Chapter 2
Living on Acetylene.
A Primordial Energy Source

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Abstract The tungsten iron-sulfur enzyme acetylene hydratase catalyzes the conversion of acetylene to acetaldehyde by addition of one water molecule to the C≡C triple bond. For a member of the dimethylsulfoxide (DMSO) reductase family this is a rather unique reaction, since it does not involve a net electron transfer.
transfer. The acetylene hydratase from the strictly anaerobic bacterium *Pelobacter acetylenicus* is so far the only known and characterized acetylene hydratase. With a crystal structure solved at 1.26 Å resolution and several amino acids around the active site exchanged by site-directed mutagenesis, many key features have been explored to understand the function of this novel tungsten enzyme. However, the exact reaction mechanism remains unsolved. Trapped in the reduced W^{IV} state, the active site consists of an octahedrally coordinated tungsten ion with a tightly bound water molecule. An aspartate residue in close proximity, forming a short hydrogen bond to the water molecule, was shown to be essential for enzyme activity. The arrangement is completed by a small hydrophobic pocket at the end of an access funnel that is distinct from all other enzymes of the DMSO reductase family.

**Keywords**  acetylene • hydration • iron sulfur • tungsten


1 Introduction

Acetylene (C\_2H\_2, IUPAC name ethyne) is only a minor trace gas in the composition of the Earth’s atmosphere, but notably it can be used as carbon and energy source by several bacteria. One of these, *Pelobacter acetylenicus*, was isolated by Schink in 1985 and the acetylene-converting enzyme of *P. acetylenicus*, the acetylene hydratase (AH; EC 4.2.1.112), has been studied in great detail over the years. AH is a hydrolyase, that catalyzes the addition of one molecule of water to the C≡C triple bond of acetylene-forming acetaldehyde. Therefore, the conversion of acetylene by AH is distinct from the only other known enzymatic reaction of acetylene, the reduction of acetylene to ethylene by nitrogenase [1]. Although the addition of a molecule of water to acetylene is formally not a redox reaction, AH activity depends on the presence of a strong reducing agent like titanium(III) citrate or sodium dithionite.

2 Acetylene

In biological systems, acetylene is well known as inhibitor of microbial processes by interaction with the metal sites of several metallo enzymes, such as nitrogenase, hydrogenase, ammonia monooxygenase, methane monooxygenase, assimilatory nitrate reductase or nitrous oxide reductase [2]. Thus, acetylene has been employed for the quantification of several important biological processes for a long time. For instance, nitrogen fixation can be measured by determining the reduction of acetylene to ethylene (C\_2H\_4) by nitrogenase [3], and inhibition of N\_2O reductase by
acetylene can be used to quantify denitrification rates [2]. Acetylene itself is a highly flammable gas that forms explosive mixtures with air over a wide range of concentrations (2.4–83 % vol, material safety datasheet, Air Liquide GmbH, Germany). Set under pressure, it can polymerize spontaneously in an exothermic reaction [2].

2.1 Properties of Acetylene

The physical and chemical properties of acetylene are mainly determined by the carbon-carbon triple bond. Formed by the overlapping of one of the sp hybrid orbitals and two p orbitals of each of the sp hybridized carbon atoms the bond consists of one σ-bond and two orthogonal π-bonds. The H–C≡C bond angle is 180 °C with a bond length of 121 pm for the triple bond. The triple bond increases the electronegativity of the carbon atoms and thereby the acidity of the H–C bonds of acetylene compared to ethylene and ethane, resulting in a pKₐ of 24 for acetylene compared to 44 for ethylene [2]. This acidity is manifested in the formation of heavy metal acetylides (e.g., Cu(I) acetylide or Ag(I) acetylide) by reaction of acetylene with the corresponding metal cations. In addition, alkali metal acetylides (e.g., sodium acetylide) are formed by reaction of the elemental metal with acetylene. Acetylene can also serve as a ligand to transition metals. Both σ-bonded and π-bonded systems have been described. Examples include (HC≡CH)₂NiBr₂, HC≡CH–Ni(CN)₂ and [π–C₅H₅–Ni]₂C₂H₂ [2].

In general, the chemistry of acetylene is rather rich and diverse. Reactions of acetylene include reduction and oxidation as well as electrophilic and nucleophilic additions [4]. Due to the electron configuration of the C≡C triple bond, electrophilic additions to alkynes are much slower compared to additions to alkenes. On the other hand, nucleophilic additions are much faster than those on alkenes [5]. The reason for this is that the electrons of the carbon atoms are massed in the triple bond, leaving the “backside” of the cores of the carbon atoms open for nucleophiles to attack.

2.2 Sources and Bioavailability of Acetylene on Earth and Other Planets

Today, acetylene is only a minor trace gas in the composition of the Earth’s atmosphere. Depending on where the samples were taken, concentrations of acetylene between 0.02–0.08 ppbv were detected [6]. Since acetylene on Earth seems to be mainly of anthropogenic origin, with exhaust from combustion engines as main source, samples from oceanic and rural areas show less abundance of acetylene [7].
Apart from the anthropogenic acetylene on Earth, naturally occurring acetylene can be found among other prebiotic molecules in interstellar gas clouds [8]. Another place where acetylene occurs naturally in greater abundance is on Saturn’s moon Titan. Titan’s atmosphere is considered to be a cold model of our Earth’s early atmosphere approximately 4 billion years ago [6]. Photochemical processes in Titan’s upper atmosphere that create acetylene from methane are considered to be the source of this acetylene [9]. Besides an atmospheric concentration of 3.5 ppm acetylene [6], the Cassini/Huygens mission to Titan detected lakes consisting of hydrocarbons (76–79 % ethane, 7–9 % propane, 5–1 % methane, 2–3 % hydrogen cyanide, 1 % butene, 1 % butane, and 1 % acetylene) on Titan’s surface [10, 11]. The similarity between the atmosphere of Titan and the assumed composition of the Earth’s early atmosphere have led to speculations that photochemical reactions along with other sources like volcanic eruptions may have provided the developing life on Earth with sufficient amounts of acetylene to be a viable source of carbon and energy [6].

Bacterial growth on acetylene is facilitated by its rather high solubility in water of 47.2 mM (20 °C; 1 atm or 101 kPa) compared to other gaseous compounds like O₂, H₂ or N₂ that have solubilities of around 1 mM under these conditions [2].

3 Bacteria Living on Acetylene

The energy richness of the C≡C triple bond and the rather high solubility of acetylene in water make it a suitable substrate for bacteria, provided an adequate source is available. The first reports on bacteria living on acetylene were published more than 80 years ago [12]. In 1979, Nocardia rhodochrous was grown on acetylene as sole source of carbon and energy in the presence of dioxygen [13]. One year later, an acetylene hydratase activity was found in cell-free extracts from Rhodococcus A1 grown on acetylene by anaerobic fermentation [14]. In these cell-free extracts, acetylene was converted to acetaldehyde by addition of a water molecule. Boiling of the extracts or addition of dioxygen inhibited this activity, indicating that it originated from an oxygen-sensitive enzyme. In 1981, anaerobic oxidation of acetylene to CO₂ was found in enrichment cultures from estuarine sediments [15]. In this case, acetate was identified as major intermediate of the process. Further studies revealed that two groups of bacteria were responsible for the oxidation of acetylene to CO₂. Fermenting bacteria converted acetylene to acetaldehyde, which they dismutated to acetate and ethanol. These products were then further oxidized by sulfate-reducing bacteria [16]. According to Culbertson et al. [16], the morphology of these Gram-negative bacteria was described to be similar to Pelobacter acetylenicus, an acetylene-fermenting bacterium, isolated three years earlier by Schink [17]. After the isolation of the acetylene hydratase from P. acetylenicus [1], Rosner et al. tested new isolates and bacterial strains from culture collection in the presence of a similar enzyme by activity tests and antibody cross reaction [18]. Acetylene hydratase activity was discovered in several cell-free
extracts. As for \textit{P. acetylenicus}, it required a strong reductant and the presence of molybdate or tungstate in the growth media. However, a cross reaction with antibodies against the acetylene hydratase from \textit{P. acetylenicus} was not found [18].

Recently, a more systematic study was conducted on the presence of acetylene hydratase activity in anoxic sediments and waters from all over the United States of America and several deep sea sites [19]. Acetylene consumption was found in only 21% of the samples investigated, usually after an incubation time of several days to months. The lag phase was explained by selecting for and thereby enriching bacteria that thrive on acetylene. This would mean that acetylene-fermenting bacteria were rather scarce in the original samples. In a second part of the study, the authors tried to amplify genes coding for an acetylene hydratase from DNA extracted from the sediment and water samples. The use of primers synthesized from the acetylene hydratase gene of \textit{P. acetylenicus} resulted in 63 PCR products out of 645 environmental samples (9.8%) [19]. Since AH-like genes could not be amplified in all samples in which acetylene hydratase activity was found, it was argued that the primer may have been overly specific for the acetylene hydratase gene of \textit{P. acetylenicus}, hence, that Miller et al. could amplify the genes from \textit{Pelobacter}-like organisms but not from other, less closely related bacteria [19].

### 3.1 \textit{Pelobacter acetylenicus}

\textit{Pelobacter acetylenicus} was isolated 1985 by enrichment on acetylene, which can be used as sole source of carbon and energy by this bacterium [17]. Two strains are deposited at the DSMZ culture collection in Braunschweig (Germany). The type strain WoAcy1 (DSMZ 3246) was isolated from a freshwater creek sediment and strain GhAcy1 (DSMZ 3247) from a marine sediment. \textit{Pelobacter} are Gram-negative, strictly anaerobic deltaproteobacteria that ferment low-molecular-weight organic compounds, but no sugars. Characteristic substrates are gallic acid, acetoin, polyethylene glycol, and acetylene [20]. \textit{Pelobacter acetylenicus} is mesophilic and grows between pH 6.0 and pH 8.0. From over 40 different substrates tested, only acetylene, acetoin, ethanolamine, and choline supported growth directly, while 1,2-propanediol and glycerol could be used in the presence of acetate [17].

No growth was found on the acetylene derivatives acetylene carboxylate or acetylene dicarboxylate or the structurally related compounds ethylene and cyanide.

During growth of \textit{P. acetylenicus}, acetylene is fermented to nearly equal amounts of ethanol and acetate and small amounts of acetaldehyde [17]. According to the metabolic scheme proposed by Schink in 1985, the energetics of the initial hydration of acetylene can be derived (equations 1 and 2) [17]:

\[
\text{C}_2\text{H}_2 + \text{H}_2\text{O} \rightarrow \text{CH}_3\text{CHO} \quad \Delta G'_0 = -119.9 \text{ kJ mol}^{-1} \quad (1)
\]
The subsequent disproportionation of acetaldehyde to ethanol and acetate yields by far less energy, but is still exergonic:

\[
2 \text{CH}_3\text{CHO} + \text{H}_2\text{O} \rightarrow \text{CH}_3\text{CH}_2\text{OH} + \text{CH}_3\text{COO}^- + \text{H}^+ \\
\Delta G'_0 = -17.3 \text{ kJ mol}^{-1}\ 	ext{acetaldehyde}
\] (2)

In the oxidative branch of the disproportionation acetaldehyde is transformed to acetyl phosphate via acetyl-CoA. An acetate kinase then transfers the phosphate group from acetyl phosphate to a molecule of ADP, forming ATP and acetate. According to the growth yields determined by Schink, this kinase reaction is the only energy-conserving step during fermentation of acetylene by \textit{P. acetylenicus} [17].

4 Acetylene Hydratase from \textit{Pelobacter acetylenicus}

Acetylene hydratase from \textit{P. acetylenicus} is so far the only acetylene hydratase that has been intensively studied by biochemical, spectroscopic, crystallographic, and computational approaches. The first isolation and purification of acetylene hydratase was reported in 1995 [1], followed by a high-resolution crystal structure [21] and heterologous expression and site-directed mutagenesis of several amino acids around the putative active site [22].

4.1 Biochemical and Spectroscopic Properties

AH was isolated as a monomer of 73 kDa according to SDS-PAGE [1] versus 83.5 kDa in MALDI-TOF mass spectra [23] from the soluble fraction of broken cells. Activity of acetylene hydratase, in cell-free extracts or purified, was dependent on (i) the presence of tungstate or molybdate in the growth media and (ii) addition of a strong reductant like titanium(III) citrate (\(E'_0 = -480 \text{ mV}\); [24]) or dithionite (\(E'_0 = -527 \text{ mV}\); [25]) to the activity assay [1]. The pH optimum of the enzyme reduced with 2 mM titanium(III) citrate was between pH 6.0 and pH 6.5. The temperature optimum was 50 °C (2 mM Ti(III) citrate; pH 7.0), the \(K_m\) for acetylene was 14 \(\mu\)M [1].

According to its amino acid sequence, acetylene hydratase is a member of the DMSO reductase family. Consistent with this, the metal content of AH was determined by ICP-MS analysis to be 0.4 to 0.5 mol W per mol enzyme and 3.7 to 3.9 mol Fe per mol enzyme [1, 22, 23]. Like in all members of the DMSO reductase family, a bis-molybdopterin-guanine-dinucleotide cofactor coordinates the tungsten ion in AH (Figure 1).
While acetylene hydratase as isolated under N₂/H₂ (94%/6% v/v) atmosphere was EPR-silent, EPR spectra of the enzyme reduced with dithionite showed a typical signal of a low potential ferredoxin-type [4Fe-4S] cluster with $g$ values of $g_z = 2.048$, $g_y = 1.939$, and $g_x = 1.920$ [23]. After oxidization of the AH with one equivalent hexacyanoferrate(III) ($[\text{Fe(CN)}_6]^{3-}$) the EPR spectra showed the signal of a W(V) center with $g_x = 2.007$, $g_y = 2.019$, and $g_z = 2.048$. Upon further addition of hexacyanoferrate(III), the W(V) signal disappeared, as the tungsten site in the enzyme was oxidized to W(VI). Since AH isolated under N₂/H₂ needs to be reduced to be active, the dependence of AH activity on the applied redox potential was studied by Meckenstock et al. [23] in potentiometric titrations. The midpoint redox potential of the [4Fe-4S] cluster was determined to be $-410$ mV. The enzyme activity had a midpoint redox potential of $-340$ mV, meaning that AH is already active when the tungsten site is in its W(IV) state. Apparently, the redox state of the iron-sulfur center appears to be less important for enzyme activity [23].

**4.2 Molybdenum-Substituted Enzyme**

By growing *P. acetylenicus* in a medium containing only trace amounts of tungstate (2 nM instead of the original 800 nM in the tungstate medium) and elevated amounts of molybdate (2 μM instead of the original 6 nM) it is possible to exchange the central W ion in the active site of AH against Mo [26, 27]. The resulting molybdenum-substituted acetylene hydratase (AH(Mo)) is 10 times less active...
than the original acetylene hydratase in its native tungsten form (AH(W)) but still converts acetylene to acetaldehyde at a rate of 1.9 μmol min\(^{-1}\) mg\(^{-1}\) at 37 °C compared to 14.8 μmol min\(^{-1}\) mg\(^{-1}\) of the original AH(W) [27]. According to ICP-MS AH(Mo) contained 0.45–0.51 mol Mo per mol enzyme, 2.7–3.1 mol Fe per mol enzyme, and no tungsten [26–28], ruling out that the low activity of AH(Mo) derives from residual tungsten in the enzyme.

The EPR spectrum of AH(Mo), as isolated under N\(_2\)/H\(_2\) (94 %/6 % v/v) atmosphere, showed a weak signal assigned to a Mo(V) center with \(g_x = 1.978\), \(g_y = 1.99\), and \(g_z = 2.023\). The signal size increased upon oxidation with hexacyanoferrate(III), indicating that AH(Mo) was isolated in a partially oxidized state. Dithionite-reduced samples of AH(Mo) showed the identical signal of a ferredoxin type [4Fe-4S] cluster as AH(W) with \(g_z = 2.048\), \(g_y = 1.939\), and \(g_x = 1.920\) [28]. When comparing the contribution of secondary structural elements to the total fold in AH(Mo) and AH(W) by circular dichroism (CD) spectroscopy, only slight differences in the amount of \(\alpha\)-helices (14.3 % in AH(Mo) \textit{versus} 11.3 % in AH(W)) and \(\beta\)-sheets (35.4 % in AH(Mo) \textit{versus} 39.9 % in AH(W)) were found [27].

4.3 Crystallization

A high resolution X-ray structure of acetylene hydratase was solved in 2007 (PDB 2E7Z) [21]. The crystallization of AH was performed under a N\(_2\)/H\(_2\) (94 %/6 % v/v) atmosphere at 20 °C using the sitting drop vapor diffusion method. Yellow brownish, plate-shaped crystals grew within 1–3 weeks from a 10 mg/mL solution of AH in 5 mM HEPES/NaOH pH 7.5 containing 5 mM Na\(^+\) dithionite. 2 μL of the protein solution were mixed with 2.2 μL of 0.1 M Na cacodylate, pH 6.5, containing 0.3 M Mg(acetate)\(_2\), 21 % PEG 8000, and 0.04 M Na\(^+\) azide. 15 % MPD was added as cryo protectant before flush freezing of the crystals in liquid N\(_2\). The crystals belonged to space group C2 with \(a = 120.8\) Å, \(b = 72.0\) Å, \(c = 106.8\) Å, and \(\beta = 124.3\)°, and contained one monomer per asymmetric unit. The native structure was solved by single wavelength anomalous dispersion (Fe absorption edge) and refined to 1.26 Å. In total the model consisted of 730 amino acid residues, 880 water molecules, two MGD cofactor molecules, and one [4Fe-4S] cluster. Additionally, two MPD molecules, one acetate molecule, and one sodium ion have been identified in the crystal structure of AH [21].

4.4 Structural Overview

As expected, from the biochemical and spectroscopic characterization of the enzyme, the structure is a monomer of 730 amino acids, containing a bis-molybdopterin-guanine-dinucleotide (bis-MGD) and a cubane [4Fe-4S] cluster. The two cofactors are buried deep inside a four domain fold, as found typically in enzymes of the DMSO reductase family (Figure 1) [21]. Domain I (residues 4-60)
harbors the [4Fe-4S] cluster, ligated by the four cysteine residues Cys9, Cys12, Cys16, and Cys46. Domain II (residues 65–136 and 393–542) and III (residues 137–327) have an αβα fold with homologies to the NAD-binding fold in dehydrogenases. Each of these two domains provides hydrogen bonds, needed to bind one of the MGD cofactors. The interactions are mainly provided by variable loop regions at the C-termini of the strands of a parallel β-sheet. The coordination of both of the MGD cofactors is completed by domain IV (residues 590–730), which consists mainly of a seven stranded β-barrel fold [21].

The overall tertiary structure of acetylene hydratase and the position of the cofactors, with the two MGDs (P_MGD and Q_MGD) in an elongated conformation and the [4Fe-4S] cluster close to the Q_MGD is similar to all other structures of members of the DMSO reductase published so far [21]. However, the access from the surface of the protein towards the putative active site, consisting of the tungsten ion ligated by the two MGDs with the iron-sulfur cluster in close proximity is unique for an enzyme of the DMSO reductase family. In all structures of DMSO reductase family enzymes published so far, the access funnel starts at the pseudo twofold axis between domain II and III. A shift in the loop region of the residues 327–335 towards the surface of the protein and further rearrangements of the residues 336–393 seal this entrance point in AH. In other enzymes (e.g., nitrate and formate reductases), this loop region separates the [4Fe-4S] cluster from the Mo/W site. In AH, the shift of this loop opens a new access funnel towards the central tungsten ion at the intersection of domains I, II, and III, allowing the substrate to reach the central tungsten ion from a totally different direction than in other enzymes of the DMSO reductase family [21].

4.5 Active Site Setup

While the overall fold of AH is quite similar to other enzymes of the DMSO reductase family, major rearrangements are found at the active site [21]. The tungsten center in its reduced W(IV) state is coordinated by the four sulfur atoms of the dithiolene moieties of the P_MGD and Q_MGD cofactors and by one sulfur atom of a cysteine residue (Cys141), as found in the dissimilatory nitrate reductase. The sixth ligand position is taken by a tightly coordinated oxygen atom at 2.04 Å distance from the tungsten ion. Due to a rotation of the P_MGD cofactor, the geometry of the coordination in AH is not square pyramidal or trigonal prismatic, as typically found in enzymes of this family [29], but resembles more an octahedral or trigonal antiprismatic coordination (Figure 2) [21].

The access funnel opened by a shift in the loop region of residues 327–335 ends in a ring of six bulky hydrophobic residues (Ile14, Ile113, Ile142, Trp179, Trp293, and Trp472) forming a small hydrophobic pocket directly above the oxygen ligand and an adjacent aspartate residue (Asp13) (Figure 3). Asp13, a direct neighbor of the [4Fe-4S]-coordinating Cys12, forms a tight hydrogen bond of 2.41 Å to the oxygen ligand of the W ion. Although it was yet not possible to solve a crystal
Figure 2  Active site of acetylene hydratase. The W ion is octahedrally coordinated by the two MGD cofactors (P and Q), Cys141, and a water molecule. Asp13 forms a short hydrogen bond to the water molecule. Element colors: W in cyan, O in red, S in yellow, N in blue, C in gray, P in orange.

Figure 3  View down the access funnel towards the active site of AH. The six amino acid residues (Ile14, Ile113, Ile142, Trp179, Trp293, and Trp472) of the hydrophobic ring are represented as green spheres. W in cyan, O in red, S in yellow, N in blue, C in gray, P in orange, and Fe in brown.
structure with acetylene or an inhibitor bound [21, 27], an acetylene molecule
docked computationally to the AH structure gave an excellent fit in the pocket of
the hydrophobic ring with its carbon atoms positioned directly above the oxygen
ligand and the carboxylic acid group of Asp13 [21].

Crucial for deriving a reaction mechanism for AH from the X-ray structure is the
nature of the oxygen ligand of the tungsten ion. The bond length of 2.04 Å observed
in the structure falls between the values expected for a hydroxo ligand (OH−
1.9–2.1 Å) and a coordinated water molecule (2.0–2.3 Å) [21]. Seiffert et al. [21]
decided for a water molecule, since the close proximity of the heavy scatterer
tungsten may distort the distance observed in the X-ray data by Fourier series
termination and a simulation of this effect resulted in a true ligand distance of 2.25 Å.

4.6 Site-Directed Mutagenesis

The development of a protocol for the heterologous expression of AH in
Escherichia coli allowed for further studies towards the reaction mechanism by
site-directed mutagenesis [22]. Initially, the metal and cofactor content of the
expressed protein was quite low compared to that of the native AH from
P. acetylenicus (0.06 mol W and 1.22 mol Fe versus 0.4 mol W and 3.7 mol Fe
per mol enzyme) [22]. This problem was partially solved by addition of an
N-terminal chaperone-binding sequence of the soluble nitrate reductase NarG
from E. coli. According to the literature this 30 amino acid long sequence is one
of two binding sites for chaperones during insertion of the bis-MGD cofactor during
protein biosynthesis [30]. The resulting fusion variant of AH had a higher content of
W (0.14 mol per mol enzyme) and Fe (3.5 mol per mol enzyme) and therefore an
increased activity compared to the heterologously expressed AH. Overall, when
normalized to their W content, the activity of the heterologously expressed AH
was nearly identical to that of the native enzyme purified from P. acetylenicus
(Table 1) [22].

<table>
<thead>
<tr>
<th>Acetylene hydratase</th>
<th>W mol/mol AH</th>
<th>Fe mol/mol AH</th>
<th>Specific activity [μmol×min⁻¹×mg⁻¹]</th>
<th>Relative activity W^a</th>
</tr>
</thead>
<tbody>
<tr>
<td>P. acetylenicus AH</td>
<td>0.37 ± 0.04</td>
<td>3.69 ± 0.04</td>
<td>14.2 ± 0.9</td>
<td>38.4</td>
</tr>
<tr>
<td>E. coli AH</td>
<td>0.06 ± 0.02</td>
<td>1.22 ± 0.26</td>
<td>2.6 ± 0.8</td>
<td>43.3</td>
</tr>
<tr>
<td>E. coli AH D13A</td>
<td>0.09 ± 0.02</td>
<td>1.17 ± 0.29</td>
<td>0.2 ± 0.1</td>
<td>2.2</td>
</tr>
<tr>
<td>E. coli AH D13E</td>
<td>0.05 ± 0.01</td>
<td>1.11 ± 0.30</td>
<td>2.5 ± 0.3</td>
<td>50.0</td>
</tr>
<tr>
<td>E. coli NarG-AH</td>
<td>0.14 ± 0.06</td>
<td>3.17 ± 0.49</td>
<td>9.7 ± 1.9</td>
<td>69.3</td>
</tr>
<tr>
<td>E. coli NarG-AH K48A</td>
<td>0.15 ± 0.01</td>
<td>3.56 ± 0.31</td>
<td>7.2 ± 0.3</td>
<td>48.0</td>
</tr>
<tr>
<td>E. coli NarG-AH I142A</td>
<td>0.18 ± 0.02</td>
<td>3.20 ± 0.22</td>
<td>2.2 ± 0.2</td>
<td>12.2</td>
</tr>
</tbody>
</table>

^aRelative to tungsten concentration, in nmol.
Three amino acids at the active site could be exchanged by site-directed mutagenesis: Asp13, Lys48, and Ile142 (Figure 4). Asp13 forms a hydrogen bond to the oxygen ligand of the W ion and was expected to be important for the reaction of AH by helping to activate the oxygen atom for the addition on the C\(\equiv\)C triple bond. The exchange of Asp13 against alanine resulted in a dramatic loss of activity (0.2 \(\mu\)mol min\(^{-1}\) mg\(^{-1}\) for the D13A variant compared to 2.6 \(\mu\)mol min\(^{-1}\) mg\(^{-1}\) for the expressed wild-type) while the exchange of Asp13 against glutamate had nearly no effect on the activity of AH (2.5 \(\mu\)mol min\(^{-1}\) mg\(^{-1}\) for the D13E variant compared to 2.6 \(\mu\)mol min\(^{-1}\) mg\(^{-1}\) for expressed wild-type). These results underline the important role of the carboxylic acid group at this position for the reaction of AH (Figure 5) [22].

Lys48 is located between the [4Fe-4S] cluster and the Q\(_{\text{MGD}}\) cofactor. In other enzymes of the DMSO reductase family, this residue is involved in electron transfer between the two cofactors [29]. As the reaction of AH does not involve a net electron transfer, the exchange of Lys48 against alanine did not affect the catalysis rate of the enzyme (Figure 5) [22].

Ile142 is part of the hydrophobic ring that is expected to form the substrate binding site at the end of the access funnel towards the active site [22]. The exchange of Ile142 against alanine resulted in a strong loss of activity (2.2 \(\mu\)mol min\(^{-1}\) mg\(^{-1}\) for the NarG-AH I142A variant compared to 9.7 \(\mu\)mol min\(^{-1}\) mg\(^{-1}\) for the NarG-AH fusion protein with the N-terminal chaperone-binding sequence). This finding supports the idea that the cavity within the hydrophobic ring is the substrate binding site of AH (Figure 5) [22].
4.7 Density Functional Theory Calculations on the Substrate Binding Mode and Amino Acid Protonation States

Several groups directed a major computational effort towards the understanding of the reaction mechanism of AH. Seiffert et al. [21] used density functional theory (DFT) based atomic charge calculation to determine the titration curves of all residues in AH. Notably, out of 34 Asp and 58 Glu residues, only three showed a highly aberrant titration behavior: Asp298, Glu494, and Asp13. Asp13 that forms a hydrogen bond to the water ligand of the W center stayed protonated in the pH range 0–24, when the [4Fe-4S] cluster in close proximity was reduced. Whereas a partially deprotonated Asp13 was found at high pH values when the [4Fe-4S] cluster and the W center were fully oxidized [21].

Antony and Bayse [34], Vincent et al. [37], and Liao et al. [35] applied DFT methods to calculate the energies of acetylene adduct formation and the energetic barriers between intermediate states of several possible reaction mechanisms of model complexes that mimic the active site of AH. Antony and Bayse [34] used models of molybdenum- and tungsten-oxo dithiocarbamates (dtc) and dithiolates (dtl) that have been shown to form adducts with alkynes [31, 32] or even had AH-like activity [33]. Furthermore, a truncated model of the active site of AH consisting of a W ion coordinated by four dithiolene sulfur atoms, a cysteine sulfur and a water molecule was used to study the binding of water versus acetylene [34]. In these models, the free energy difference $\Delta G_{\text{complex}}$ for the formation of an acetylene metal complex favored the tungsten complexes over their molybdenum analogues, due to a more favorable interaction of the 5d W orbitals with the $\pi$ molecule orbitals of acetylene. Additionally, the models showed that a thiolate ligand (Cys141 in AH) made the complex formation more exergonic than an oxo ligand in this position would do [34]. When calculating the $\Delta G$ values for the substitution of the water by acetylene, the acetylene complex was favored by ~10 kcal/mol [34].
Vincent et al. [37] used a truncated model of the AH active site, consisting of the W ion ligated by 4 dithiolene sulfur atoms, Cys141, and a water molecule and the carboxylic acid group of Asp13 hydrogen-bonded to the water molecule, to calculate the energy barriers between intermediates of several putative reaction pathways. A very similar approach was followed by Liao et al. [35]. Here, a much larger model complex of the active site of AH was used, consisting of the W ion ligated by the two pterin cofactors, Cys141, and a water molecule. Additionally, Asp13 and several other amino acids in close proximity (Cys12, Met140, Ile142, Trp179, and Arg606) were included [35]. While the reaction pathways deriving from the DFT calculation will be discussed in Section 4.8, it is noteworthy that Liao et al. [35] calculated a $pK_a$ of 6.3 for the Asp13 residue. Thus in this model, Asp13 will most likely be deprotonated under reaction conditions in contrast to the model of Seiffert et al. [21] in which Asp13 is always protonated.

Using their established model for DFT calculations on the reaction mechanism of AH [35], Liao and Himo [36] calculated the binding and activation energies for several other compounds such as ethylene, acetonitrile, and propyne. In biochemical experiments none of these compounds was turned over by AH [22]. The DFT calculations showed that compared to acetylene, ethylene and propyne have a about ~6 kcal/mol and ~5 kcal/mol higher binding energy for the initial displacement of the water molecule at the W ion. The subsequent steps of the reaction would have a much higher barrier than in the case of acetylene, showing why these compounds are no substrates for AH (see Figure 7 in Section 4.8) [36]. Acetonitrile had a much higher binding energy than acetylene (~13 kcal/mol) but the differences of ~3 kcal/mol more for the barriers of the subsequent steps were too low to draw a firm conclusion whether acetonitrile is a substrate of AH or not [36].

### 4.8 Towards the Reaction Mechanism

When the crystal structure of AH was solved at a resolution of 1.26 Å, Seiffert et al. [21] proposed two alternative reaction mechanisms on the basis of their structural data. Depending on the nature of the oxygen ligand of the W ion (OH$^-$ or H$_2$O), either a nucleophilic addition or an electrophilic Markovnikov-type addition were proposed [21]. In both cases, acetylene, located in the pocket formed by the hydrophobic ring, would not interact directly with the W ion but only with the oxygen ligand activated by the W$^{IV}$ center and Asp13. A hydroxo ligand (OH$^-$) would constitute a strong nucleophile that would yield a vinyl anion with acetylene. The basicity of the vinyl anion would be sufficient to deprotonate Asp13 to form a vinyl alcohol that would tautomerize to acetylene. A water molecule would then bind to the W ion and get deprotonated by the now basic Asp13 to restore the active site for the next reaction cycle [21].

A bound water molecule would gain a partially positive net charge by the proximity of the protonated Asp13, turning it into an electrophile that could directly attack the C≡C triple bond with a vinyl cation as intermediate (Figure 6) [21].
Figure 6  Pathways of several reaction mechanisms proposed for AH. (a) Nucleophilic attack of the activated water ligand of the W ion [21] (b) First shell mechanism involving a $\eta^1$-acetylene complex followed by a nucleophilic attack of a water molecule [34]. (c) First shell mechanism with a tungsten vinylidene complex as intermediate [37]. (d) Mechanism with a $\eta^2$-acetylene complex followed by several proton transfers to and from Asp13 [35]. Data adapted from [34, 35, 37].
The second shell mechanism was supported by the loss of activity when Ile142 was exchanged against alanine in the site-directed mutagenesis experiments [22]. However, in all DFT calculations performed so far, a first shell mechanism with a direct binding of acetylene to the W ion gave much lower energy barriers [34, 35, 37]. Antony and Bayse [34] calculated the substitution of the water molecule by acetylene to form a $\eta^2$-acetylene complex to be favorable by a $\Delta G$ of $-10$ kcal/mol. Therefore, a reaction mechanism starting with the formation of a $\eta^2$-acetylene complex followed by a nucleophilic attack by a water molecule was proposed, yielding either a $\eta^2$-complex of a vinyl alcohol or a $\beta$-hydroxovinylidene (Figure 6) [34]. Vincent et al. [37] calculated the energetic barriers for the intermediates of the reaction mechanism proposed by Seiffert et al. [21] and Antony and Bayse [34]. The nucleophilic attack of a water ligand on the C$_\alpha$ of acetylene to form a vinyl alcohol via a vinyl anion and deprotonation of Asp13 [21] was calculated to be exothermic in total ($-21.4$ kcal/mol) but the barrier of $43.9$ kcal/mol was quite high [37]. The results for the nucleophilic attack of a water molecule on a $\eta^2$-acetylene complex were quite similar, the overall reaction was slightly exothermic ($-1.9$ kcal/mol) but the barrier was also quite high ($41.0$ kcal/mol) [37]. The nearly identical barriers for both mechanisms could be explained by the similarities between both pathways, involving a nucleophilic attack of a water molecule, a cyclic intermediate structure and a proton shuttle from Asp13 [37].

Therefore, Vincent et al. [37] proposed a new reaction mechanism with overall lower barriers. The mechanism starts with a $\eta^2$-acetylene complex that forms an end-on bound vinylidene complex ($W=\text{C}=$CH$_2$) by deprotonation of Asp13. The attack of a water molecule activated by a hydrogen bond to Asp13 leads to the formation of a carbene complex that will isomerize to form acetaldehyde (Figure 6) [37]. The barriers for the formation of the end-on bound vinylidene complex and the formation of the carbene complex were calculated to be $28.1$ kcal/mol and $34.0$ kcal/mol respectively. The isomerization to acetaldehyde via a tungsten hydride complex requires the breaking of a W-C bond with a barrier of $29$ kcal/mol and the decomposition of the product (3 kcal/mol barrier) (Figure 7) [37].

Liao et al. [35] postulated another reaction mechanism based on DFT calculations. Notably in this model, Asp13 is in a deprotonated state because the model includes three hydrogen bonds of Asp13 to Cys12, Trp179, and the H$_2$O ligand of the W ion, lowering the calculated pK$_a$ of Asp13 to 6.3. In the first step of this mechanism acetylene forms a $\eta^2$-complex with the W ion by displacing the water ligand. The displaced water molecule is activated for a nucleophilic attack on the $\eta^2$-acetylene complex by a proton transfer to the ionized Asp13. The resulting vinyl anion will be protonated by Asp13, yielding a vinyl alcohol. The tautomerization needed to form acetaldehyde from the vinyl alcohol, can either occur spontaneously after release of the alcohol from the active site or will be assisted by the W ion and Asp13 [35]. The assisted tautomerization starts with a proton transfer from the OH group of the vinyl alcohol to Asp13, yielding an enolate that binds with the oxygen atom instead of a carbon atom to the W ion. The proton is then delivered back to C2 yielding the product acetaldehyde (Figure 6) [35]. The displacement of the water molecule by acetylene in the first reaction step was calculated to be exothermic
The barrier of the subsequent reaction steps were calculated to be 16.9 kcal for the nucleophilic attack, the protonation of the vinyl anion by Asp13 yields another 6 kcal/mol, raising the total barrier for the \( \eta^2 \)-acetylene complex to 23 kcal/mol. The following deprotonation to form an enolate has a barrier of 9.3 kcal/mol and the final reprotonation at C2 to form vinyl alcohol transition to 23 kcal/mol. The following deprotonation to form an enolate has a barrier of 9.3 kcal/mol and the final reprotonation at C2 to form vinyl alcohol transition to 23 kcal/mol.

**Figure 7** Potential energy profiles of transition states (TS) and intermediates (Int) in reaction mechanisms proposed for AH. **Top:** Energy profiles for the hydration of acetylene via a vinylidene complex (black line) [37] or a \( \eta^2 \)-acetylene complex (grey line) [35]. **Bottom:** Energy profiles for the first hydration steps in the hydration of propyne (black line) and ethylene (grey line) [36]. Data adapted from [35–37].
acetaldehyde has a barrier of 14.1 kcal/mol (Figure 7) [35]. When Liao et al. [35] used their model to calculate the barriers for the nucleophilic attack of a water ligand on acetylene [21] they found a barrier of 45.4 kcal/mol (compared to 43.9 reported by Vincent et al. [37]).

So far, a first shell mechanism is clearly favored in all DFT calculations. But since the hydrophobic ring and therefore the putative binding site for acetylene in the second shell mechanism [21] was not included in any of the models, it would be interesting to see a calculation with this feature included.

5 Conclusions

The isolation of \textit{P. acetylenicus} [17] and the purification of a first tungsten-dependent acetylene hydratase [1] led to numerous speculations with regard to the physiological role and origin of this enzyme. Hydrolytic transformations of toxic compounds such as cyanides and nitriles were brought forward as one possibility [17]. However, neither growth of \textit{P. acetylenicus} nor a reaction of AH with one of these compounds could be observed [1, 17, 22]. When the X-ray structure of AH was solved [21], major structural rearrangements became obvious in comparison to other members of the DMSO reductase family. The different location of the access funnel and the absence of a loop region separation of the [4Fe-4S] from the MGD cofactor in formate and nitrate reductases leads to a new active site on the opposite face of the W ion [21]. At the end of the access funnel, a ring of 6 bulky hydrophobic residues forms a binding pocket ideally suited for a small hydrophobic molecule like acetylene [21, 22].

Taking everything into account, AH appears to be an enzyme that is highly adapted to the conversion of acetylene, thus, it might be a rather old enzyme, from a past when acetylene was more abundant in the Earth’s atmosphere [16]. With regard to its reaction mechanism, a clear statement is not yet possible. Unfortunately, a high resolution structure of AH with acetylene or an intermediate of the reaction bound at the active site, could not be obtained so far [21, 27]. Computational attempts to model the reaction pathway led to new insights and a deeper understanding of the atomic structure and possible substrate-active site interaction on the atomic level. For example, it was possible to demonstrate why certain potential substrates, such as ethylene, acetonitrile, and propyne do not react with AH [36], under the assumption of a reaction pathway. However, with the rapidly increasing computer power and shorter computing times, future calculations will take into account important features of AHs active site including the hydrophobic ring and the putative binding pocket for acetylene within it.

Note that an acetylene molecule can be modeled into this pocket located directly above the water ligand of the W ion at a distance of \(\sim 4 \, \text{Å} \) [21, 22]. Once these important features have been included into the calculations, a second shell mechanism might become favorable, which is clearly supported by the X-ray data.
and the results from site-directed mutagenesis. In the case of a first shell mechanism, the more polar reaction product, acetaldehyde will have to pass through a narrow hydrophobic gate upon leaving the active site, energetically not a favorable event. On the other hand, in a second shell mechanism, acetaldehyde would just be expelled into the substrate channel, assisting the product release by the repellant interaction of a polar molecule with a hydrophobic surrounding [27].

**Abbreviations and Definitions**

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
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<tbody>
<tr>
<td>Å</td>
<td>Ångström; 1 Å = 10^{-10} m</td>
</tr>
<tr>
<td>acetyl-CoA</td>
<td>acetyl-coenzyme A</td>
</tr>
<tr>
<td>ADP</td>
<td>adenosine 5’-diphosphate</td>
</tr>
<tr>
<td>AH</td>
<td>acetylene hydratase</td>
</tr>
<tr>
<td>AH(Mo)</td>
<td>acetylene hydratase, molybdenum-substituted</td>
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<td>AH(W)</td>
<td>acetylene hydratase, in its native tungsten form</td>
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<td>ATP</td>
<td>adenosine 5’-triphosphate</td>
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<td>CD</td>
<td>circular dichroism</td>
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<td>Da</td>
<td>Dalton; 1 Da = 1 g · mol^{-1}</td>
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<td>DFT</td>
<td>density functional theory</td>
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<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
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<tr>
<td>DSMZ</td>
<td>Deutsche Sammlung von Mikroorganismen und Zellkulturen</td>
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<td>dtc</td>
<td>dithiocarbamate</td>
</tr>
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<td>dtl</td>
<td>dithiolate</td>
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<tr>
<td>EPR</td>
<td>electron paramagnetic resonance</td>
</tr>
<tr>
<td>HEPES</td>
<td>2-[4-(2-hydroxyethyl)piperazine-1-yl]ethanesulfonic acid</td>
</tr>
<tr>
<td>ICP-MS</td>
<td>inductively coupled plasma-mass spectrometry</td>
</tr>
<tr>
<td>MALDI-TOF</td>
<td>matrix-assisted laser desorption/ionization time of flight analysis</td>
</tr>
<tr>
<td>MGD</td>
<td>molybdopterin-guanine-dinucleotide</td>
</tr>
<tr>
<td>MPD</td>
<td>2-methyl-2,4-pentanediol</td>
</tr>
<tr>
<td>NAD^+/NADH</td>
<td>β-nicotinamide adenine dinucleotide (oxidized/reduced)</td>
</tr>
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<td>NarG</td>
<td>nitrate reductase G subunit from <em>E. coli</em></td>
</tr>
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<td>NarG-AH</td>
<td>acetylene hydratase with a fused chaperone binding sequence from NarG</td>
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<td>PDB</td>
<td>Protein Data Bank</td>
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<tr>
<td>PEG</td>
<td>polyethylene glycol</td>
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<tr>
<td>SDS-PAGE</td>
<td>sodium-dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>U</td>
<td>unit; 1 U = 1 μmol·min^{-1}</td>
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<tr>
<td>UV/Vis</td>
<td>ultraviolet/visible</td>
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<tr>
<td>v/v</td>
<td>volume per volume</td>
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References

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