Mechanism of tungsten-dependent acetylene hydratase from quantum chemical calculations

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Acetylene hydratase is a tungsten-dependent enzyme that catalyzes the nonredox hydration of acetylene to acetaldehyde. Density functional theory calculations are used to elucidate the reaction mechanism of this enzyme with a large model of the active site devised on the basis of the native X-ray crystal structure. Based on the calculations, we propose a new mechanism in which the acetylene substrate first displaces the W-coordinated water molecule, and then undergoes a nucleophilic attack by the water molecule assisted by an ionized Asp13 residue at the active site. This is followed by proton transfer from Asp13 to the newly formed vinyl anion intermediate. In the subsequent isomerization, Asp13 shuttles a proton from the hydroxyl group of the vinyl alcohol to the α -carbon. Asp13 is thus a key player in the mechanism, but also W is directly involved in the reaction by binding and activating acetylene and providing electrostatic stabilization to the transition states and intermediates. Several other mechanisms are also considered but the energetic barriers are found to be very high, ruling out these possibilities.

enzyme catalysis | metalloenzyme | cluster approach

Tungsten is the heaviest metal in biology and plays prominent roles in carbon, nitrogen, and sulfur metabolisms (1–6). In all known tungstoenzymes, the tungsten ion coordinates to the dithiolene group of two pterin cofactors and has oxidation numbers ranging from +4 to +6. Three families of enzymes have been discovered to be tungsten-dependent, namely aldehyde oxidoreductase, formate dehydrogenase, and acetylene hydratase (AH) (1). The former two are involved in oxidative reactions, whereas the third one catalyzes the nonredox hydration of acetylene (Scheme 1) in the anaerobic unsaturated hydrocarbon metabolism (7). This reaction is exothermic by about 27 kcal/mol, and the resulting acetaldehyde can be further oxidized to provide a significant source of carbon and energy for certain bacteria (8).

The crystal structure of AH from *Pelobacter acetylenicus* has been solved by Seiffert et al. at 1.26-Å resolution, and it reveals a mononuclear tungsten center in the active site and a nearby iron–sulfur cubane cluster (9). In the active site, the tungsten ion is ligated by the four sulfur atoms of the two pterin dithiolene moieties, Cys141, and an oxygen species, which was assigned to be a water molecule. A second-shell residue, Asp13, is found to hydrogen-bond to the oxygen species and was suggested to be in the protonated form, based on pH titration calculations using continuum electrostatics and statistical thermodynamics (9). However, the ionized form of Asp13 cannot be ruled out because the crystal structure shows that it has two additional hydrogen bonds, to the peptide bond of Cys12–Asp13 and the side chain of Trp179, which would lower the pK_a significantly. An arginine residue (Arg606) is also located close to the two pterins, providing electrostatic stabilization to the cofactors. Based on studies of biomimetic complexes of AH (10) and redox titration (11), it has been demonstrated that W^{IV} participates in the catalysis, whereas W^{VI} is inactive. Molybdenum, tungsten's lighter congener, can also be incorporated into the active site, but it results in 60% activity (12). The iron–sulfur cluster is not required for enzyme activity if strong reducing agents are available (11).

Scheme 1. Reaction catalyzed by AH.

The catalytic mechanism of AH is presently poorly understood. The mechanistic proposals can be divided into two groups, first- and second-shell mechanisms. Based on the crystal structure, Seiffert et al. proposed a second-shell mechanism in which the W-bound water functions as an electrophile to deliver a proton to the acetylene substrate (9). In this mechanism, Asp13 is assumed to be protonated and donate a hydrogen bond to the water molecule, which would stabilize the negative charge on the water/hydroxide during the proton transfer. On the other hand, using density functional theory (DFT) calculations on a small model consisting of the tungsten and its first-shell ligands, Antony and Bayse have shown that the displacement of water by an acetylene molecule is exothermic by more than 20 kcal/mol (13). Based on this, they suggested that the water molecule performs a nucleophilic attack on the W-bound acetylene, assisted by the neutral Asp13 residue. Most recently, Vincent et al. showed, also using DFT calculations, that both these mechanisms have very high barriers, more than 40 kcal/mol, ruling them out (14). Instead, they speculated that the reaction could proceed through a $W=C=CH_2$ vinylidene intermediate. However, the barrier for the formation of this intermediate and also the barriers for the following steps were found to be quite high (28.1 and 34.0 kcal/mol, respectively), suggesting that some other mechanism is in action.

In recent years, quantum chemical methods have proven very successful in the study of enzymatic reaction mechanisms (15–19). In particular, the mechanisms of Mo- (20–24) and W-dependent (13, 14) enzymes have been investigated and a wealth of mechanistic insight has been obtained, especially on the role of the metal ions in the reactions. Here, density functional calculations are employed to elucidate the mechanism of AH. On the basis of the crystal structure, a large model of the active site is designed, and the hybrid functional B3LYP (25) is used to calculate the potential energy profiles for several mechanistic scenarios. We propose a previously undescribed mechanism involving five steps, which all have feasible energy barriers. All other mechanisms considered are found to be associated with much higher barriers.

Results and Discussion

Active Site Model. A model of the active site (Fig. 1) was designed on the basis of the crystal structure of native AH (PDB ID code 2E7Z) (9). The model comprises the tungsten ion with

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its first-shell ligands, including two pterin molecules, Cys141, and a water molecule. The peptide chains formed between Cys141 and Met140 as well as between Cys141 and Ile142 were also included to prevent rotation of Cys141. Several second-shell residues, Cys12, Asp13, Trp179, and Arg606 were also included. The amino acid residues and the pterin cofactors were truncated so that in principle only the functional groups, extended by one or two carbons, were kept in the model; see Fig. 1. Hydrogen atoms were added manually. The catalytically important Asp13 residue was modeled in the ionized form. It is engaged in three hydrogen bonds, with the peptide bond of Cys12-Asp13, Trp179, and the tungsten-bound water molecule. These interactions will lower the pK_a of Asp13 considerably, which could render the residue in the ionized form. Rough pK_a estimation using PROPKA (26) gives a value of 6.3, further corroborating this idea. As will be discussed in the final section of the paper, we have also considered a number of mechanisms involving a neutral Asp13, but they were all associated with much higher barriers. To keep the optimized structures resembling the experimental one, the truncation atoms were kept fixed at their X-ray positions during the geometry optimizations. This is a standard procedure in the cluster approach for modeling enzymes (27, 28). The locked atoms are indicated by asterisks in the figures below. The total size of the model is 116 atoms and the overall charge is -1 .

Reaction Mechanism. To arrive at a mechanism that has plausible energies and barriers, we examined a number of mechanistic scenarios. We first describe the results concerning the preferred reaction mechanism (summarized in Scheme 2). Some of the other alternative mechanisms that are shown to have higher barriers will be discussed at the end.

The mechanism starts with a ligand exchange step (Step 1, Scheme 2), in which the acetylene substrate displaces the tungsten-bound water molecule and binds to the metal in an η^2 fashion (see Fig. 2 for optimized structures). In the current active site model, this step (from React to Inter1) is calculated to be exothermic by 5.4 kcal/mol, already here suggesting an energetic preference for the first-shell mechanism. We have not specifically calculated the transition state for this ligand exchange step, but the barrier is expected to be quite low because preliminary calculations show that full dissociation of the water molecule requires less than 9 kcal/mol.

Fig. 1. Optimized structure of the active site model of AH. Atoms marked with asterisks were fixed at their X-ray structure positions during the geometry optimizations. Distances are given in angstrom (Å).

At React, the water molecule coordinates to the tungsten ion with a distance of 2.26 Å. The coordination of the W^{IV} ion is close to octahedral, which reproduces the X-ray structure (9). At Inter1, the acetylene molecule binds to the tungsten ion with two equal W-C distances (2.04 Å). Acetylene acts as a 4e donor through its two π -orbitals interacting with two empty d orbitals of tungsten (29). In addition, the occupied d orbital of tungsten donates two electrons to one of two unoccupied π^* -orbitals of acetylene (13). This back donation weakens the C-C bond, resulting in an increase of the bond length, from 1.21 Å in React to 1.30 Å in Inter1. Also, the two H-C-C angles are bent to *ca.* 140° in Inter1. Furthermore, we can see that one of the remaining unoccupied d orbitals of tungsten interacts with the other unoccupied π^* -orbital of acetylene to form a δ -like orbital, which can facilitate the electron transfer to this empty molecular orbital during a nucleophilic attack on the acetylene (next step). This analysis shows that the d^2 configuration of the metal is required to bind and weaken the triple bond and also to facilitate the nucleophilic attack, which could explain why only W^{IV} and Mo^{IV} can be used in enzymatic acetylene activation. Detailed orbital interaction diagram for Inter1 is given in *SI Appendix*.

At Inter1 the substrate is thus set up for a nucleophilic attack by the displaced water molecule, which can be activated by a proton transfer to Asp13. The optimized structures of the concerted nucleophilic attack-proton transfer transition state (TS1) and the resulting vinyl anion intermediate (Inter2) are shown in Fig. 2. The barrier for this step (Step 2) is calculated to be 16.9 kcal/mol (19.8 kcal/mol without inclusion of solvation effects) relative to Inter1, whereas Inter2 is 17.0 kcal/mol (17.8 kcal/mol without solvation) higher than Inter1, i.e., essentially equienergetic with TS1. At TS1, the critical O-C1 distance is 1.88 Å and the proton is transferring from water to Asp13 with H-O_{water} and H-O_{Asp13} distances of 1.05 and 1.43 Å, respectively. It is very interesting to note that the nucleophilic attack takes place nearly perpendicularly to the W-C-C plane, indicating the involvement of the empty δ -like orbital resulting from the $d-\pi^*$ interaction discussed above.

Because of the negative charge developed at the vinyl anion intermediate in Inter2, the two carbons now are bound asymmetrically to the tungsten ion, with W-C1 and W-C2 distances of 2.17 and 1.93 Å, respectively. The C1-C2 bond is elongated from 1.30 Å in Inter1 to 1.35 Å in TS1 and 1.40 Å in Inter2. From Inter2, the protonated Asp13 can now function as a general acid to deliver a proton to the C2 center of the vinyl anion, creating a vinyl alcohol intermediate, Inter3 (Step 3, Scheme 2). This turns out to be the rate-limiting step of the reaction. The barrier is 6.0 kcal/mol relative to Inter2, and the accumulated barrier relative to Inter1 is thus 23.0 kcal/mol. Step 3 is very exothermic, by 28.5 kcal/mol. At the transition state, TS2, the key distances of the proton to O_{Asp13} and C2 are 1.10 and 1.58 Å, respectively. The proton transfer neutralizes the negative charge on C2, weakening thus the W-C2 bond, which is elongated from 1.93 Å in Inter2 to 2.02 Å in TS2 and 2.33 Å in Inter3. At Inter3, the vinyl alcohol coordinates weakly to the tungsten ion, with only one of the two carbon atoms, and the hydroxyl group forms a rather short hydrogen bond to the ionized Asp13 residue (O...H distance of 1.53 Å).

To complete the reaction, the vinyl alcohol now needs only to tautomerize to acetaldehyde. At this point, the vinyl alcohol can be released and the interconversion can take place outside the active site. However, we have here also examined how it could be affected at the tungsten center. The tautomerization can be achieved with the help again of Asp13 over two steps (Steps 4 and 5, Scheme 2), first a proton transfer to Asp13 via TS3 to form an enolate (Inter4) and then the back delivery of the proton to C2 via TS4 to form the acetaldehyde (Prod). The barrier for Step 4 is 9.3 kcal/mol, whereas the barrier for Step 5 is 14.1 kcal/mol. We note that once the proton is transferred to the aspartate, the

Scheme 2. Suggested mechanism for acetylene hydratase based on the present calculations.

Fig. 2. Optimized structures of the intermediates and transition states along the reaction pathway. For clarity, only the core of the model is shown. For full model, see Fig. 1.

enolate binds to the tungsten ion with the oxygen atom instead of the carbons (W—O distance of 2.10 Å at Inter4). The final product complex Prod is calculated to be 13.0 kcal/mol lower than Inter1. The overall potential energy graph for all steps is given in Fig. 3.

In contrast to previous mechanistic suggestions, this mechanism has thus feasible barriers. It requires the Asp13 residue to be in the ionized form, and as discussed above, this is quite reasonable considering that it is hydrogen-bonded to three other groups in the active site.

Alternative Mechanisms. In this section we briefly discuss some other mechanisms that we also investigated and that turned out to have high barriers. The mechanism proposed above (Scheme 2) requires that the Asp13 is in the ionized form to

Fig. 3. Calculated potential energy profile for the mechanism proposed in this study.

Fig. 4. Potential energy profile and optimized structures for electrophilic attack by neutral Asp13. For full active site model, see Fig. 1.

start with. All previous suggestions considered Asp13 to be neutral. We have examined three different mechanisms with a neutral Asp.

First, in the case of neutral Aps13, the initial ligand exchange step (React' to Inter1'; see Fig. 4), in which the acetylene substrate displaces the tungsten-bound water, is now exergonic by as much as 21.2 kcal/mol (19.9 kcal/mol without solvation). This is quite close to the result of Antony and Bayse (23.7 kcal/mol) (13), but quite different from the 8.8 kcal/mol found by Vincent et al. (14). The difference could originate from the size of the model employed in the calculations. This large driving force for substrate binding at the metal indicates a first-shell mechanism also here. When Asp13 now is protonated, and cannot function as a general base as in our suggested mechanism, it could instead act as an electrophile to protonate the tungsten-bound acetylene, as suggested by Vincent et al. (14). With their model, the barrier was calculated to be 28.0 kcal/mol for the first step.

Here, we also considered this possibility to investigate whether the model size can affect the energetics. We have located the transition state for the proton transfer from Asp13 to C1 (TS', Fig. 4). It turns out that concertedly with this proton transfer, the Cys141 ligand performs a nucleophilic attack on C2, and thus no W^{VI} -vinyl anion (or W^{IV} -vinyl cation) intermediate could be located using the larger active site model employed here. The barrier is calculated to be 31.6 kcal/mol, providing additional evidence that this pathway is not a viable option.

Another option is that the W-bound acetylene undergoes a [1,2] hydride shift to directly form the $\text{W}=\text{C}=\text{CH}_2$ vinylidene intermediate. In a theoretical study on the tungsten-catalyzed cycloisomerization of 4-pentyn-1-ol, the barrier for a similar hydride shift catalyzed by $\text{W}^0(\text{CO})_5$ was calculated to be 31.0 kcal/mol (30). In our active site model (with an W^{IV} ion) we found that the barrier for this step is as high as 48.7 kcal/mol, ruling out this possibility as well. See [SI Appendix](#) for optimized structures of the transition state and the vinylidene intermediate.

One final alternative discussed here is based on the second-shell mechanism suggested by Seiffert et al. in which the W-bound water protonates the substrate to give a vinyl cation intermediate (9). As mentioned in the Introduction, later calculations showed that it has a high barrier (14). In our model, we found that concertedly with the proton transfer, a nucleophilic attack by the thiolate moiety of the Cys141 ligand takes place on the other carbon of the substrate. According to our calculations, the barrier is 24.2 kcal/mol relative to React', which might sound reasonable. However, considering that React' is 21.2 kcal/mol higher than Inter1', the total barrier becomes 45.4 kcal/mol and this mechanism can be ruled out as well. The optimized stationary points for this mechanism are also given in *SI Appendix*.

Conclusions

We have in this work designed a large model of the active site of tungsten-dependent acetylene hydratase and used density functional theory calculations to explore its reaction mechanism. Based on these calculations, we have proposed a previously undescribed, energetically feasible reaction mechanism. The mechanism assumes that the important active site residue Asp13 is in the ionized form and can act as a general base in the reaction.

The reaction starts with the displacement of the water molecule bound to the W^{IV} ion by the acetylene substrate. Asp13 then deprotonates the water molecule, which performs a nucleophilic attack on the acetylene, resulting in a vinyl anion stabilized by coordination to the metal. The proton is then transferred to the vinyl anion, generating a vinyl alcohol intermediate. This step is calculated to be rate-limiting, associated with a barrier of 23.0 kcal/mol. The two final steps involve tautomerization of the vinyl alcohol to aldehyde, with help again of Asp13. In this mechanism, both Asp13 and the tungsten metal have thus central roles. The aspartate is essential for activating the nucleophilic water and for shuttling the proton, whereas the metal is essential for binding and activating the acetylene substrate and for stabilizing the anionic intermediates and transition states. We have discussed that the d^2 configuration of the metal is of importance for this mechanism, which could explain why only W^{IV} or Mo^{IV} can be used for enzymatic acetylene activation. Finally, all other mechanisms examined assuming a protonated Asp13, both first- and second-shell variants, have too high barriers.

Methods

The quantum chemical calculations presented herein were accomplished with the B3LYP functional as implemented in the Gaussian03 program package (31). For geometry optimizations, the LANL2TZ(f) (32) pseudopotential was used for W, 6-311 + G(d) for S, and 6-31G(d,p) for the C, N, O, H elements. Based on these optimized geometries, single-point calculations were performed using larger basis sets, where all elements, except W, were described by 6-311 + G(2d,2p).

The polarization effects of the protein surrounding on the active site model were taken into account by performing single-point calculations on the optimized structures using the conductor-like polarizable continuum model method (33). The dielectric constant was set to 4. Recent studies on several different classes of enzymes from our group have shown that the dielectric effects from the environment decline as the model size increases (34–36). When the chosen model contains ca. 150–200 atoms, the solvation effects almost vanish and the choice of the dielectric constant becomes less significant.

Analytic frequency calculations were carried out at the same level of theory as the geometry optimizations to obtain zero-point energies (ZPE) and to establish the nature of the various stationary points. Several atoms were kept fixed to their crystal structure positions during the geometry optimizations, to mimic the steric constraints by the protein matrix. This coordinate locking procedure gives rise to a few small imaginary frequencies, normally on the order of 10–40i cm⁻¹. These frequencies contribute little to the ZPE and can be disregarded.

To examine the sensitivity of the results to the choice of functional, we have performed single-point calculations (with the larger basis sets) for our proposed mechanism using the B3LYP* (with 15% exact exchange) (37), TPSS (38), and BB1K (39) methods based on the B3LYP-optimized geometries. For the rate-limiting step, the calculated barriers are 21.7, 18.0, and 19.5 kcal/mol using B3LYP*, TPSS, and BB1K, respectively. The values are stable and quite close to the B3LYP barrier (23.0 kcal/mol), further corroborating the adequacy of the B3LYP functional.

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