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Articles

Substrate Oxidation in the Active Site of Xanthine Oxidase and Related Enzymes. A Model Density Functional Study

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Gradient-corrected density functional calculations have been performed on a model for the reductive half-reaction of the molybdenum center of xanthine oxidase related enzymes, enabling a discussion of structural details of the Mo coordination sphere in various species involved in the reaction. On the basis of a calculated stable intermediate, we suggest that the substrate is bound to the molybdenum site before rather than after its oxidation. The activation barrier for hydride transfer from the model substrate formaldehyde to the Mo site is calculated to be 7.7 kcal/mol. Since this reaction is predicted to be thermoneutral, the hydride transfer may occur in either direction. The complex formed by the oxidized substrate and the active site is rather weakly bound, supporting the postulated facile replacement of the product from the Mo site by a water molecule.

Introduction

The structures of molybdenum centers in pterin-containing enzymes and the reaction mechanisms involved have been extensively studied in recent years.[1−6] These oxomolybdenum enzymes catalyze the transfer of an oxygen atom between water and a substrate X, which may range from an aromatic heterocycle (e.g., xanthine) to a simple aldehyde:

$$X + H_2O \leftrightarrow XO + 2H^+ + 2e^-$$

This oxygen transfer is coupled to a two-electron redox reaction. The catalytic reaction can be thought of as proceeding in two half-cycles. Within the first (reductive) half-cycle, oxidation of X occurs at the Mo site, accompanied by a reduction of the metal center from Mo(VI) to Mo(IV). In the course of the second (oxidative) half-cycle, the Mo(VI) state is regenerated due to two one-electron transfer processes from the molybdenum center to other redox-active cofactors (e.g., to the FAD cofactor in xanthine oxidase).[5−7] During the past decade a number of catalytic models based on dioxo−Mo(VI) and oxo−Mo(IV) complexes have been developed.[2] However, xanthine oxidase and related enzymes contain an MoOS unit, which is quite unstable, and consequently, their chemical modeling remains a difficult problem. EXAFS,[8,9] EPR,[10,11] and MCD spectroscopy[12] have been important techniques for investigating these enzymes and their models. Thanks to massive efforts involving chemical, structural, and spectroscopic studies of these enzymes, the structure of the molybdenum site has been established and a general enzymatic mechanism for oxo transfer has been proposed.[5,13,14] Enzymes of the xanthine oxidase type possess a molybdenum-containing cofactor (Moco) in which a single molybdenum atom is ligated by a dithiolene side chain to a pterin ring. The structure of Moco is depicted as[13,14]

The oxidized form of Moco contains an Mo(VI) center coordinated by a single pterin cofactor and by three other ligands: an oxo and a sulfido group and either a hydroxo or a water ligand. The reduced form of the molybdenum site has a similar structure, but instead of the sulfido group, an SH^- ligand, formed by hydride $[H^+ + 2e^-]$ transfer, is bound to Mo(IV).[13,14]

Structural features found for xanthine oxidase and related

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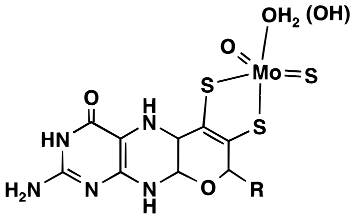

enzymes suggest the following reaction mechanism.[5,6,14] In the case of aldehyde oxidoreductase, an aldehyde substrate binds to Moco through its carbonyl oxygen, facilitating nucleophilic addition to the carbonyl carbon. Then the oxygen of a catalytically labile water molecule or a hydroxo group is transferred to the substrate. A crucial step of the reaction is a hydride anion transfer from the substrate to Moco resulting in the reduction Mo(VI) → Mo(IV). However, definitive conclusions concerning the reaction mechanism are hardly possible in the absence of structural and energetic characteristics of the stationary points (reactants, transition state, intermediate, and products) for these transformations.

In this context, theoretical studies can help to elucidate some basic aspects of the structure and the reactivity of the molybdenum-containing active site. Recently, two density functional calculations on the structure of models for the molybdenum center have been performed.[15,16] Alternative structures for the Mo(VI) and Mo(IV) states of Moco have been predicted.[15] Recently, Bray and Deeth have considered a mechanism for the transformation of a substrate at the Mo center (Scheme 1, adapeted from ref 17) where they assumed that hydride transfer from the substrate to the sulfido ligand of Moco occurs *before* the postulated intramolecular rearrangement of the hydroxyl group.[17] However, they neither evaluated nor discussed the activation barrier for the hydride transfer, but studied only the nucleophilic attack of a coordinated hydroxyl on the CHO ligand.[17]

Thus, the crucial aspect of the catalytic half-cycle, namely, hydride transfer between the substrate and the Mo center, has not been theoretically investigated so far. The present work was undertaken with a view to modeling this hydride transfer in the active site of xanthine oxidase and related enzymes (Scheme 2).

Model

The geometries of the complexes modeling the oxidized and reduced states of the Moco site are displayed in Figures 1 and 2. The ligand L = [−SCR═CRS−] (R = H) represents the dithiolene group of the enzyme pterin moiety. Formaldehyde is chosen as a model substrate. This computational model has been formulated following closely the proposals of Hille[5] and Huber et al.[14] for the reductive half-cycle of the oxygen transfer reaction catalyzed by Moco-containing enzymes; the main aspects are as follows (Scheme 2): (1) a hydroxo group or a coordinated water molecule rather than the oxo ligand of Moco is the labile oxygen to be transferred onto the carbonyl carbon of the substrate in the course of the nucleophilic attack; (2) the substrate replaces the hydroxo (or water) ligand at the metal center; (3) hydride transfer occurs from the substrate to the sulfido group of Moco; (4) and the oxidized substrate, a carboxylic acid, is bound to the reduced molybdenum center and its release from the active site may be facilitated by an incoming water molecule.

Computational Details

The calculations reported in this study were carried out with the help of the nonrelativistic variant of the all-electron linear combination of Gaussian-type orbitals density functional (LCGTO-DF) method,[18] employing an energy functional that incorporates density gradient corrections to the exchange[19] and the correlation terms[20] (generalized gradient approximation, GGA). This quantum chemical method is known to provide accurate results for transition-metal complexes.[21,22] The following orbital basis sets were used:[23] Mo (19s,14p,9d) → [8s,6p,5d], S (12s,9p,2d) → [6s,5p,2d], C, O (9s,5p,1d) → [5s,4p,1d], H (6s,1p) → [4s,1p] (for details see ref 23). The fitting basis sets used in the LCGTO-DF method to represent the electron charge density were constructed in a standard fashion.[18] The exchange-correlation integrals were evaluated by a numerical quadrature scheme.[24,25] All structure determinations were carried out with full geometry optimization using analytical energy gradients.[26] The transition-state structure was located by the eigen mode-following method[27,28] starting from an average geometry of the two minima that are separated by the transition state. For each stationary point a full vibrational analysis was performed to check that all normal eigenvalues of the Hessian matrix were positive for a proposed minimum structure and only one negative eigenvalue existed for a transition state.

Results and Discussion

We start by briefly considering the geometries of the complexes modeling the Mo(VI) and Mo(IV) states of Moco (see Figures 1 and 2, respectively). A detailed discussion was given in our previous work.[15] The structure LMoOS (**1**) has C_s symmetry. Calculated metal−ligand bond lengths and bond angles are in line with experimental values found in crystal structures for related compounds.[29] A significant deviation from planarity is found for the chelated subunit LMo manifested by the dihedral angle MoSCC $\delta = 37.2°$. The coordination sphere of Mo in LMoOS can be easily extended due to interaction with the environment, for example, by a water molecule or a substrate. In fact, the binding energy of a water molecule to Mo in LMoOS is calculated to be 1.2 kcal/mol. The resulting aqua complex **2** has a distorted trigonal bipyramidal structure (Figure 1). The three equatorial positions are occupied by the oxo ligand, the sulfido group, and an S_L atom while another S_L atom and the coordinated water molecule are coordinated at the axial sites. The calculated Mo−OH_2 distance amounts to 2.33 Å. The five-membered LMo ring of **2** is more planar than

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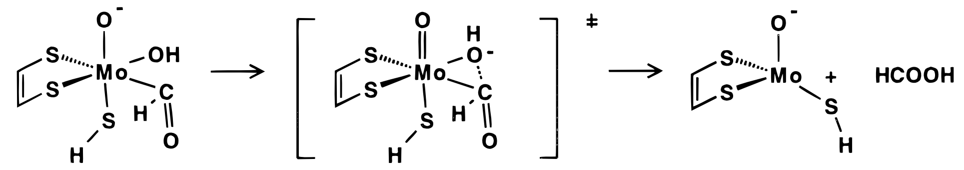
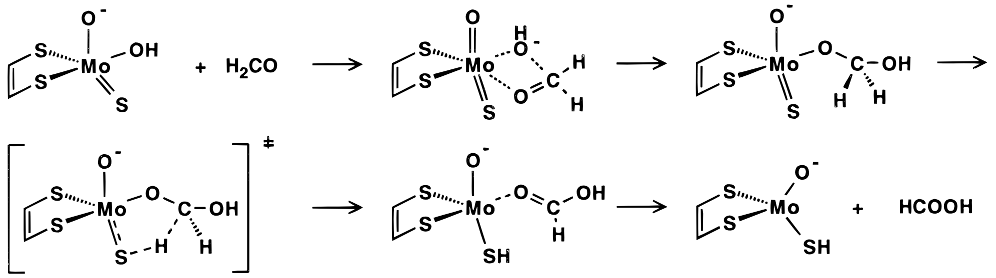
Scheme 1



Scheme 2



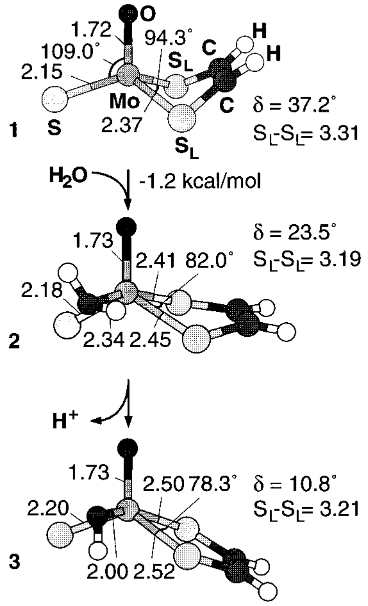


Figure 1. DF-GGA calculated geometrical parameters and binding energy of the H_2O ligand for model complexes of the oxidized state of Moco: distances in Å; angles in deg; δ designates the dihedral angle MoSSC.

the one of the parent complex **1** ($\delta = 23.5°$). In the enzyme crystal structure[13] the carboxylic group of Glu869 is located close to (but not bound to) the Mo site, in a position where it is ready to accept a proton from the coordinated water molecule, thus generating the anionic complex **3**. The loss of a proton increases the nucleophilicity of this (hydroxo) ligand and leads to a considerable shortening of the Mo−O bond length as well as to a further flattening of the LMo ring ($\delta = 10.8°$); other structural parameters of the complex are not significantly affected. Thus, from the calculated data a substantial alteration of the Mo−OH bond length and, consequently, of the bond strength can be expected by proton transfer.

Within the reductive half-cycle the Mo(VI) center is reduced to Mo(IV) by a coupled two-electron and proton transfer (formally, a hydride ion transfer). There are three conceivable possibilities for such a hydride transfer: (i) as proposed earlier,[14] to the sulfido group yielding complex **4** shown in Figure 2, (ii) to the oxo group; and (iii) directly to the metal atom. The resulting three structures have very similar total energies according to the DF calculations.[15] In the current study we will consider solely case i, which is in line with experimental data.[30,31] As expected, the bond between Mo and sulfur is considerably elongated (by 0.2 Å) after hydride transfer to the sulfido group. Complex **4** has a distorted tetrahedral structure in which the LMo fragment is much more planar than that of **1** ($\delta = 7.7°$). Compound **4** binds a water molecule, resulting in the aqua complex **5**. The distance Mo−OH_2 in **5** is unusually long, 2.51 Å, indicating a weak metal−ligand covalent bonding. The interaction energy is calculated to be −8.7 kcal/mol; thus, it seems that the formation of **5** is mainly due to an ion−dipole interaction between the negatively charged oxo group ($Q_O = -0.54$) and the protons ($Q_H = +0.33$) of the water ligand (the corresponding O−H distances are 2.45 and 3.02 Å). The calculated decrease of the Mo−S_L bond length on reduction of Moco, Mo(VI) → Mo(IV), is in accord with EXAFS data,[32] but was previously considered as an unexpected effect.[6] To rationalize the experimentally found trend, a significant change in the coordination sphere had been assumed.[6] However, comparing the calculated geometries of **2** and **5** one notes that the Mo−S_L bonds become shorter on reduction (on the average by 0.04 Å) even when the coordination sphere of Mo remains unchanged.

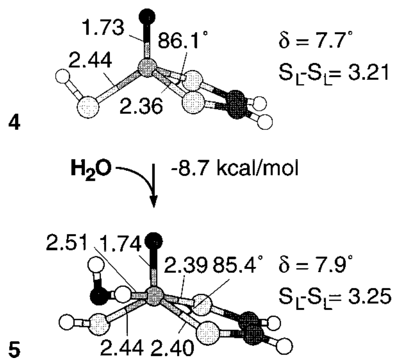


Figure 2. DF-GGA calculated geometrical parameters and binding energy of the H_2O ligand for model complexes of the reduced state of Moco; distances in Å; angles in deg; δ designates the dihedral angle MoSSC.

Let us now consider the reaction of the molybdenum site with the model substrate (Figure 3). The nucleophilic attack of the

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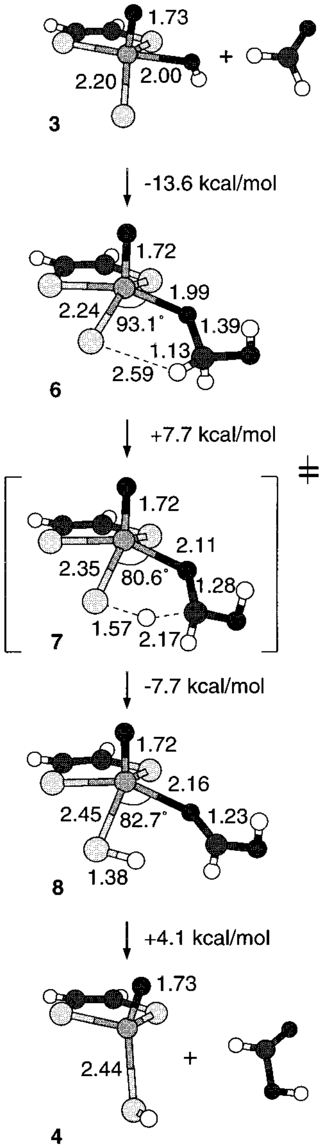

Figure 3. Relative energies and structural features of the equilibrium and transition-state structures (layout as in Figures 1 and 2) along the reaction pathway for the model substrate oxidation calculated at the DF-GGA level.

coordinated hydroxide group of **3** on the carbonyl carbon of the substrate is exothermic. The resulting intermediate **6** is relatively stable; the reaction energy of its formation is calculated to be −13.6 kcal/mol. The intermediate features a rather strong covalent metal−substrate interaction. The corresponding Mo−O bond length in **6** is 1.99 Å, almost equal to that of Mo−OH (2.00 Å) in the parent complex **3**. Note that the coordination number of the molybdenum center remains unchanged in the course of the nucleophilic attack. We failed to localize a transition structure corresponding to a hydroxo group transfer from the molybdenum site to the substrate. Such a transfer is not the only possible pathway for this reaction. The transferred oxygen may be one of an outer-sphere hydroxo group formed due to a proton transfer from a water molecule to the Glu residue in the vicinity of Moco.[13,14] In any case, the existence of the stable intermediate **6** (Figure 3) found in the present calculations may induce speculations about a modified mechanism for the catalytic reaction which would also be in line with structural features found for the oxotransferase enzymes.[13,14] Currently, on the basis of the EPR measurements, one assumes that before oxidation the substrate is located in the second, rather than in the first, coordination sphere of molybdenum and that its binding to the metal center occurs only after hydride transfer.[33] Taking into account the present results, we suggest that the substrate is bound to Mo(VI) before oxidation; substrate binding may occur in the course of the nucleophilic attack of the hydroxo group (see Scheme 2). However, other mechanisms of the formation of the intermediate are also plausible, for example, a nucleophilic attack of a water molecule assisted by the carboxylic group of Glu located in the vicinity of the Moco.[14]

The most important feature for the enzymatic reaction considered is a hydride transfer from the substrate to the sulfido group of Moco. A key to the understanding of this process is the location of the transition structure determining the activation barrier for the reaction. According to our calculation the transition state **7** of this reaction lies 7.7 kcal/mol above the anionic intermediate. In the transition structure the S−H distance is elongated by 0.2 Å compared to a normal S−H bond length (e.g., in **8**). An unusually large distance of 2.17 Å is calculated in **7** between the carbonyl carbon of the substrate and the hydrogen atom to be transferred. There is also a considerable elongation of the Mo−O bond between the metal atom and the oxygen center of the substrate (by 0.12 Å) as compared to the intermediate. The Mo−S distance falls in between those in **6** and **8** and corresponds to a partial loss of the double-bond character. The C−O bond of the substrate is changed remarkably in the course of the reaction: its length decreases from 1.39 Å in **6** to 1.28 Å in **7** and further to 1.23 Å in **8**. The activation barrier is found to be symmetric; in other words, the intermediate **6** and the active site−product complex **8** are almost isoenergetic. Since the transformation of **6** into **8** is thermoneutral, the hydride transfer reaction can proceed also in the opposite direction (transforming **8** into **6**), resulting in a reduced substrate and the oxidized state of Moco. This finding is in line with the fact that some oxymolybdenum enzymes catalyze the reduction of substrates.[1,5] In the transition state the Mulliken charge of the H atom to be transferred is +0.05 au; for comparison, we note the charge on H of the hydroxo group in **3** to be +0.29 au, and that of H of formaldehyde to be +0.10 au. Thus, it may be more appropriate to describe the oxidation reaction as a $[H + e^-]$ transfer rather than a hydride or $[H^+ + 2e^-]$ transfer reaction. In the course of the reaction a single electron is transferred from the carbonyl group of the substrate to the molybdenum center simultaneously with the hydrogen transfer (the charge on this group is decreased by −0.26 au in the course of the reaction **6** → **8**).

The geometry of complex **8**, reduced Moco with a product (here: formic acid), is presented in Figure 3. The relatively long distance between the molybdenum center and the product (Mo−O: 2.16 Å; elongated by 0.17 Å compared to **6**) reflects a weak bonding interaction between the two moieties in the final complex. In fact, **8** is favored in energy only slightly, by −4.1 kcal/mol, over the isolated Mo(IV) center and formic acid. Note that a water molecule features a stronger interaction energy with Moco (−8.6 kcal/mol) and thus is able to extrude the product from the active site. Overall, this substitution reaction is slightly exothermic (−4.5 kcal/mol).

Summarizing, we would like to emphasize the following points. First, a stable structure has been located on the potential energy surface, which corresponds to a covalent complex of the molybdenum site with an anionic species formed after nucleophilic attack of the hydroxo group on the substrate. Second, the activation barrier of the hydride transfer from the

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carbonyl carbon of the substrate to the sulfido group of the molybdenum site is found to be 7.7 kcal/mol. Third, the resulting complex of Moco with the oxidized substrate is predicted to be almost of the same energy as the parent intermediate; this makes it possible that the hydride transfer proceeds in either direction. Fourth, a water molecule squeezes the product from the reduced Moco with a small energy gain. Clearly, the results of the calculation have to be considered with care due to possible environmental effects and steric restrictions that are present in the enzymes, but are not taken into account by the model employed in this study.

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