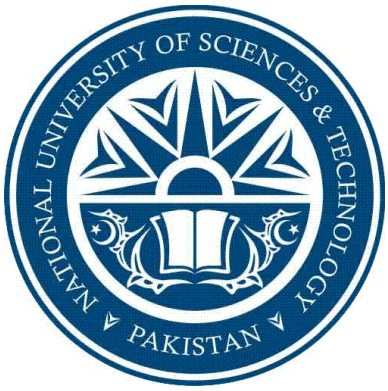
DFT STUDY ON THE REACTION MECHANISM OF ACETYLENE HYDRATASE FROM *PELOBACTER ACETYLENICUS*



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Master of Science
in
Computational Science and Engineering

Research Centre for Modeling and Simulation

National University of Sciences and Technology

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Islamabad, Pakistan

DFT STUDY ON THE REACTION MECHANISM OF ACETYLENE HYDRATASE FROM *PELOBACTER ACETYLENICUS*

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A thesis submitted to Research Centre for Modeling and Simulation National University of Sciences and Technology in partial fulfillment of the requirements for the degree of

Master of Science
in
Computational Science and Engineering

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STATEMENT OF ORIGINALITY

I hereby certify that the work embodied in this thesis is the result of original research and has not been submitted for a higher degree to any other University or Institution.

Date

Mahum Riaz

DEDICATION

This thesis is dedicated to my parents who have always been a source of inspiration for me

ACKNOWLEDGEMENTS

"Recite in the name of your Lord who created - Created man from a clinging substance. Recite and your Lord is the most Generous - Who taught by the pen - **Taught** ***man that which he knew not."*** (Sura Al-Alaq)

Firstly, I would acknowledge that this work would never have been completed without the blessings and belief of Allah and guidance of Holy Prophet Muhammad Sallalahu alaihi waalihi wassalam.

I express a heartfelt thanks to Principal RCMS who has guided and helped me through the toughest of the times. I express a deep sense of appreciation for my supervisor, Dr. Uzma Habib, for her valuable time and guidance at each and every step of the research work. She has taken me from the very basics to the point where I have been able to produce a significant research work.

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DFT STUDY ON THE REACTION MECHANISM OF ACETYLENE HYDRATASE FROM *PELOBACTER ACETYLENICUS*

ABSTRACT

Introduction: Acetylene Hydratase (AH) is a unique tungsten containing enzyme that catalyzes a non-redox hydration reaction; where acetylene is converted to acetaldehyde under anaerobic conditions. The X-ray crystal structure of AH from *Pelobactor acetylenicus* provides important insight into its active site as the proposed catalytic mechanism of AH depends on the nature of oxygen specie bound to the tungsten center. Hypothetically, two possible mechanisms, electrophilic or nucleophilic, for hydration of acetylene were suggested by Seiffert et. al. Computational studies were performed by Antony and Bayse, Vincent, and Himo et al., however, they ruled out both the mechanisms suggested by Seiffert et al and proposed a reaction mechanism where hydration reaction starts with the displacement of a water molecule with the acetylene. Later on, based on the computational results for AH active site model complexes the most likely single step nucleophilic mechanism for the hydration of acetylene was suggested by Uzma et al, where initially, small model complexes (3 Å) were computed but to get more reliable results surrounding amino acid residues (6 Å) were considered. Although the relative energies for the formation of vinyl alcohol products are comparable with the results from the small model complexes, the energy barriers were considerably higher for both mechanistic options. These energy barriers were decreased when solvent molecules present in selected area (8 Å) of AH were considered. We sought to focus on identifying the importance of solvent molecules in the catalytic process of AH and

validating the suggested most probable nucleophilic reaction mechanism for hydration of acetylene.

Method: Active site model complexes of Acetylene Hydratase (AH) (6 Å), considering the solvent molecules, based on the X-ray crystal structure were computed for hydration of acetylene at the COSMO-B3LYP/SDDp//B3LYP/Lanl2DZ(p) level of density functional theory (DFT). Activation energies have been calculated for both the electrophilic and nucleophilic reaction mechanisms in gas as well as in solvent phase.

Results: Nucleophilic reaction mechanism shows energy barrier of 15.1 kcal/mol (20.8 kcal/mol in gas phase) which is in concordance with the results obtained by Uzma et al. This energy barrier is lower than those for the electrophilic pathway i.e.29.4 kcal/mol (33.4 kcal/mol) or for the other mechanisms suggested in literature

Conclusion: The results of the study suggest that the water/solvent molecules play key role in the catalytic reaction of AH and it is also validated that nucleophilic mechanism is the most probable reaction mechanism for hydration of acetylene to acetaldehyde.

1. Introduction

1.1 Metalloenzymes

Life is a system of very complex, self-regulatory set of chemical reactions also called biochemical or metabolic reactions. To maintain the high rate of these biochemical reactions, enzyme catalysis is needed [1]. Enzymes are proteins, most of which also contain metal ions as part of their structures, therefore called metalloenzymes. Metals ions are necessary for the function of these enzymes, acting either as electron donors or acceptors (Lewis acids) or structural regulators. Examples of some metalloenzymes are mentioned in **Error! Reference source not found.**

Table 1.1 Metalloenzymes[1]

Metal Ion	Enzyme
Ca^{2+}	amylase, galactosyltransferase, thermolysin
Co^{2+}	dialdehydrase, glycerol dehydratase, ribonucleotidereductase
Cu^{2+}	cytochrome c oxidase, superoxide dismutase
Fe^{2+}	catalase, NADH dehydrogenase, peroxidase, xanthine oxidase
Mn^{2+}	arginase, histidine-ammonia lyase, pyruvate carboxylase
Mo^{2+}	nitrogenase, xanthine oxidase
Ni^{2+}	urease, Ni-Fe hydrogenase
Zn^{2+}	alcohol dehydrogenase, carbonic anhydrase

1.1.1 General Properties of Enzymes

Following are some general features and properties associated with enzymes [2]:

a. Enzyme Function

The function of enzyme is to catalyze the multitude of chemical reactions inside the biological systems. These chemical reactions are of various different types, therefore enzymes are categorized according to the type of reaction they catalyze i.e. [1]

1. **Oxidoreductases:** Carry out oxidation and reduction reactions.
2. **Transferases:** Transfer functional groups from one molecule to another.
3. **Hydrolases:** Catalyse hydrolysis i.e the breaking of single bonds through addition of water.
4. **Lyases:** Involved in formation or breaking of double bond through group transfer.
5. **Isomerases:** Change the position of functional group within the molecule, without any change in number and kind of atoms in the molecule.
6. **Ligases:** Catalyse the joining of two molecules deriving energy from the breakage of high energy phosphate bonds in ATP or other nucleotides.

b. Enzyme Structure

Enzymes are the protein macromolecules, with well-defined primary, secondary and tertiary structures. Primary structure of the enzyme is the polypeptide chain with a specific amino acid sequence which is typically 62-2500 amino acids long.[3,4] The polypeptide chain can form hydrogen bonds through its amide and carboxyl groups twisting around other chains or folding on itself forming the secondary structures such as the alpha helices or beta sheets. Further folding of the secondary structure of the enzyme results in a three dimensional structures i.e. the tertiary structure.

Most of the enzymes are much larger in size than the substrate they catalyze, however, only a small portion of the enzyme i.e. the active site is involved in catalysis. Enzymes also have other binding sites for molecules including cofactors. Cofactors are either inorganic ions or

complex metallo-organic molecules i.e. coenzymes, needed for the proper functioning of enzyme [5, 6, 7].

c. Enzyme Catalysis

Enzymes act as catalysts in biochemical reactions where substrates are converted into products. These biochemical reactions are very important in the cell as without enzymes rate of reaction decreases with the increase in activation energy. For a biochemical reaction, substrate must have to pass the transition state of potential energy i.e. the activation energy. This activation energy is usually higher for the reaction without any catalyst/enzyme. Enzymes provide an alternative pathway for the reaction to proceed, therefore, reducing the energy required for the transition state (Figure **1.1**).

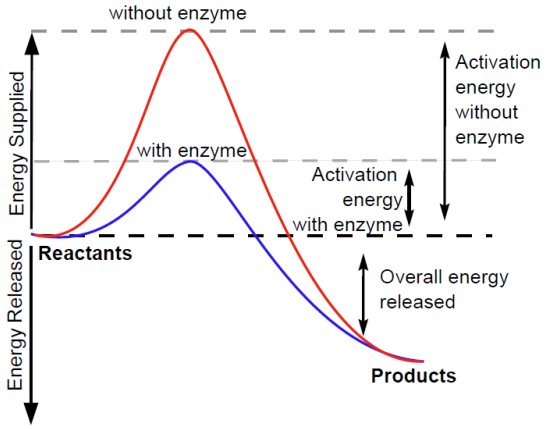

Figure 1.1 Activation energy with and without enzyme[8]

A simple enzyme catalyzed reaction can be represented as:

$$E+S \leftrightarrow ES \leftrightarrow EP \leftrightarrow E+P \quad [9] \qquad \textbf{(Eq. 1)}$$

Where

E is Enzyme

S is Substrate

P is Product

ES is Enzyme substrate complex

EP is Enzyme product complex

A chemical reaction consists of the formation and decay of many transient chemical species between reactants and products, called **Reaction Intermediates**. Reaction intermediates are those transient species which have a lifetime longer than a molecular vibration i.e. ~10^{-13} seconds. In an enzyme catalyzed reaction, ES and EP can be considered as the reaction intermediates, along with others as well. The inter-conversion of two sequential intermediates constitutes a step. The overall rate of reaction is determined by the step with the highest activation energy. This step is known as the **Rate Limiting Step**. [8]

d. Substrate specificity

Enzymes are highly specific to their reactants (substrates) and the type of reaction they catalyzed, providing a specific environment in which reaction can take place more rapidly. This specificity is due to the unique active site structure of each enzyme.[1] Any change in the active site structure can affect the enzyme activity e.g.

- An increase in temperature increases the reaction rate but beyond an optimum level it disturbs the structure of the enzyme, destroying their active site structure [1].
- Enzymes cannot function properly below or above their optimum pH. A change in pH alters the ionization state of the amino acids i.e. affecting the ionic bonds in the protein 3D structure. This results in either a change or complete destruction of the active site structure.
- Presence of inhibitors completely stops or slows down the catalytic activity. Depending on the type of inhibitor, they either attach themselves to the active site or to another site on the enzyme, blocking the formation of enzyme substrate complex.

1.1.2 Tungsten and Molybdenum metals

The two metals tungsten (W) and molybdenum (Mo) with atomic number 74 and 42, respectively, are biologically very important (Table 1.2). They are incorporated into the active sites of most metalloenzymes over the course of evolution [10].

Role of Mo is known for many decades[17] but only after 1983 first naturally occurring W enzyme was purified and after 1990 progress has been made in this regard[18]. This has provided chances of comparison between the Mo and W enzymes and to study their structural and functional similarities.

W and Mo metals are rare in nature [13, 14, 15, 16] but their enzymes are chemically versatile and have high bioavailability [10]. They exist in -II to +VI oxidation states but only +IV, +V and +VI oxidation states are biologically important. Mo is mostly present in jordesite and molybdite ($Mo^{IV}S_2$) and W in oxo-rich minerals as schelite ($CaWO_4$) or wolframite ($[Fe/Mn]WO_4$). [12]

Table 1.2 Physical and Chemical properties of Mo and W metals[10]

Property	Mo	W
Atomic Number	42	74
Atomic Weight	95.94	183.85
Electronic configuration of the outer shell	$4d^5 5s^1$	$4f^{14} 5d^4 6s^2$
Atomic radii	1.40	1.40
Electronegativity	1.8	1.7
Concentration in seawater	≈100nM	≈1pM
Concentration in fresh water	≈5-50nM	≈500pM
M=O bond length (Å)	1.76	1.76

Mo and W are commonly present in biological systems because they are soluble in water [10, 11]. For almost all W enzymes there is an analogous Mo enzyme in the same organism or closely related specie. [16] They are frequently involved in redox reactions of biological systems [10]. Molybdoenzymes occur in all aerobic systems while tungstoenzymes only occur in obligate, thermophilic anaerobes. W enzymes have low reduction potentials and bonding of sulphur with W is much more stable than bonding of sulphur with their Mo counterparts [10].

Most of the Mo and W enzymes are mononuclear and contain pterin cofactor in their active site which coordinates with the metal through their ene-dithiolate moeity [11] (Figure **1.2**). Mo and W enzymes can be grouped into 3 families according to their structure and functions[10].

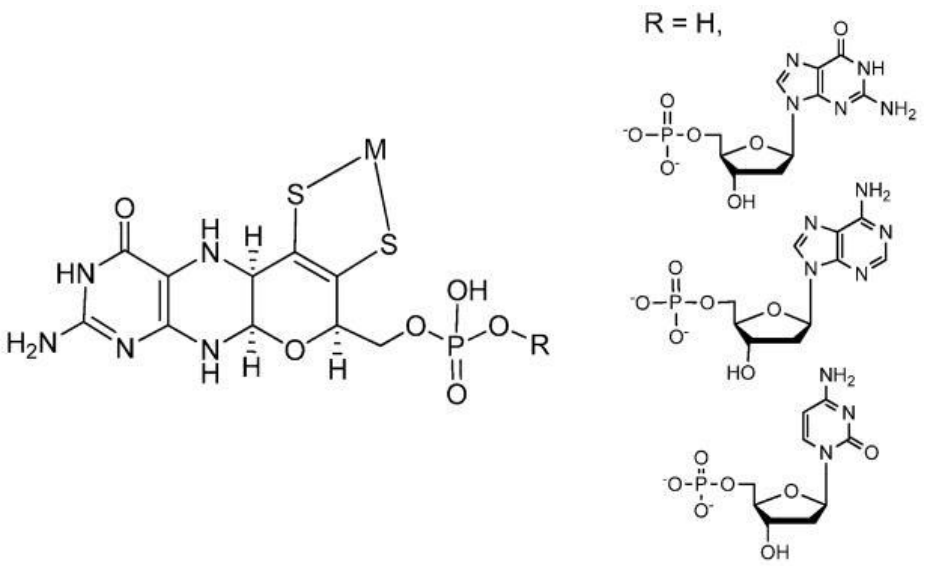

Figure 1.2 The pterin cofactor[11]

Table 1.3 Families of Mo and W enzymes; L refers to the pterin cofactor [10]

Mo Enzymes	**W Enzymes**
Xanthine oxidase family: $LMo^{VI}OS(OH)$ Catalyze hydroxylation of carbon centers	**Aldehyde ferredoxinoxidoreductase family** Catalyze oxidation of aldehydes to carboxylic acids
Sulphite oxidase family: $LMo^{VI}O_2$(S-cys) Catalytic transfer of oxygen atom to or from the substrate.	**Formate dehydrogenase family** Catalyze the oxidation of formate ($HCOO^-$) to CO_2.
DMSO reductase family: $L_2Mo^{VI}O(X)$ X→*ser , cys, selenocystein, OH/H_2O* Catalysediverse reactions due to structural variability including dissimilatory reduction of certain toxic Oxo anions.	**Acetylene Hydratase** Hydration of acetylene to acetaldehyde

1.1.3 Tungstoenzymes

The majority of tungstoenzymes belong to the aldehyde ferredoxinoxidoreductase (AOR) family; AOR from the hyperthermophile, *Pyrococcusfuriosus* is the best studied example from this family, others being from different microbial species having somewhat different properties. The second family F(M)DH includes the two types formate dehydrogenase (FDH) and the N-formylmethanofuran dehydrogenase (FMDH). The third family, acetylene hydratase (AH) has just one member named acetylene hydratase purified from acetylene utilizing anaerobe *Pelobacter acetylenicus.* [16, 19]

1.1.4 Acetylene Hydratase from *Pelobactor Acetylenicus:*

Acetylene hydratase (AH) from *Pelobactor Acetylenicus* is a unique tungsten containing enzyme that catalyses the hydration of acetylene to acetaldehyde: [19,20]

$$\mathbf{C_2H_2 + H_2O \rightarrow CH_3CHO} \qquad \textbf{(Eq. 2)}$$

Acetylene exists as a highly soluble and highly flammable gas and enters the environment through exhausts from combustion engines. It is involved in microbial processes like nitrogen fixation and methanogenenisis. Microrganisms have acquired ways to make use of such compounds as growth substances. Aerobic utilization of such compounds has been known since 20th century. But oxygen is not available in all enviornments such as reservoirs and deep sediments. Unlike other AH enzymes, AH from *Pelobactor Acetylenicus* carries out an anaerobic degradation of acetylene and utilizes it as a sole carbon and energy source. [16]

AH from *Pelobactor Acetylenicus,* is the only member of the Acetylene Hydratase family and the only acetylene hydratase yet crystallized and characterized. It is also sometimes referred to be a member of DMSO reductase family of Mo enzymes[21, 22, 23] due to its protein sequence homology and metal coordination.[20] The molecular properties of AH also closely resemble that of Pf *(Pyrococcus furiosus)* AOR (aldehyde ferredoxin oxidoreductase) family of W enzymes, but it catalyzes a totally different type of chemical reaction i.e. hydration in contrast to the oxidoreductase type reactions catalyzed by all other W and Mo enzymes.[16]

1.2 Computational Methods

In order to analyze molecules and molecular systems or to predict their molecular, chemical and biochemical properties, there are a number of computational techniques used. These techniques, collectively referred to as computational chemistry or molecular modeling techniques, are based on theoretical chemistry methods and experimental data. They help to compare experimental and theoretical data for the model as well as understand and interpret the experimental observations.[24, 25] These techniques are categorized into two main group of methods; Classical Mechanical methods and Quantum Mechanical methods (Figure 1.3). Both the classical and quantum mechanical methods are widely used to predict energies associated with a particular conformation of a molecule.[25, 26, 27, 28]

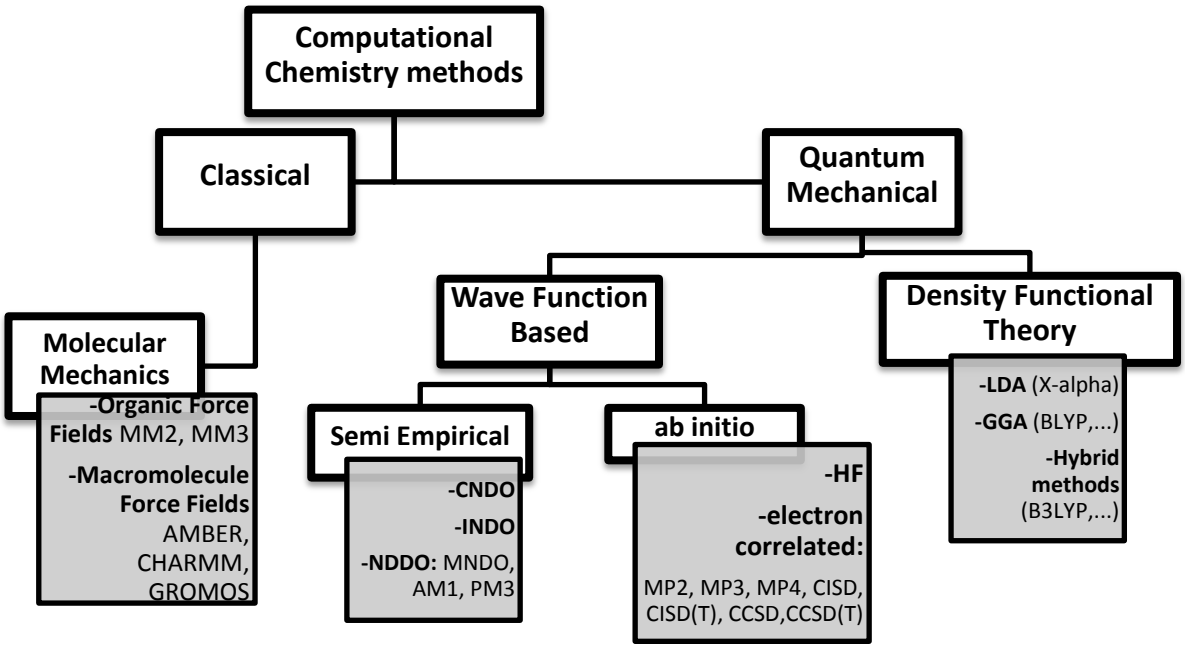

Figure 1.3: Classification of Computational methods [25, 26, 31]

a. Classical Mechanical Methods

Classical Mechanical methods are based on Newtonian Mechanics where atoms and molecules are treated as rubber balls of different sizes joined together by springs of varying length (bonds). These methods consist of the molecular mechanical methods followed by molecular dynamic simulations.[26] In molecular mechanical methods, the potential energy (energy due to the position of a particle/object) calculation is based on Hooke's Law i.e. P.E= 1/2 k x^2 where k is the spring constant and **x** is the amount of compression relative to equilibrium position [26, 29].

A force field or a potential function (Eq. 3) calculates the potential energy (E) of a molecular system in a specified conformation as a sum of other energy terms. Each term is a function of the nuclear coordinate and a number of parameters. The values for absorption of energy in the IR spectroscopy fall in almost similar ranges for a certain bond stretch or angle deformation. Therefore in force fields the energy is expressed as a function of these geometric parameters i.e. bond length, bond bending, van der Waal interactions etc.[24, 27, 29]

$$\mathbf{E_{FF} = E_{str} + E_{bend} + E_{tors} + E_{vdW} + E_{elst} + \ldots} \qquad \textbf{(Eq. 3)}$$

Where

E_{str} is Bond Stretching Energy (Bonded),

E_{bend} is Bond Bending Energy (Bonded),

E_{tors} is Torsional Energy (Bonded),

E_{vdW} is Van der waal interactions (Non-bonded interactions),

E_{elst} is Electrostatic interactions (Non-bonded interactions).

b. Quantum Mechanical Methods

The Quantum mechanical methods have been based on the fundamental concept of wave-particle duality i.e. all particles show both wave and particle like properties. Molecular Orbital Theory (MO theory) and Valence Bond Theory (VB theory) have been the earlier basis for all quantum mechanical methods. [25, 26,27]

The MO theory begins with the Schrodinger's Equation, which refers to a single particle of mass m, moving through space and time under the influence of an external field. The non-relativistic (Time-independent) form of Schrödinger equation is also known as the wave equation (Eq. 4) and gives the total energy of the system. [24, 26, 27, 31]

$$\mathbf{E\Psi(r) = (-h^2/2m)\,\nabla^2\,\Psi\,(r) + V\,(r)\,\Psi\,(r)} \qquad \textbf{(Eq. 4)}$$

Where

E is Energy of the system,

V is Potential Energy,

∇^2 is Laplacian,

m is Particle's mass,

Ψ is Wave function,

h is Reduced Planck's constant.

Exact solutions to Schrodinger's equation are needed to achieve the required goal of QM calculations. However, the exact solution can only be achieved for atoms with one electron, for more than one electron it gives an approximation only. To solve this problem various amendments have been made to the equation giving rise to new theories including Born-Oppenheimer Approximation of wave equation, Hartree Fock Theory etc. [24, 26, 31]

Many QM based methods have been developed over time and are categorized into ab-initio and semi empirical methods. Ab-initio methods are based on the basic principles while the empirical or semi-empirical methods make use of the experimental results. [26, 28, 29, 31]

Hogenberg and Kohn in 1964 showed that the ground state energy and other properties of the system were uniquely defined by the electron density. This gave rise to the Density Functional Theory (DFT) based methods for electron structure level calculations. The main advantage of DFT is that density is a physical property that exists in real molecules and not a mathematical equation like the wave equation.[26, 30]

1.2.1 Density Functional Theory Methods

DFT states that energy of the system is the functional (E) of the electron density i.e., as the wave equation becomes more complicated with the increase in number of electrons; electron density is used instead which depends only on the x, y, z coordinates of the individual electron i.e.

Electron density= $\rho(x, y, z)$
Energy =E (Electron density)
Energy =E ($\rho(x, y, z)$)

Therefore, the goal of DFT becomes finding the value of E(ρ). [24, 26, 32]

$$\mathbf{E[\rho] = T[\rho] + E_{ne}[\rho] + J[\rho] + E_{xc}[\rho]} \qquad \textbf{(Eq. 5)}$$

Where

T is kinetic energy of the electrons,

E_{ne} is nucleus electron attraction,

J is electron-electron repulsion,

E_{xc} is electron-electron exchange correlation energy.

$T[\rho]$, $E_{ne}[\rho]$, $J[\rho]$ can be reasonably found by ab-initio and semi empirical methods. But $E_{xc}[\rho]$ needs to be approximated. This is the reason why many DFT methods have been used such as the Thomas-Fermi-Dirac expression, Kohn-Sham implementation followed by the Local Density Approximations and the Generalized Gradient Approximation. [24, 33] Some methods are also formed from a combination of different approximations i.e. the hybrid methods. DFT methods are complex and of many different types and can be broadly divided into three classes:

a. Local Density Approximation (LDA) Based Methods

These methods assume a uniform density of molecule with many electrons in a gaseous state. Density is not uniform for all molecules, so this method doesn't work well in all cases.

b. Gradient Correction Factor Based Methods

These methods account for non-uniformity of electron density using gradient correction.

c. Hybrid Methods

These methods combine Hartree-Fock approximation with a DFT approximation.

DFT has found its advantages as being more computationally cost effective. Although QM methods are more suitable for small molecules, some DFT based methods are also suitable for large molecules including bio-molecules. Combining DFT with MD simulations has also proved to be a very useful technique in molecular biology. [26, 31, 34]

1.2.2 Basis Sets

To represent the molecular orbitals, QM and DFT methods use Basis Sets. A basis set is a set of mathematical functions called basis functions (representing atomic orbitals or atom electrons). These basis functions are combined in linear combinations (LCAO- Linear Combination Of Atomic Orbits) to create molecular orbitals. [35]

Basis functions (atomic orbitals), which are mathematical functions, were first described by John C.Slater in 1930[36], using Slater type orbitals (STOs). STO's are very accurate but computationally too expensive, therefore Frank Boys suggested that STO's can be approximated as a linear combination of Gaussian-Type Orbitals (GTOs) [37]. GTO's are easier to calculate leading to low computational cost. [32]

Hundreds of basis functions consisting of GTOs have been developed over time. The smallest of these are called minimal basis sets, and they are typically composed of the minimum number of basis functions required to represent all of the electrons on each atom e.g., STO-3G i.e. Slater-Type-Orbitals simulated by 3 Gaussians added together. The *minimal* basis sets have only as many orbitals as are needed to accommodate the electrons of the neutral atoms and retain spherical symmetry. But these do not accommodate the changing molecular environment e.g. protonation which can increase electron nuclear attraction and electron-electron repulsion, making comparison between charged and uncharged species unreliable. This led to the need for split valence basis sets [38, 39] where the atomic orbitals are split into two e.g. the simplest one is 3-21G i.e. the core orbitals are represented by Gaussian functions while the inner and outer valence shells are represented by two and one Gaussians respectively. Moreover, there are also polarization functions (*) and diffuse functions (+) which can be added to the basis sets e.g. 3-21+G*. There are further complicated notations for the basis sets as they become more complex and efficient. [40,41] Selection of a basis

function is a difficult but key step in QM and DFT calculations, which depends on the hardware availability, available time and the molecular size.[42]

2 Literature Review

2.1 Acetylene Hydratase from *Pelobacter acetylenicus*

Pelobacter acetylenicus is an anaerobic bacterium, isolated in 1985 by Schink. [43] It was found to metabolize acetylene with the first highly exergonic ($\Delta G^{o'}$ = -111.9 kJ/mol) step i.e. the hydration of acetylene to acetaldehyde by acetylene hydratase (AH).[43] It was purified and characterized to be an iron-sulphur containing tungstoenzyme in 1995 by Schink and Rosner[43] (Figure 2.1).

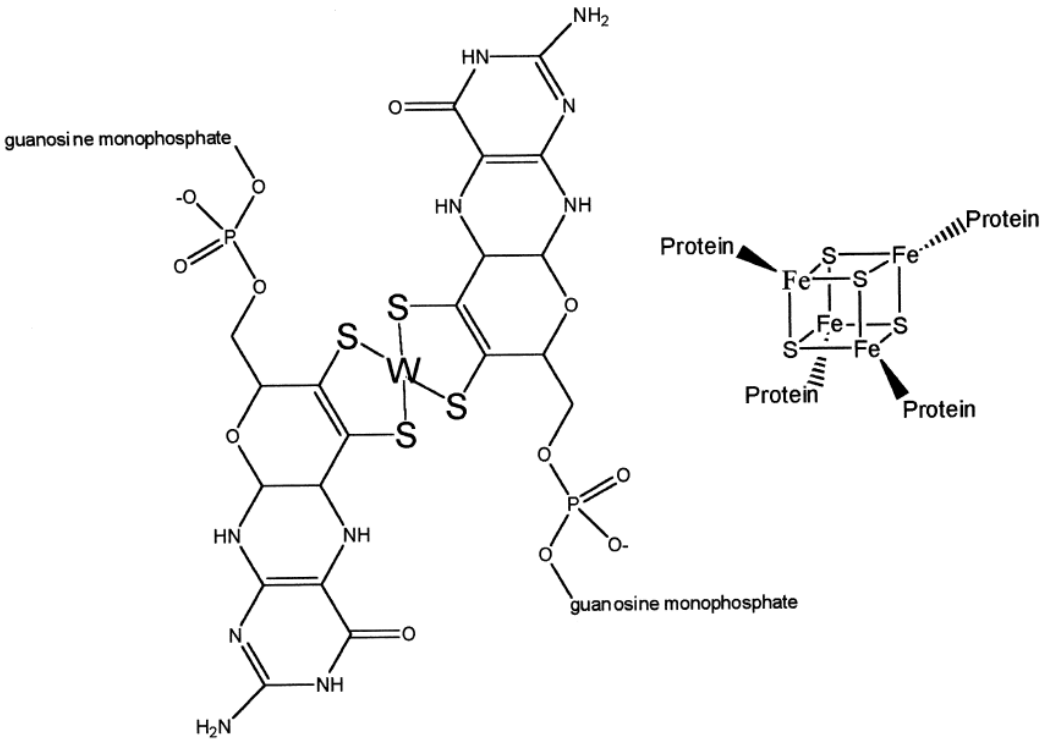

Figure 2.1 Acetylene Hydratase characterized to be a Fe/S containing Tungstoenzyme [43]

In further studies by Schink, active site model of the AH was proposed in 1999. It was found to contain one [4Fe:4S] cluster and a tungsten center co-ordinated by two molybdopterin guanine dinucleotide (MGD) ligands (Figure 2.2).

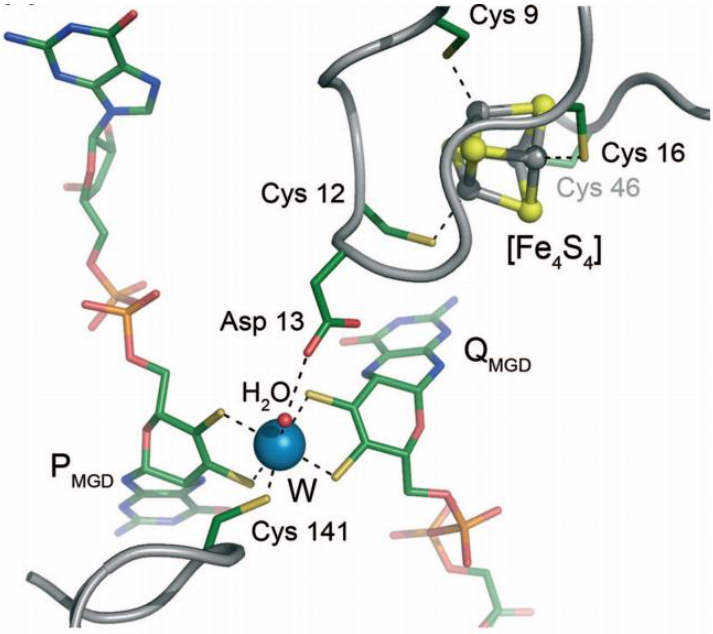

Figure 2.2 Active Site structure of Acetylene Hydratase[44]

The enzyme is a monomer with molecular mass of 73 kDa (SDS/PAGE) or 83 kDa (matrix-assisted laser-desorption ionization MS).[44] It was purified and crystallized in 2005 under strict exclusion of dioxygen, in its active reduced state. [45] Its crystal structure was determined at resolution of 1.26Å by Seiffert et al in 2007. [23]It is a monomer of 730 a.a residues and the peptide chain folds into a tertiary structure with four domains:

1. **Domain 1:** residues 4-60: It holds the [4Fe:4S] cluster of protein bound to Cys-9, Cys-12, Cys-16 and Cys-46

2. **Domain 2:** residues 65-136, residues 393-542: It has a αβα-fold which provides multiple hydrogen bonding interactions to one of the MGD cofactors**.**

3. **Domain 3:** residues 137-327: Like domain 2, it has a αβα-fold which provides multiple hydrogen bonding interactions to one of the MGD cofactors**.**

4. **Domain 4:** residues 590-730: It has a dominance of seven stranded β-barrel structure and participates in the coordination of both MGD ligands. [23]

In the active site, a mononuclear W center is present which is connected to two sulfur atoms of the two MGD cofactors, a sulfur atom of cysteinate and oxygen specie (Figure **2.2**).A nearby [4Fe:4S] cluster also exists.[20] Access to the active site is provided by a funnel like entrance at the junction of domains I, II and III (Figure 2.3).[23]

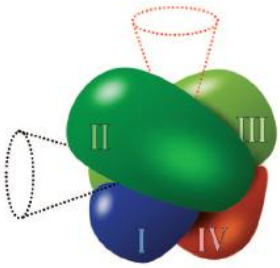

Figure 2.3 Active Site Access[23]

At the end of the substrate access funnel, a ring of hydrophobic residues containing tungsten atom and the coordinated oxygen specie are originating from domains I, II and III. These residues combine to create a small binding cavity with dimensions that make it a perfect mold for binding acetylene (Figure 2.4).[23] The binding pocket positions an acetylene molecule directly above the oxygen specie and Asp-13.

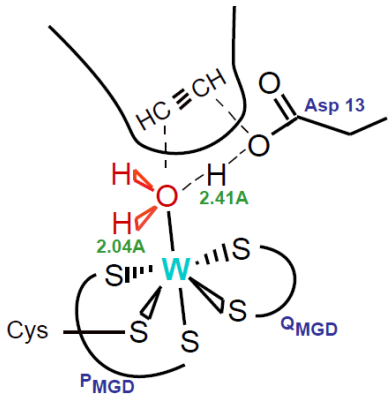

Figure 2.4 Acetylene binding to the active site [23]

It has been demonstrated that W only in IV oxidation state participates in the reaction. In VI oxidation state, the enzyme needs to be activated using a strong reductant to convert it to IV

oxidation state.[44] The [4Fe:4S] cluster is thought to facilitate this activation.[20] This reaction doesn't involve electron transfer or change in oxidation state of W or [4Fe:4S] cluster. [45] Moreover, AH is extremely oxygen sensitive, its [4Fe:4S] cluster is converted to [3Fe:4S] if exposed to air and its activity is irreversibly lost. [20, 44]

2.2 Suggested Mechanisms for Acetylene Hydration

The X-ray crystal structure provides important insights into the reaction mechanism of acetylene hydration. A number of reactions have been suggested in literature based on these observations.

In 2007, Seiffert et al [23] hypothetically proposed nucleophilic (Figure 2.5) and electrophilic (Figure 2.6) reaction mechanisms for the acetylene hydration, based on the observation that the oxygen moiety attached to the W atom has a W – O bond distance of 2.04Å which is in between the values expected for a hydroxo ligand (1.9-2.1 Å) or a water molecule (2.0-2.3 Å). A hydroxo ligand would constitute a strong nucleophile while a water molecule would act as an electrophile leading to two different reaction pathways.[20]

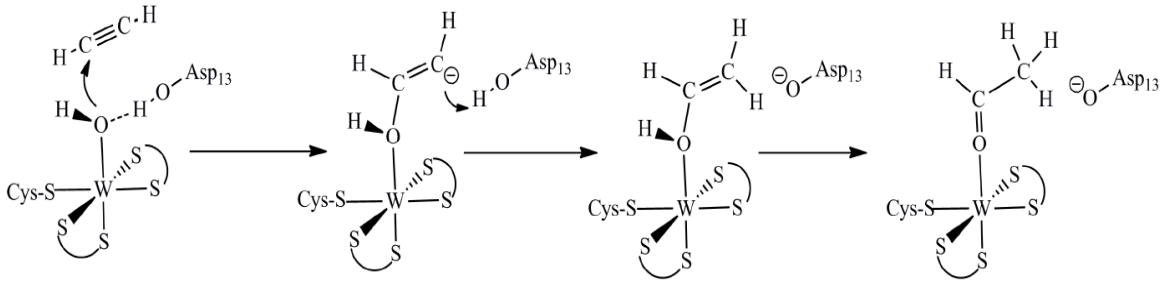

Figure 2.5Nucleophilic Pathway by Seiffert [46]

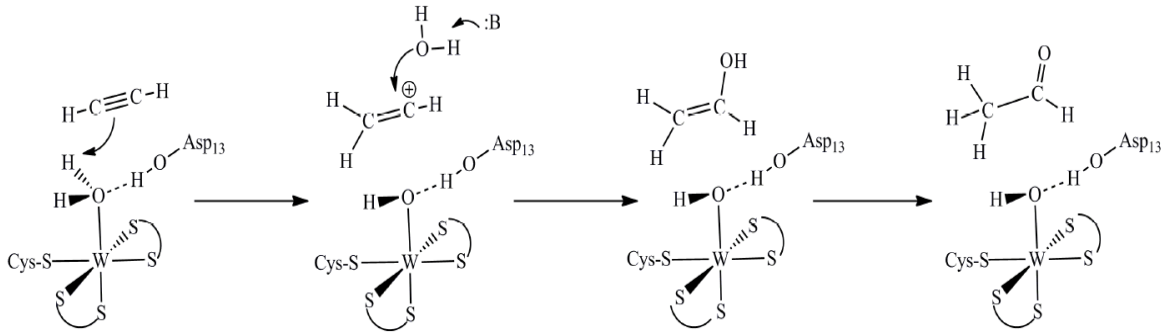

Figure 2.6 Electrophilic Pathway by Seiffert [46]

In 2009, Antony and Bayse[46] suggested a mechanism which starts with the nucleophilic attack of water molecule at the acetylene, η^2 bound to W, to form vinyl alcohol assisted by protonated Asp_{13} (Figure 2.7).

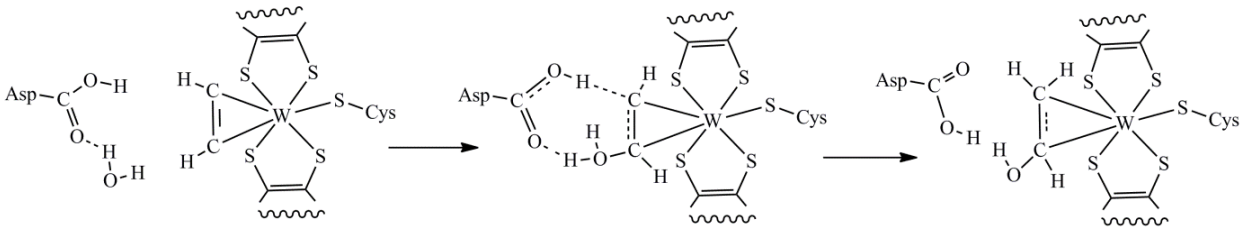

Figure 2.7 Suggested pathway by Antony and Bayse [46]

Vincent et al in 2010[47] performed DFT study on electrophilic pathway suggested by Seiffert and found quite high energy barrier of 43.9 kcal/mol. He also calculated energy barriers for mechanism suggested by Antony and Bayse[46] and found it to be too high as well i.e. 41.0 kcal/mol. Based on their computational results, they suggested a new mechanism (Figure 2.8) in which the reaction starts with the displacement of water molecule by acetylene bound to W center in an η^2 fashion. Vinylidene ($W=C=CH_2$) and carbine ($W=C(OH)CH_3$) complexes are formed during the reaction. Rate determining step in formation of vinylidene complex has energy barriers 28 kcal/mol and cabine complex has 34 kcal/mol.

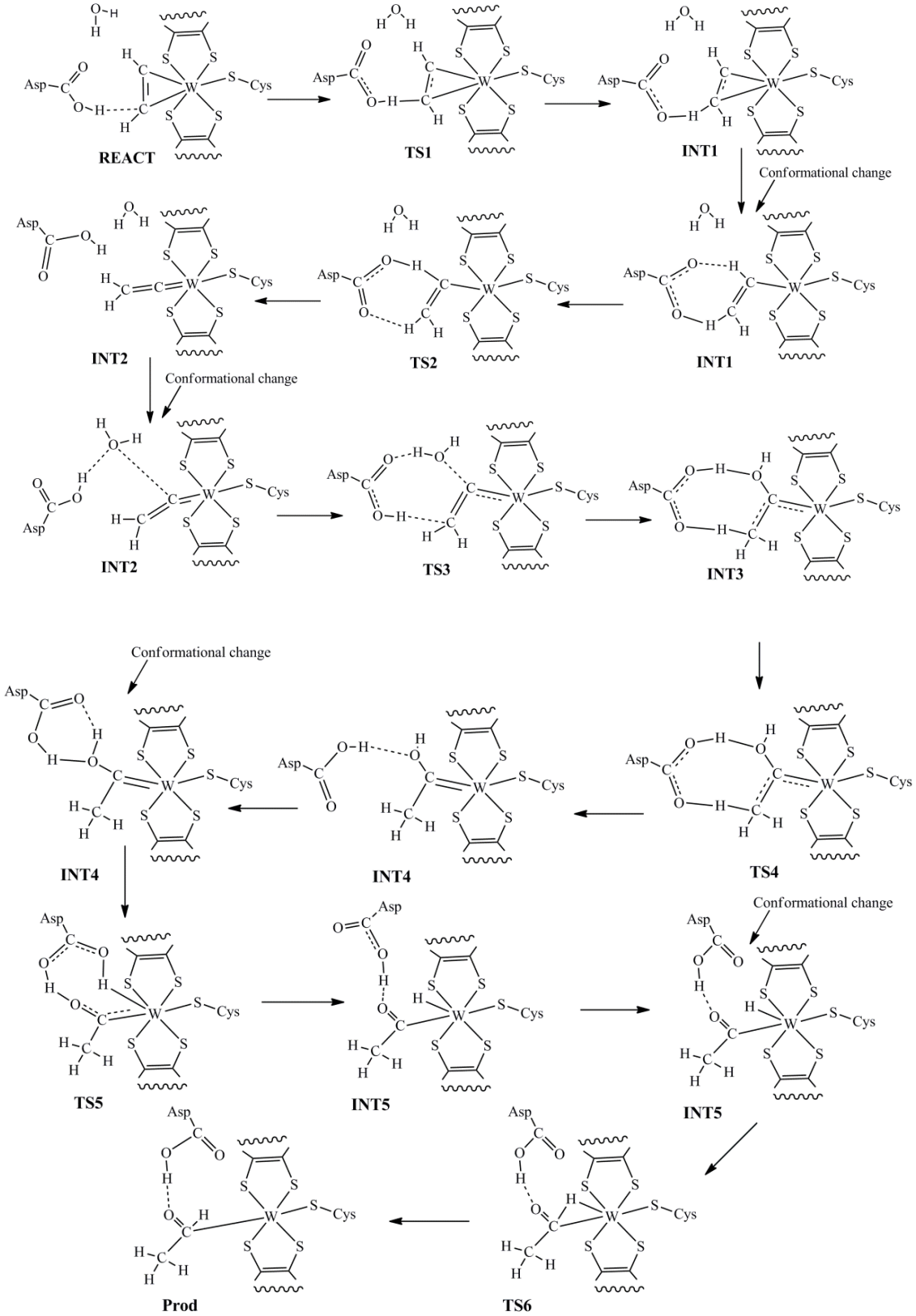

Figure 2.8 Suggested pathway by Vincent et. al [47]

Based on larger active site models for acetylene hydratase, In 2010, Himo et.al[48] suggested a mechanism (Figure 2.9) which starts with displacement of W bound water molecule with η^2 acetylene in an exothermic step. DFT studies were carried out and the rate limiting step was found to have energy barrier of 23 kcal/mol.

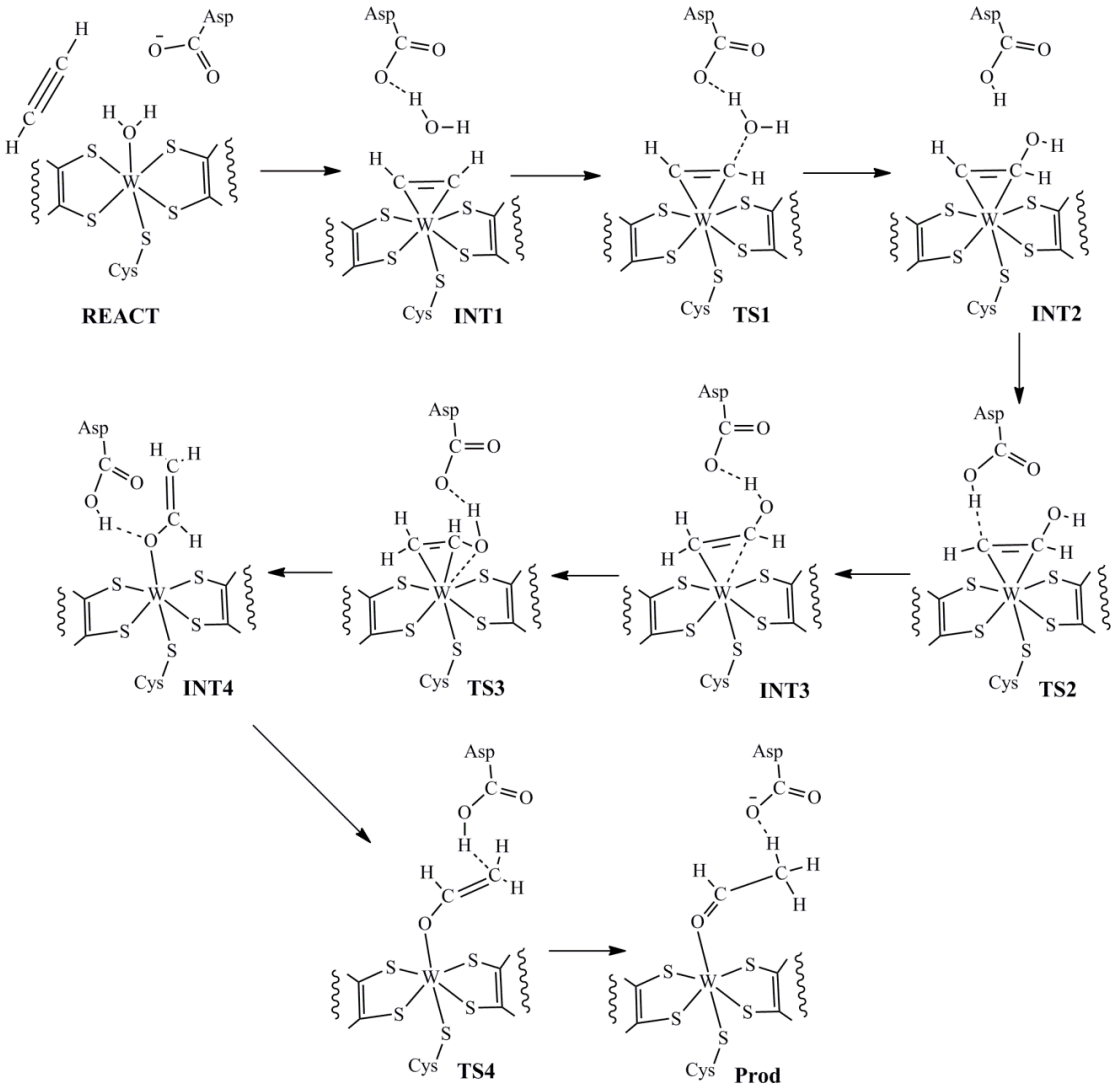

Figure 2.9 Suggested Pathway by Himo et al. [48]

Uzma et al. in 2012[20] performed DFT calculations on active site model complexes considering both the nucleophilic and electrophilic reaction mechanisms suggested by Seiffert et. al.and found that the nucleophilic pathway is more preferable with an energy barrier of 17.0 kcal/mol and 35.2 kcal/mol, respectively for the transition state.

2.3 Problem Statement

Considering the two proposed reaction mechanisms, nucleophilic and electrophilic, suggested by Seiffert et.al[23] for hydration of acetylene by Acetylene Hydratase, Uzma et al. in 2012[20] initially performed DFT calculations on small active site model complexes (3Å) and found that the nucleophilic pathway is more preferable with an energy barrier of 17.0 kcal/mol and 35.2 kcal/mol, respectively for the transition state. To identify the most probable reaction mechanism, large model complexes were then analyzed. However, the computational results from the large model complexes (6Å) also favour the nucleophilic pathway, energy barriers (35.2 kcal/mol for nucleophilic and 54.6 kcal/mol for electrophilic reaction pathways) are considerably higher for both mechanistic options. Further calculations were performed on larger active site model complexes, including four of the water molecules (8Å) found in close proximity to the W center. The transition state energy for nucleophilic pathway was found to be remarkably low in the presence of water molecules, i.e 14.4 kcal/mol.

It was also revealed from the X-ray crystal structure data that there are at least 16 well defined water molecules in a vestibule directly adjacent to the active site.[60] Therefore, In this study, we focused on identifying the importance of solvent molecules in the catalytic process of AH and validating the most probable nucleophilic reaction mechanism, suggested by Uzma et al.,[20] for hydration of acetylene.

3 Methodology

This study employs Density Functional Theory (DFT) to elucidate the reaction mechanism of hydration of acetylene using Acetylene Hydratase. DFT calculations were carried out using Gaussian 09[49] for both suggested pathways i.e. the electrophilic and nucleophilic pathways for the hydration of acetylene. Pathways are schematically represented in Figure 3.1 and 3.2.

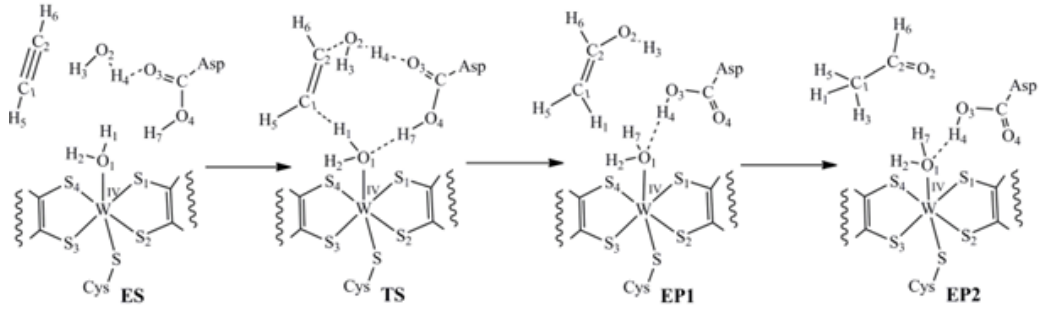

Figure 3.1 Schematic Representation of the electrophilic mechanism

where, ES is Educt Substrate Complex, TS is Transition State, EP1 is Alcoholic Product, EP2 is Tautomerized (to aldehyde) Product.

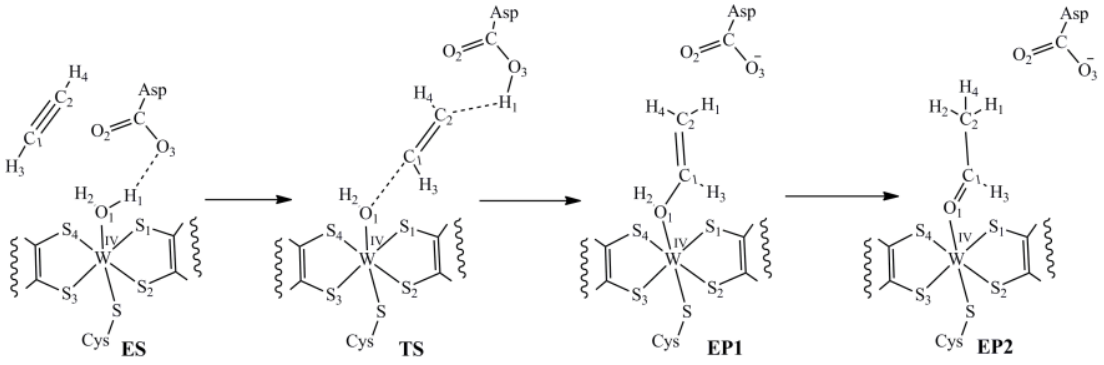

Figure 3.2 Schematic Representation of the Nucleophilic Mechanism

where ES is Educt Substrate Complex, TS is Transition State, EP1 is Alcoholic Product, EP2 is Tautomerized (to aldehyde) Product

3.1 Active Site Model Complexes

Active site model complex (Figure 3.3) and the data of educt active site, educt substrate, educt product model complexes obtained from the computational study by Uzma et al., [20] has

been utilized for this study. The Transition state complexes have been regenerated for the electrophilic and nucleophilic pathways, and their energies were calculated of acetylene hydration.

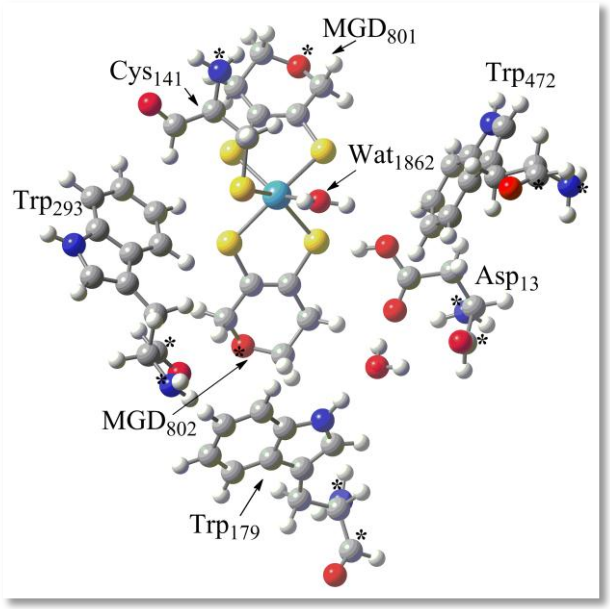

Figure 3.3 Optimized active site model complex.[20]
Atoms fixed for the optimization are labeled (*)

The active site model complex includes the W metal center coordinated with two molybdopterin (MGD), a metal bound water molecule (Wat_{1862}), a nearby Asp_{13} and a cysteinate (Cys_{141}) ligand. Four water molecules, (Wat_{1209}, Wat_{1212}, Wat_{1424}, and Wat_{1432}) which were in the proximity of W metal center, were considered from 16 water molecules present in the X-ray crystal structure. These water molecules were connected together through the hydrogen bonding. (Figure 3.4)

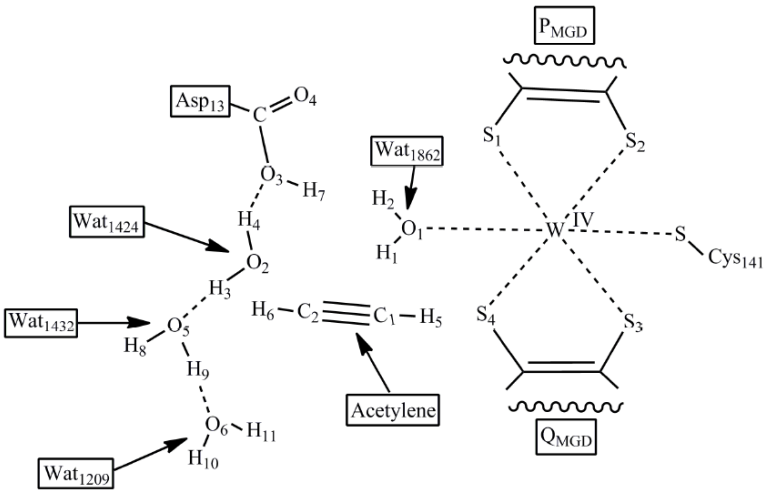

Figure 3.4 Chemical Structure of the active site model complexes derived from X-ray crystal structure of AH.[20]

3.2 Computational Methods

Transition state is the highest potential energy state along the reaction pathway. It is very difficult to observe this state physically due to the presence of other reaction intermediates along with the transition state in a very short time interval. The starting geometries for transition state (TS) search were generated by shortening and lengthening of forming and breaking bonds, respectively. All the active site model complexes were first pre-optimized, validated by the frequency calculations and then transition state was searched. Single point energies were then computed in the gas and solvent phase.

3.2.1 Geometry Pre-optimization

3.2.2 Frequency Calculation

3.2.3 Transition State Search

3.2.4 Single point Energy calculations

3.2.5 Self-Consistent Reaction Field calculations

3.2.1 Geometry Pre-Optimization

Pre-optimization of transition state geometries were performed, freezing the crucial atoms directly involved in the reaction, in order to find the most stable molecular arrangement, i.e. the lowest energy geometry, for transition state search. Series of iterations are performed on the molecule to find the most stable molecular arrangement, until the energy of the molecule reaches a minimum.

In this study, the active site model complex geometries for transition state were optimized using hybrid density functional method, B3LYP[50] and LANL2DZ basis set[51] enlarged with polarization function on sulfur atoms ($\varsigma = 0.421$)[52]. Self-Consistent Field method (SCF)[53] was used with IntRep option to account for integral symmetry and NoVaracc for full integral accuracy. QC[53] option was also used with the SCF procedure whenever there was a convergence problem. The oxidation state of W at the active site was IV and overall charge was kept -1 for the electrophilic model complexes and -2 for the nucleophilic model complexes.

3.2.2 Frequency Calculations

Frequency calculations were performed on the pre-optimized geometries, checkpoint file of pre-optimized geometries were used to get the internal force constants which were then supplied as starting values in the TS search.

3.2.3 Transition State Search

Transition state was searched using hybrid density functional method, B3LYP[50] and LANL2DZ basis set[51] enlarged with polarization function on sulfur atoms ($\varsigma = 0.421$)[52]. Self-Consistent Field method (SCF)[53] was used with IntRep option to account for integral symmetry and NoVaracc for full integral accuracy. TS (Berny optimization) were used to optimize geometries to their transition state rather than local minimum. EigenTest was used

to request testing the curvature in TS search. CalcAll was used to specify the force constants to be computed at every point.

3.2.4 Single Point Energy Calculations

Single Point energies were calculated for the optimized geometries in gas phase, using Stuttgart-Dresden effective core potential basis set (SDD)[54,55] augmented by polarization function for all atoms except W and H (ς = 0.600, 1.154, 0.864, 0.421 for C, O, N and S, respectively)[52]

3.2.5 Self-Consistent Reaction Field Calculations (SCRF)

SCRF calculations were performed on all the optimized active site geometries. These calculations were performed in order to model the effect of protein surrounding the active site by a conductor like polarizable continuum method (CPCM) with a dielectric constant of 4 and solvent radius of 1.4 Å. The molecular cavity was specified using a minimum radius (RMin) of 0.5 Å and an overlap index (OFac) of 0.8.

4 Results and Discussion

4.1 Transition State Search for the Electrophilic Pathway

In electrophilic reaction pathway, water bound to the W, at the active site of AH, is considered to be activated by the protonated Asp_{13} present nearby. This activated water molecule acts as an electrophile, and attacks the triple bond of acetylene; as a result, a proton (H_1) is transferred from the O_1 attached to the W to C_1 of acetylene, one proton (H_7) is transferred from Asp_{13} to O_1. From another water molecule present near Asp_{13}, one proton (H_4) is transferred to Asp_{13} and its electron donating part (OH) is transferred to C_2 of acetylene resulting in the formation of vinyl alcohol. This vinyl alcohol is then tautomerized to acetaldehyde (Figure 4.1).

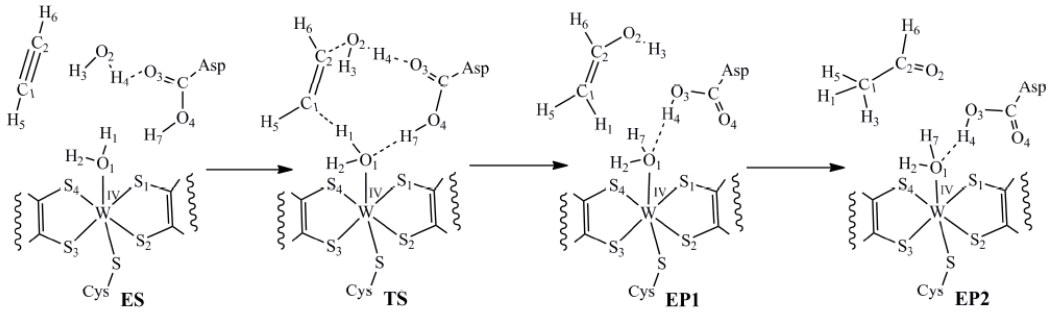

Figure 4.1 Schematic Representation of the electrophilic mechanism[20]

Results of transition state search obtained from DFT studies (Figure 4.2) were compared with the results produced by Uzma et. al[20] which shows a decrease in dihedral angle of S_1-S_2-S_3-S_4 from -12.6 to -26.4˚ and in the bond distance of W-O_1 from 4.314 to 2.121 Å (in Uzma et al results W-O_1 bond (4.314 Å) was broken). O_1-H_1 bond distance is increased while C_1-H_1 and O_2-C_2 bond distances are reduced (Table 4.1), showing the bond breaking and bond formation between these atoms in the transition state. The energy barrier for the formation of this

transition state is 29.4 kcal/mol in the polarizable continuum and 33.4 kcal /mol in the gas phase (Table 4.3).

Table 4.1 Geometrical Parameters of the optimized model complexes of electrophilic reaction mechanism for acetylene hydration

	X	LE-E	LE-ES	LE-TS	E-TS	LE-EP1
W-S_1 (Å)	2.432	2.429	2.374	2.359	2.419	2.380
W-S_2 (Å)	2.489	2.366	2.362	2.358	2.379	2.367
W-S_3 (Å)	2.511	2.356	2.357	2.333	2.363	2.355
W-S_4 (Å)	2.336	2.400	2.362	2.348	2.362	2.359
S_1-S_2-S_3-S_4 (˚)	-31.4	-17.4	-2.3	-12.6	-26.4	6.5
W-O_1 (Å)	2.041	2.182	4.229	4.134	2.121	4.688
O_1-H_1 (Å)	-	0.976	0.979	1.097	1.165	3.303
H_1-C_1 (Å)	-	-	2.385	1.540	1.475	1.086
C_1-C_2 (Å)	-	-	1.225	1.278	1.352	1.346
C_2-O_2 (Å)	-	-	3.104	1.930	1.479	1.406
O_2-H_4 (Å)	-	0.987	0.999	1.045	0.999	5.021
H_4-O_3 (Å)	-	1.788	1.683	1.477	1.693	1.014
O_4-H_7 (Å)	-	1.068	1.097	1.314	1.026	1.859
H_7-O_1 (Å)	-	2.729	1.341	1.113	1.007	0.982
H_5-C_1-C_2 (˚)	-	-	178.9	125.6	111.1	120.3
C_1-C_2-H_6 (˚)	-	-	178.7	155.0	127.9	123.6

where, **X** protein X-ray crystal structure data, **LE-E** is Educt complex, **LE-ES** is Educt-substrate complex, **LE-TS** is Transition state complex without water molecules, **E-TS** is Transition state complex with water molecules, **LE-EP1** is Alcoholic product complex. (Results for LE-E, LE-ES, LE-TS and LE-EP1 from Uzma et. al.[20] were included for reference)

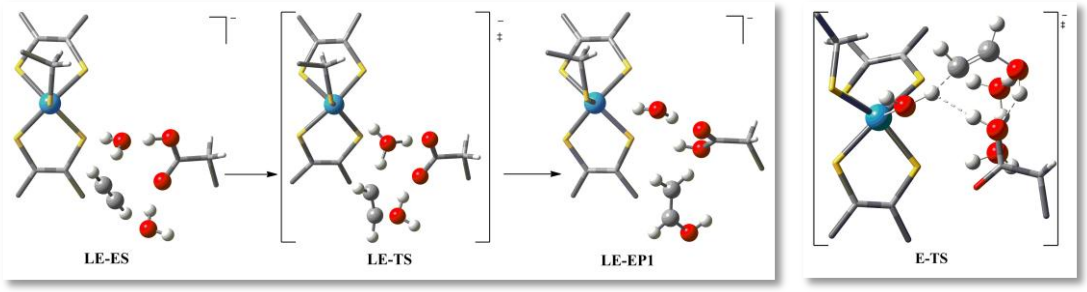

Figure 4.2 Optimized Transition State geometries, LE-ES, LE-TS and LE-EP1 from Uzma et. al.[20] for reference, whereas E-TS is the optimized Transition state geometry for the Electrophilic reaction

4.2 Nucleophilic Pathway

In nucleophilic reaction pathway, Asp_{13} is considered to be present in the form of an anion. In this mechanism W present at the active site accepts electrons (acts as a Lewis acid) from the oxygen (O_1) attach to it resulting in the formation of W bound OH ligand and protonated Asp_{13}. The OH ligand attached to the W center then acts as a nucleophile and attacks the acetylene at its carbon, C_1. At the same time a proton from Asp_{13} is transferred to C_2 of acetylene forming W bound vinyl alcohol. This vinyl alcohol is then tautomerized to acetaldehyde (Figure 4.3).

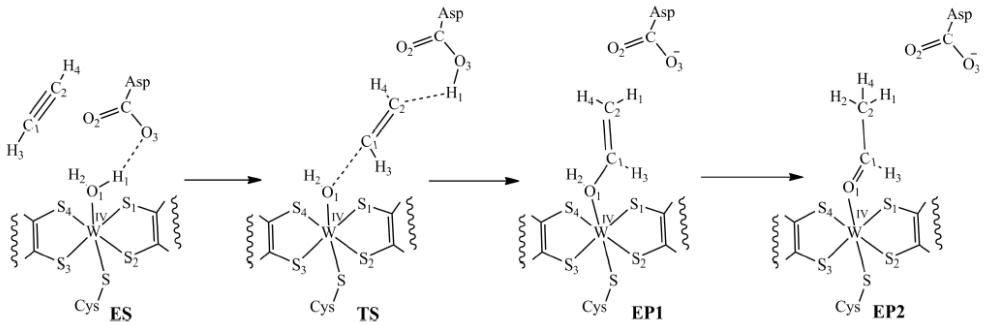

Figure 4.3 Schematic Representation of the Nucleophilic Pathway[20]

Transition state search for the nucleophilic pathway under study resulted in a geometry (Figure 4.4) where distance between W and S of ene-dithiolene ligands are increased from ~2.383 to ~2.416 Å and dihedral angle of S_1-S_2-S_3-S_4 is increased to -27.3˚ in comparison with the results obtained by Uzma et al[20]. Decreased W-O_1 bond distance shows the nucleophilic nature of OH. Reduced O_1-C_1 and C_2-H_1 bond distances represent the formation of transition state (Table 4.2). The energy barrier for the formation of the transition state complex is 15.1 kcal/mol in the continuum (20.8 kcal/mol in the gas phase) (Table 4.3).

Table 4.2 Geometrical Parameters of the optimized model complexes of nucleophilic reaction mechanism for acetylene hydration

	X	LN-E	LN-ES	LN-TS	N-TS	LN-EP1
W-S_1 (Å)	2.432	2.405	2.379	2.411	2.427	2.403
W-S_2 (Å)	2.489	2.367	2.340	2.346	2.372	2.353
W-S_3 (Å)	2.511	2.383	2.369	2.391	2.404	2.346
W-S_4 (Å)	2.336	2.388	2.373	2.387	2.461	2.397
S_1-S_2-S_3-S_4 (°)	-31.4	-22.1	-26.6	-24.0	-27.3	-24.4
W-O_1 (Å)	2.041	2.203	2.196	2.067	2.008	2.306
O_1-H_1 (Å)	-	1.022	1.022	-	-	-
H_1-O_3 (Å)	-	1.559	1.544	1.158	1.053	2.676
O_1-C_1 (Å)	-	-	3.779	2.212	2.26	1.381
C_1-C_2 (Å)	-	-	1.223	1.266	1.244	1.353
C_2-H_1 (Å)	-	-	4.483	1.433	1.735	1.086
H_3-C_1-C_2 (°)	-	-	179.6	117.8	164	122.5
C_1-C_2-H_4 (°)	-	-	177.8	138.1	154.1	119.1

where, **X** protein X-ray crystal structure data, **LN-E** is Educt complex, **LN-ES** is Educt-substrate complex, **LN-TS** is Transition state complex without water molecules, **N-TS** is Transition state complex with water molecules, **LN-EP1** is Alcoholic product complex. (Results for LN-E, LN-ES, LN-TS and LN-EP1 from Uzma et. al.[20] have been included for reference)

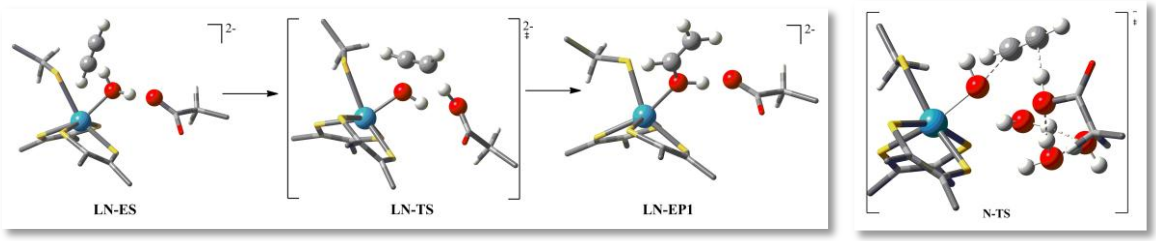

Figure 4.4 Optimized Transition State geometries, LN-ES, LN-TS and LN-EP1 from Uzma et. al.[20] for reference, whereas N-TS is the optimized Transition state geometry for the Nucleophilic reaction

Table 4.3 Computed Energies relative to the educt-substrate complex for stationary points relevant in the hydration of acetylene.

	Electrophilic Pathway, LE	**Nucleophilic Pathway, LN**	
L-ES[20]	0.0	0.0	//B3LYP[a] SDD[b] COSMO[c]
L-TS[20]	47.1 50.8 54.6	47.2 44.6 35.2	//B3LYP[a] SDD[b] COSMO[c]
N-TS	31.7 33.4 29.4	21.5 20.8 15.1	//B3LYP[a] SDD[b] COSMO[c]
L-EP1[20]	-18.3 -28.9 -32.5	-21.5 -26.8 -27.8	//B3LYP[a] SDD[b] COSMO[c]

Where, **L-ES** is Educt-substrate complex, **L-TS** is Transition state complex without water molecules, **N-TS** is Transition state complex with water molecules, **L-EP1** is alcohol product complex. a) B3LYP/Lanl2DZ(p), b) B3LYP/SDDp//B3LYP/Lanl2DZ (p), c) COSMO-B3LYP/SDDp//B3LYP/Lanl2DZ(p). (Results for L-ES, L-TS and L-EP1 from Uzma et.al.[20] have been included for reference)

4.3 Discussion

Acetylene hydratase (AH), purified from mesophillic anaerobe *Pelobacter Acetylenicus*, has a unique active site structure as compared to other tungstoenzymes. Its mononuclear tungsten (W) centre is coordinated by two metallopterin guanine dinucleotide cofactors (MGD), a sulfur atom of the cyteinate and an oxygen specie. The bond distance (2.04Å) of the oxygen specie from the W center makes it likely to be either a hydroxo ligand (bond distance: 1.9-2.1 Å) or a water molecule (bond distance: 2.0-2.3 Å). The hydroxo ligand will lead to a nucleophilic reaction pathway while a water molecule will lead to electrophilic reaction pathway (as proposed by Seiffert et. al.)[23].

Uzma et.al[20] performed DFT calculations on both these mechanisms for small (3Å) (without water molecules), large (6Å) (without water molecules) and large (8Å) (with water molecules) model complexes and found that the nucleophilic reaction mechanism has a lower energy barrier i.e. 17.0kcal/mol, 35.2kcal/mol, 14.4kcal/mol respectively than the electrophilic reaction mechanism 28.5kcal/mol, 54.6kcal/mol, 23.1kcal/mol respectively. Uzma et.al's[20] study also highlighted that the water molecules were may be importance as the energy barrier decreases from 35.2kcal/mol to 14.4kcal/mol for the nucleophilic reaction and from 54.6kcal/mol to 23.1kcal/mol for the electrophilic reaction when water molecules were considered.

Transition state search was done for both the electrophilic and nucleophilic reaction mechanisms. DFT calculations were performed on the transition state model complexes where initial model structures were generated by including the active site structure (6Å) from Uzma et.al's[20] study. Water molecules (Wat_{1209}, Wat_{1212}, Wat_{1424}, and Wat_{1432}) which are in the proximity of W metal center and connected together through the hydrogen bonding and the substrate are also considered. Shortening and lengthening of forming and breaking bonds are then done in order to mimic the transition state. For the electrophilic pathway, transition state involved the electrophilic attack of activated Wat_{1862} molecule on the triple bond of acetylene with the simultaneous transfer of protons among Asp_{13}, Wat_{1862}, Wat_{1424} and acetylene. The proton from the Asp_{13} residue (-COOH) is transferred to the Wat_{1862} molecule and one proton of the Wat_{1862} molecule is transferred to the alpha carbon atom (C_α or C_1) of acetylene. From the Wat_{1424}, one proton is transferred to Asp_{13} while its electron donating part (-OH) is transferred to the second carbon atom (C_β or C_2) of acetylene. The transition state for nucleophilic pathway involved the nucleophilic attack of W-bound hydroxide at the alpha carbon atom (C_α or C_1) of acetylene together with the simultaneous transfer of a proton from the Asp_{13} to the second acetylene carbon atom (C_β or C_2).

According to the computational results of this study, the transition state for the electrophilic reaction is found to have an energy barrier of 29.4 kcal/mol which is 14.3 kcal/mol higher than the energy barrier for the nucleophilic reaction i.e. 15.1 kcal/mol. This validates the findings of Uzma et. al[20] that nucleophilic pathway is a preferable pathway for this reaction. The energy barriers are also found to be lower than the other suggested mechanisms for this reaction i.e. by Vincent et. al. (34.0 kcal/mol) and Himo et. al (23.9 kcal/mol).

Moreover at 6Å without water molecules, Uzma et.al[20] observed an energy barrier of 35.2kcal/mol for the nucleophilic reaction pathway which was quite high. In this study water molecules have been included at 6Å, and the energy barrier has been found to be 15.1kcal/mol which is ~20kcal/mol lower than without the inclusion of water molecules. Therefore water molecules were actually the reason for a lower energy barrier and not the presence of any other amino acid residues at 8Å in Uzma et. al's[20] study.

4.4 Conclusion

The results of this study validate Uzma et.al's[20] findings that the nucleophilic reaction pathway for this reaction is preferable due to lower energy barrier i.e. 15.1 kcal/mol as compared to the electrophilic reaction pathway i.e. 29.42 kcal/mol (solvent phase). The results also highlighted the role of water molecules in lowering the energy barriers for this reaction; which were found to be ~20 kcal/mol less in the presence water molecules as compared to Uzma et.al's[20] findings without water molecules.

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