyme active site is difficult at this stage, we have evaluated the effect of a bulk dielectric environment on the calculated potential energy surface shown in Figure 1. Therefore, the energy of each structure in Figure 1 was recalculated with the PCM[20] with a dielectric constant of 5.6 and without geometrical optimization. The calculated results are given in parentheses in Figure 1.[27] With this model, the complexation energy for COM disappears. This is likely due to the overestimation of the solvent stabilization for the bimolecular system over the complex. The calculated activation energies of TS1 and TS-C both increase with respect to REAC but decrease with respect to COM. However, the concerted pathway is still more favorable by about 4.3 kcal/mol. For the stepwise mechanism, the barriers to the hydride transfer and the dissociation of formic acid product both are reduced to some extent. Nevertheless, the product inhibition is still predicted. Therefore, the conclusion that the concerted mechanism for the oxidation of formaldehyde is more favorable than the stepwise mechanism stands in this "enzyme solvent model" calculation.

Stabilization from the Neighboring Water. We next examined the role of the adjacent water that is believed to be very important for catalysis. A complex between Moco

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(26) We also calculated this reaction with the MP2 method using the same basis set. The calculated activation energy with the concerted transition structure is about 53.3 kcal/mol. Thus, the MP2 method appears to overestimate the reaction activation energy. Further study also indicated that the MP2 method underestimates the stability of the intermediate INT-C, which is about 21.6 kcal/mol less stable than REAC.
(27) We thank one of the reviewers for a suggestion on the calculations.

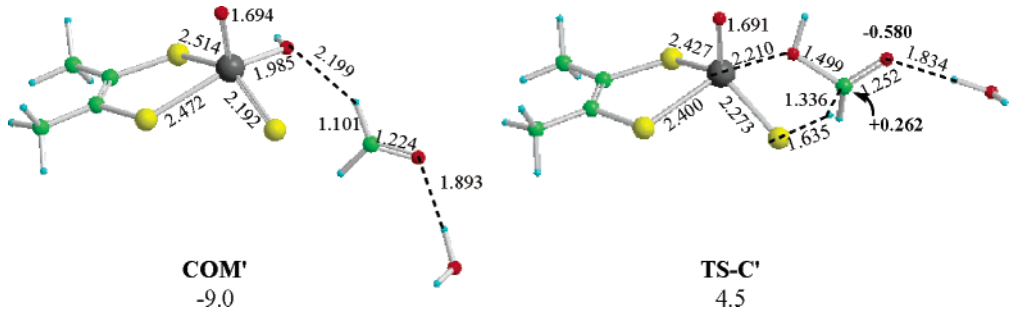

Figure 2. Calculated "water-assisted" complex COM′ and transition structure TS-C′ for the concerted pathway of the reaction of Moco model **2** with formaldehyde. Bond distances are in angstroms and enthalpies (with respect to isolated reactants) in kilocalories per mole.

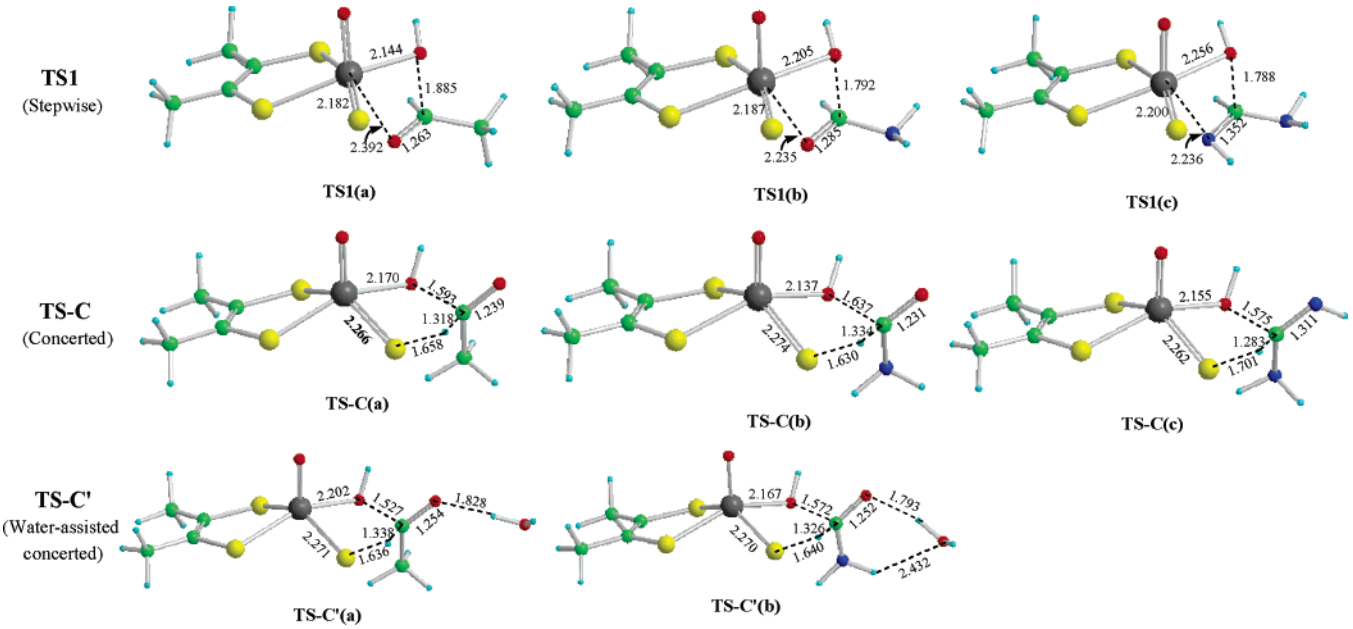

Figure 3. Optimized transition structures TS1, TS-C, and TS-C′ for the reactions of Moco model **2** with acetaldehyde (a), formamide (b), and formamidine (c). Bond distances are in angstroms.

1 and $H_2C{=}O{---}H_2O$ was located. As shown in Figure 2, the hydrogen bond by the water molecule causes a polarization of the C═O bond. As a result, the complexation interaction of Moco **2** with $H_2C{=}O{---}H_2O$ becomes considerably stronger (−9.0 kcal/mol) than with $H_2C{=}O$ (−4.9 kcal/mol). Starting from this complex, we tried to locate a nucleophilic addition transition structure (corresponding to TS1) for the stepwise mechanism. However, all of our efforts failed. Starting from the geometry of TS1 and adding H_2O to the formaldehyde oxygen, geometric optimization leads to the departure of the formaldehyde oxygen from the Mo center. This means that formaldehyde has a stronger interaction with water than with Mo. Thus, with the water molecule, TS1 cannot exist. More careful geometrical optimization leads to the location of TS−C′ as the only transition structure. As shown in Figure 2, this transition structure is basically a water-coordinated concerted transition structure (TS-C). TS-C′ and TS-C have very similar geometries except that TS-C′ has a slightly shorter forming O---C bond length. There is a stronger hydrogen bond between formaldehyde and water in TS-C′ than in COM′, as indicated by a shorter O---C distance (1.834 Å vs 1.893 Å). The hydrogen-bonding by the water molecule apparently serves as a Lewis acid, and it stabilizes the nucleophilic addition transition structure. As a result, the calculated relative enthalpy of "water-assisted" TS-C′ with respect to the complex is reduced to 13.5 kcal/mol, while that of TS-C is about 16.8 kcal/mol. That is, the hydrogen bond by the water molecule reduces the activation energy of the concerted mechanism by about 3.3 kcal/mol. Thus, we can conclude that with the presence of the nearby water molecule the stepwise mechanism disappears and the concerted mechanism is promoted and its activation energy is reduced.

Different Reactivities of Substrates. It is known that xanthine oxidase can catalyze the oxidation of a variety of substrates.[28] Is the above mechanism for reactive formaldehyde suitable for other less reactive substrates? Acetaldehyde, formamide, and formamidine were next examined. The calculated transition structures for the rate-determining step of both the concerted and stepwise mechanisms of these reactions are shown in Figure 3. The geometries of these transition structures are similar to those of the formaldehyde reaction. The calculated activation enthalpies with respect to the catalyst−substrate complex (COM or COM′ for water-assisted reactions) are listed in Table 2.

(28) (a) Morpeth, F. F.; Bray, R. C. *Biochemistry* **1984**, *23*, 1332−1338. (b) Morpeth, F. F.; George, G. N.; Bray, R. C. *Biochem. J.* **1984**, *220*, 235−242.

Table 2. Calculated Activation Enthalpies (kcal/mol) of the Reactions between Moco Model **2** and Different Substrates with Respect to the Catalyst−Substrate Complex COM or COM′

	formaldehyde	acetaldehyde (a)	formamide (b)	formamidine (c)
TS1[a]	22.0	24.5	38.6	49.1
TS-C[a]	16.9	21.2	40.0	45.4
TS-C[a]	13.5	18.4	38.5	
TS1(sol)[b]	17.8	20.1	29.5	34.4
TS-C(sol)[b]	13.5	18.5	32.6	41.6
TS-C(sol)[b]	12.6	17.5	34.9	

[a] In the gas phase. [b] In a bulk solvent with a dielectric constant of 5.6.

As expected, acetaldehyde is less reactive than formaldehyde. The calculated activation enthalpies of the stepwise pathway (TS1(a)) and the concerted pathway (TS-C(a)) of the acetaldehyde reaction are higher than those of the formaldehyde reaction by 3.5 and 4.3 kcal/mol, respectively. The concerted mechanism (TS-C(a)) is still more favorable than the stepwise mechanism (TS1(a)). For the reaction of formamide, the calculated activation enthalpies are 38.6 and 40.0 kcal/mol with the stepwise and concerted mechanisms, respectively. Compared to those of the formaldehyde reaction, these barriers are increased by about 16.6 and 23.1 kcal/mol, respectively. Such a large increase in activation energy is due to two factors: one is the less reactive substrate; the other is the more stable starting complex (COM) with stronger electrostatic interaction between formamide and Moco **2**. While both mechanisms are quite unreactive, the stepwise mechanism becomes slightly more favorable than the concerted mechanism for formamide. For the formamidine, the simplest model of xanthine, we were also able to locate transition structures for the stepwise and concerted mechanisms. The reaction barriers are 49.1 and 45.4 kcal/mol for the two mechanisms, respectively. These high barriers are also understandable because C═N is less reactive than C═O toward nucleophilic addition.

The calculated effect of water on the stabilization of the concerted transition structure is the largest (3.4 kcal/mol) for the formaldehyde reaction. This is reduced to about 2.8 kcal/mol for the acetaldehyde reaction. It has less stabilization effect (1.4 kcal/mol) for the reaction of formamide. For the reaction of formamidine, we were unable to locate a water-assisted concerted transition structure. A closer examination indicates that while the overall stabilizations of the water molecule for the three reactions are similar, the water binds to formaldehyde (2.6 kcal/mol) much weaker than to acetaldehyde (4.9 kcal/mol) and to formadehyde (7.9 kcal/mol). The solvent effect on these transition structures was also calculated by the PCM method using a dielectric constant of 5.6. The results are listed in Table 2. The solvent effect reduces the calculated activation enthalpy for each reaction. However, the preference for the concerted pathway over the stepwise pathway is still apparent for each reaction.

Summary

Density functional theory calculations have been carried out for the concerted and stepwise nucleophilic addition/hydride-transfer mechanisms of the reductive half-reaction of the xanthine oxidase model with the substrates formaldehyde, acetaldehyde, formamide, and formamidine. The results can be summarized as follows: (1) The potential energy surfaces of both the concerted and stepwise mechanisms of the reaction of formaldehyde have been obtained. It is found that the concerted mechanism has a lower activation energy than the stepwise mechanism. In addition, the concerted mechanism gives a more reasonable overall potentional energy surface. (2) The role of a nearby water molecule has been explored. It has been found that it makes the stepwise mechanism disappear, and it stabilizes the concerted transition structure by serving as a Lewis acid for the nucleophilic addition of the molybdenum-bound hydroxide. (3) The effect of the enzyme environment on the potential energy surface has been modeled by the PCM solvent model with a dielectric constant of 5.6. The presence of the dielectric medium does not change the conclusions obtained in the gas phase. (4) The calculations indicate that anionic Moco catalyst model **2** has its characteristic reactivity. That is, it is featured in the nucleophilic addition of the molybdenum-bound hydroxide. Thus, it is reactive toward aldehydes, which have high reactivities toward a nucleophile. It is also important that a nearby water molecule can facilitate this catalytic reaction. The substrates formamide and formamidine (a model of xanthine) are much less reactive toward nucleophilic molybdenum-bound hydroxide. Therefore, the anionic form of Moco model **2** might not be good for these substrates. Two alternative pathways might be considered. It has been shown by Voityuk et al. that a water-bound form of Moco can be in equilibrium with a dihydroxide form.[13a] Such a catalyst, which is neutral, would prefer a hydride abstraction by the sulfido group. Alternatively, for these substrates, the enzyme might need to generate a more powerful nucleophile, such as a dianionic form that is derived from further deprotonation of the hydroxide in Moco **2** by a nearby Glu residue.[5] Further investigations of these pathways are currently being carried out.

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Supporting Information Available: Calculated total energies and structures with Cartesian coordinates (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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