Exploring the Active Site of the Tungsten, Iron-Sulfur Enzyme Acetylene Hydratase

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The soluble tungsten, iron-sulfur enzyme acetylene hydratase (AH) from mesophilic Pelobacter acetylenicus is a member of the dimethyl sulfoxide (DMSO) reductase family. It stands out from its class as it catalyzes a nonredox reaction, the addition of H₂O to acetylene (H=C≡C−H) to form acetaldehyde (CH₃CHO). Published ahead of print on 30 December 2010.

Molybdenum and tungsten are the only transition metals of the second (Mo) and third (W) row of the periodic table of elements with known biological functions (7). In their biologically active form, both metals are bound to the cofactor molybdopterin (Moco), which is present in all molybdenum and tungsten enzymes with the exception of nitrogenase, where molybdenum is coordinated to a large iron-sulfur cluster, MoFe₇S₉ (9). Virtually all organisms including plants and mammals use either molybdenum or tungsten proteins in important metabolic pathways (35). Microorganisms carry a wide variety of molybdenum enzymes, such as nitrate reductase (NAR), formate dehydrogenase (FDH), dimethyl sulfoxide reductase (DMSOR), or trimethylamine-N-oxide reductase (TMAOR) (7). These enzymes are involved in either oxygen atom transfer reactions or in reductive hydroxylations. By this means, the metal shuttles between the oxidation states +IV and +VI (16). Notably, the tungsten, iron-sulfur enzyme acetylene hydratase ([AH] EC 4.2.1.112), isolated from the soluble fraction of the mesophilic anaerobe Pelobacter acetylenicus, is an exception (26). It catalyzes the hydration of acetylene to acetaldehyde via an enol intermediate as an initial step for the fermentation of acetylene by Pelobacter acetylenicus, clearly a nonredox reaction (equation 1):

\[
\begin{align*}
\text{H} & \rightarrow \text{C} & \rightarrow \text{H} & \rightarrow \text{H}_2\text{O} \\
& + \text{H}_2\text{O} & \rightarrow [\text{H}_2\text{C} & \rightarrow \text{CHOH}] & \rightarrow \text{H}_2\text{C} & \rightarrow \text{CHO} \\
\text{(ΔG° & = & -111.9 KJ mol}^{-1})
\end{align*}
\]

(1)

Except for nitrogenase, which reduces acetylene to ethylene (H₂C=CH₂), AH is the only enzyme known to accept acetylene as a substrate. However, acetylene is well known to act as an inhibitor for numerous metal-dependent enzymes (10). AH is a member of the DMSOR family and carries one [4Fe-4S] cluster and two molybdopterin-guanosine-dinucleotide (referred to as P- and Q-MGD) ligands that coordinate the tungsten atom (Fig. 1) (18). The enzyme is sensitive toward dioxygen, and its [4Fe-4S] cluster is converted to a truncated [3Fe-4S] cluster upon exposure to air, as shown by electron paramagnetic resonance (EPR) (18). In AH prepared under the exclusion of dioxygen, the EPR signal of the [3Fe-4S] cluster was absent, and reaction with sodium dithionite led to a rhombic EPR signal (gₓ of 2.048, gᵧ of 1.939, and gₜ of 1.920) originating from a [4Fe-4S]⁺ cluster. Upon oxidation with hexacyanoferrate(III), a new signal appeared (gₓ of 2.007, gᵧ of 2.019, and gₜ of 2.048; average g value [gav] of 2.022), which was assigned to a W(V) center (18).

For catalytic activity, AH requires a strong reductant, such as sodium dithionite or titanium(III) citrate (18). Recently, the X-ray structure of AH in the reduced state could be solved at 1.26-Å resolution (28) which gave a first view of its active site: W(IV) is coordinated by four sulfur atoms delivered by the two dithiolene ligands (MGD), one cysteinyI sulfur (Cys141), and one oxygen ligand at a distance of 2.04 Å (Fig. 1). Mechanistically, the nature of this oxygen ligand is critical. The observed W-O distance of 2.04 Å is right between the values expected for a hydroxide ligand (1.9 to 2.1 Å) and a coordinated water (2.0 to 2.3 Å), thus not allowing an unequivocal assignment of the sixth ligand of the WS₃O core. Two different reaction mechanisms have been proposed: (i) nucleophilic attack of the hydroxide group and (ii) electrophilic attack of a polarized water molecule, on the C,C triple bond of acetylene (28). As a consequence of theoretical calculations, and in agreement with the observed bond distances, the active W(IV) state should favor a water ligand and therefore an electrophilic addition.

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Acetylene hydratase (AH) is suggested to be important for catalysis at the active site of the nitrate reductase NarG, from the bacterium Pelobacter acetylenicus. To better understand the reaction mechanism of AH, a study was carried out by site-directed mutagenesis to exchange several amino acids which have been suggested to be important for catalysis at the active site of the enzyme. The goal was to develop a suitable procedure for the heterologous expression of AH in Escherichia coli. Notably, E. coli uses a chaperone system for the insertion of Moco into its enzymes. The first experiments were carried out with E. coli JM109 (Stratagene) for expression, using the Rosetta (DE3) system.

Expression of AH was carried out using 1-liter cultures of E. coli Rosetta (DE3) in anaerobic mineral medium (100 mM KPi, 0 mM H4Cl, 2 mM MgCl2, and 0.5 g/liter protein hydrolysate), supplemented with 1 ml/liter SL10 (33), 10 mM Na2WO4, 1 mM Na2S, 1 ml/liter seven-vitamin solution (33), 15 mg/ml kanamycin sulfate, and 17 mg/ml chloramphenicol. Glyceral (0.5%) was used as a carbon source, and 50 mM Na-fumarate was used as an electron acceptor.

The 108-bp N-terminal chaperone binding site of the AH gene was amplified by high-fidelity PCR from genomic DNA of P. acetylenicus strain WoAcy1 (DSMZ 3246) and was cloned into the NheI/XhoI restriction sites of the pET24a(+) vector by Trenzyme GmbH and ligated into the NdeI/NheI restriction sites of the pPET24a(+) vector already containing the AH gene. The resulting vector was called pPET24_AH.

To gain further information about the reaction mechanism of AH, a study was carried out by site-directed mutagenesis and exchanged several amino acids which have been suggested to be important for catalysis at the active site of the enzyme. The first experiments were carried out with E. coli JM109 (Stratagene) for expression, using the Rosetta (DE3) system.

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Materials and Methods

Bacterial strains, plasmids, and cultivation. Pelobacter acetylenicus strain WoAcy1 (DSMZ 3246) was grown in freshwater mineral medium at 30°C, as described previously (24). E. coli JM109 (Stratagene) was used for plasmid proliferation, and E. coli Rosetta (DE3) (Novagen) was used for expression of AH; the pPET24a(+) vector was from Novagen. Two-milliliter cultures of E. coli JM109 cells were grown aerobically in DYT medium (16 g/liter tryptone, 10 g/liter yeast extract, and 5 g/liter NaCl), supplemented with 50 mg/ml kanamycin sulfate. Expression of AH was carried out using 1-liter cultures of E. coli Rosetta (DE3) in anaerobic mineral medium (100 mM KPi, 10 mM NH4Cl, 2 mM MgCl2, and 0.5 g/liter protein hydrolysate), supplemented with 1 ml/liter SL10 (33), 10 mM Na2WO4, 1 mM Na2S, 1 ml/liter seven-vitamin solution (33), 15 mg/ml kanamycin sulfate, and 17 mg/ml chloramphenicol. Glyceral (0.5%) was used as a carbon source, and 50 mM Na-fumarate was used as an electron acceptor.

Cloning of the AH gene. The AH gene was amplified by high-fidelity PCR from genomic DNA of P. acetylenicus strain WoAcy1 and ligated into the NheI/XhoI restriction sites of the pPET24a(+) vector by Trenzyme GmbH and ligated into the NdeI/NheI restriction sites of the pPET24a(+) vector already containing the AH gene. The resulting vector was called pPET24_AH.

Addition of NarG chaperone binding site. The 108-bp N-terminal chaperone binding site of the E. coli nitrate reductase NarG was amplified by high-fidelity PCR from genomic DNA of P. acetylenicus strain WoAcy1 and ligated into the NheI/XhoI restriction sites of the pPET24a(+) vector already containing the AH gene. The resulting vector was called pPET24_NarG-AH.

Site-directed mutagenesis. Exchange of single amino acid residues was done by PCR. The mismatch primers are listed in Table S1 in the supplemental material. The vectors pET24_AH and pET24_NarG-AH were used as templates. High-fidelity PCR enzyme mix was obtained from Fermentas; deoxynucleoside triphosphate (dNTP) bundles were from Jena Bioscience. The PCR was performed in a Master cycler gradient thermocycler (Eppendorf). DNA polymerase (0.05 U/μl), 0.2 mM dNTPs, 10× high-fidelity PCR buffer (Fermentas), 2 mM MgCl2, 1 μM primer I, 1 μM primer II, and 0.2 ng/μl template were used in the PCR. After a test restriction with NheI/XhoI, PCR products with the correct restriction pattern were amplified in E. coli JM109. The plasmids were then isolated using a GeneElute Plasmid miniprep kit (Sigma) and sequenced at GATC (Konstanz, Germany). Plasmids with the correct amino acid exchange were then transformed into E. coli Rosetta (DE3) for expression, using the method of Inoue et al. (12).

Expression of AH in E. coli. The first experiments were carried out with E. coli BL21(DE3), E. coli BL21(DE3) pLys, and E. coli Rosetta (DE3) under aerobic conditions, but only insoluble protein was obtained (32). Soluble AH could be obtained by heterologous expression in E. coli Rosetta (DE3) using the medium...
described above; cells carrying the pet24_AH or the pET24_NarG-AH vector grew anaerobically at 37°C to an optical density at 600 nm (OD_{600}) of 1.0 within 2 days (1-liter batch cultures). The cultures were cooled to 25°C, and expression of AH was induced by addition of 100 μM IPTG. For the expression of AH with the chaperone binding site of E. coli NarG, 100 μM NaNO_2 was added to the culture 1 h prior the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to induce the formation of the chaperones. After induction with 100 μM IPTG and 24 h of expression at 25°C, typically 1.5 ± 0.5 g of wet cells/liter was harvested.

### Enzyme purification
AH from *P. acetylenicus* was purified under the exclusion of dioxygen, as described previously (28). Heterologously expressed AH was also purified under the exclusion of dioxygen; cells were disrupted by passage (three times) through a French press at 110 MPa. The soluble and insoluble fractions were separated by ultracentrifugation (100,000 × g). The soluble fraction was subjugated to two steps of ammonium sulfate precipitation (2.0 and 3.2 M). The pellet of the second step was dissolved in 50 mM Tris, pH 8.0, and loaded on a Co^2+^-chelating Sepharose Fast Flow column (Amersham). Bound protein was eluted by applying a pH (8.0 to 7.5) gradient, followed by an imidazole (0 to 500 mM) gradient. AH-containing fractions were identified by SDS-PAGE, pooled, concentrated by ultra-centrifugal filter devices (30-kDa cutoff; Millipore), and loaded on a Superdex 200 gel filtration column (Amersham). Fractions containing pure AH were analyzed by SDS-PAGE, pooled, and concentrated to a final concentration of 10 mg/ml protein.

### SDS-PAGE
Electrophoresis was performed according to Laemmli (17), using 12% gels; proteins were fixed with 12% trichloroacetic acid (1 h) and stained overnight with 0.1% Cooamassie brilliant blue G250, 10% (NH_4)_2SO_4, 20% methanol, and 3% H_3PO_4 (21).

### Protein concentration
Protein was determined by the biocinchonic acid method, with bovine serum albumin as a standard (30).

### Molybdopterin cofactor
Molybdopterin was determined fluorometrically (excitation wavelength \(\lambda_{ex} \), 375 nm; emission wavelength \(\lambda_{em} \), 445 nm) on a Perkin Elmer LS50 luminescence spectrometer (15). Fifty microliters of AH in 50 mM Tris, pH 7.5 (1 to 2 mg/ml), was added to 200 μl of 55 mM KMO_2 in 0.1 M NaOH. The samples were boiled at 100°C for 20 min to oxidize molybdopterin to the fluorescent form A. Excess KMO_2 was precipitated by addition of 700 μl of ethanol (EtOH; 99%). After centrifugation, the fluorescence of the supernatant was measured; commercially available pyrroline-5-carboxylic acid (Fluka) served as a reference.

### Metal analysis
The metals of AH and variants were analyzed by inductively coupled plasma mass spectroscopy (ICP-MS) at the Spurenanalytisches Laboratorium Dr. Baumann (Maxhütte-Haidhof, Germany). Iron, molybdenum, and tungsten were determined in samples from different cultivations and purifications (200-μl samples; 2.5 mg/ml protein).

### CD spectroscopy
The secondary structure elements of AH from *P. acetylenicus* and heterologously expressed AH were compared by circular dichroism (CD) spectroscopy. Spectra were recorded on a J-810 spectropolarimeter (Jasco) in cuvettes of 1.0-mm and 0.1-mm path lengths. Samples were prepared in 10 mM Tris, pH 7.5, with 0.4 mg of protein/ml. The secondary structure elements were calculated for the range 195 to 260 nm, with the program CD Spectra Deconvolution, version 2.1 (3).

### Electron paramagnetic resonance spectroscopy
X-band EPR spectra were recorded with Suprasil quartz tubes (outer diameter of EPR tube \(d_{ext} \), 4 mm; sample volume, 250 μl) on a Bruker Elexys 500 instrument equipped with an ER 049X microwave bridge, a 4122 SHQE cavity (perpendicular mode, 9.38 GHz microwave frequency, 100 kHz modulation frequency, and modulation amplitude of 0.1 to 1 mT), and an Oxford ESR 900 helium cryostat connected to an ITC 503 temperature controller (Oxford Instruments). Spectra were evaluated with the Bruker software and simulated with the program WEPR (20).

### Enzyme activity
The activity of AH and variants was determined in a coupled assay, with alcohol dehydrogenase (ADH) from *Saccharomyces cerevisiae* (18, 24). The assay is based on the formation of acetaldehyde (AH) and the subsequent NADH-dependent reduction of acetaldehyde to ethanol (ADH). Briefly, 960 μl of the reducing buffer [50 mM Tris, pH 7.5, 1.5 mM Tr[II]-citrate] was mixed with 20 μl of 30 mM NADH, 10 μl of 2.000 U/ml ADH, and 10 μl of AH (10 mg/ml) in quartz cuvettes under an N_2/H_2 (94%/6%, vol/vol) atmosphere, and cuvettes were sealed with rubber stoppers. The mixture was incubated for 30 min at 30°C; thereafter, the reaction was started by the addition of 2 ml of acetylene. Oxidation of NADH was measured photometrically at 365 nm, and the activity was calculated using a ε_{340} (NADH) of 3.4 mM⁻¹ cm⁻¹ (36). AH and variants were also tested with ethylene as a substrate, but no activity was found.

### Crystallization of AH and variants
Crystallization experiments were done under exclusion of dioxygen following the protocol developed by Seiffert et al. (28). Briefly, crystallization screens of heterologously expressed AH were performed applying both the sitting- and the hanging-drop vapor diffusion methods. Small crystals began to grow in both cases over a period of 3 to 4 weeks from a solution of E. coli NarG-AH (6.5 to 10 mg/ml) in 5 mM HEPES-NaOH, pH 7.5, containing 7.5 mM Na_2SO_4 as a reductant. For diffraction experiments, crystals were transferred to a cryoprotectant solution containing all substances of the reservoir solution and 20% (vol/vol) 2-methyl-2,4-pentanediol (MPD). After incubation with the cryoprotectant solution, crystals were flash frozen in liquid nitrogen. So far, no suitable X-ray diffraction data could be collected from these crystals, most likely due to their size and irregular shape.

### RESULTS
Several procedures were explored to express AH in *E. coli* under aerobic and anaerobic conditions and to produce active-site variants. The purified proteins have been characterized with regard to the molybdopterin content, the occupancy of the metal sites, and their specific activity, as compiled in Table 1. Activities are reported as μmol of C_2H_2 converted per minute and mg of protein; in addition, activity values have been normalized to the tungsten content (given in parentheses).

### Purification of heterologously expressed AH
The purification of AH heterologously expressed in *E. coli* typically yielded 3.25 ± 0.7 μmol of pure AH/g of wet cells. Compared to a yield of ~0.8 ± 0.1 μg of AH/g of wet cells purified from *P. acetylenicus*, a 4-fold overexpression was achieved in *E. coli*. *E. coli* AH had a specific activity of 2.6 ± 0.8 (43.3) μmol of C_2H_2 min⁻¹ mg⁻¹, which is lower than the value of 14.2 ± 0.9 (38.4) μmol of C_2H_2 min⁻¹ mg⁻¹ found for *P. acetylenicus* AH. However, when normalized to the tungsten content, the activity values were similar (38.4 versus 43.3 μmol of C_2H_2 min⁻¹ mg⁻¹) (Table 1).
Purification of the heterologously expressed AH with the NarG chaperone binding site. Addition of the N-terminal chaperone binding site of the *E. coli* nitrate reductase NarG increased the yield of AH to \(\approx 4.2 \pm 0.3\) mg/g of wet cells. Notably, the specific activity of the recombinant enzyme also increased significantly to 9.7 \(\pm 1.9\) (69.3) \(\mu\)mol of \(\text{C}_2\text{H}_2\) min\(^{-1}\) mg\(^{-1}\) (Table 1).

The homogeneity of the individual preparations was checked by SDS-PAGE (Fig. 2), and protein folding was controlled by CD spectroscopy as discussed below. None of the protein samples purified under the exclusion of dioxygen showed any EPR signal; incubation with sodium dithionite led to the appearance of the rhombic EPR signal described earlier \((g_i, g_h, g_h = 2.048, 1.939, 1.920)\) originating from the iron-sulfur cluster in the \([4\text{Fe}-4\text{S}]^+\) redox state (data not shown) (18, 24, 27).

The N-terminal chaperone binding site of *E. coli* TMAO reductase, TorA, was also cloned in frame into the AH expression vector in front of the AH gene, as described for the NarG sequence, producing TorA-AH (32). The protein could be purified to homogeneity and was active; however, the yield of TorA-AH was significantly lower than that of NarG-AH. Consequently, investigation of this expression system was not pursued (32).

**Molybdopterin cofactor in heterologously expressed AH.** The enzyme isolated from *P. acetylenicus* carries two MGD ligands/mol of enzyme, as documented in the crystal structure (Fig. 1) (28). Fluorimetric analysis of standard preparations of wild-type AH used in this work and for the crystallization of the enzyme could not be achieved for AH as isolated (18). Re-combinant AH from *E. coli* contained 0.17 \pm 0.08 mol of MGD; fusion of the NarG chaperone binding site increased the content of MGD to 0.31 \pm 0.09 mol per mol of enzyme, corresponding to 33% of the value found in standard preparations of wild-type AH (Table 1).

**Metals in heterologously expressed AH and variants.** The content of metals in AH from *P. acetylenicus* and protein heterologously expressed in *E. coli* was determined by ICP-MS. Typically, standard preparations of wild-type AH contained 3.69 \pm 0.04 mol of Fe and 0.37 \pm 0.03 mol of W/mol of AH (ratio of MGD/W, 2.54). The metal content of recombinant AH from *E. coli* was lower, 1.22 \pm 0.26 mol of Fe and 0.06 \pm 0.02 mol of W/mol of AH. However, after attachment of the N-terminal chaperone site of NarG, the content of iron increased to 3.17 \pm 0.49 mol of Fe/mol of AH, representing 86% of the value determined for AH purified from *P. acetylenicus*. In line with this result, the content of tungsten increased to 0.14 \pm 0.06 mol of W/mol of AH (ratio of MGD/W of 2.14) (Table 1). Molybdenum was absent in all samples.

**Control of protein folding.** One measure for the quality of heterologously expressed AH and variants is the proper folding of the protein, which can be checked by CD spectroscopy (Fig. 3). With regard to the secondary structure elements, heterologously expressed AH proteins exhibited a slightly lower content of antiparallel \(\beta\)-sheets than the native *P. acetylenicus* enzyme. On the other hand, the recombinant proteins had a slightly higher content of \(\alpha\)-helices. The only region with antiparallel \(\beta\)-sheets within the structure of AH is the N-terminal domain I, which harbors the \([4\text{Fe}-4\text{S}]\) cluster (Fig. 1) (28). Most likely, the minor changes in the secondary structure result from conformational changes induced by the lower occupancy of the metal sites. In the NarG-AH fusion proteins, antiparallel \(\beta\)-sheets were even less abundant, according to CD...
spectroscopy. Here, 30 amino acids were attached to the N-terminal end of the protein. The secondary structure of this tail and its influence on the conformation of domain I are currently not known. However, the increase in iron content indicates that the structure of the [4Fe-4S] cluster has not been disturbed by the addition of the chaperone binding site, which is also supported by the EPR properties described above.

**Site-directed mutagenesis.** Three amino acid residues at the active site of AH were successfully exchanged by site-directed mutagenesis to investigate their functional role in the reaction mechanism of AH. Residue Asp13, which forms a hydrogen bond to the oxygen ligand of the tungsten atom (28), was replaced with glutamate (D13E) and alanine (D13A). Lys48, which has been shown to play a critical role in electron transfer between the [4Fe-4S] cluster and the Mo(MGD)$_2$ center in enzymes of the DMSOR family (5), was replaced by alanine (K48A) (Fig. 4A). Finally, Ile142, a constituent of the hydrophobic ring between the tungsten active site and the substrate channel (Fig. 4B) (28), was successfully replaced with alanine (I142A), whereas attempts to exchange a second residue of the hydrophobic ring, Trp472, remained unsuccessful.

**AH activity of variants.** In both reaction mechanisms based on the crystal structure of AH (28), Asp13 plays a critical role, either by donating a second proton after the nucleophilic attack of the hydroxide group on the acetylene C=C triple bond or by activation of the coordinated water molecule to perform an electrophilic attack (28). In the coupled reaction assay, the activity of the D13A variant was reduced to close to zero, while the D13E variant exhibited nearly the identical activity as the wild-type enzyme, documenting the important role of the carboxylic group of Asp13 in AH catalysis (Table 1).

Lys48 is located between the [4Fe-4S] cluster and the Q-MGD ligand (Fig. 4A). As the reaction of AH does not involve a net electron transfer, it was not too surprising that the K48A variant had practically the same activity as reported for the wild-type enzyme (Table 1).

Ile142 is part of a hydrophobic ring of six bulky amino acid residues (Fig. 4B) (28), forming a small cavity for binding the substrate at the end of the channel. Acetylene placed in this cavity would be positioned directly above the oxygen ligand, in close proximity to both the tungsten atom and Asp13. The activity of the I142A variant amounted to $2.2 \pm 0.2$ $(12.2) \mu$mol of C$_2$H$_2$ min$^{-1}$ mg$^{-1}$ (Table 1). The marked loss of activity upon exchanging Ile142 for alanine supports the idea that the cavity formed by the hydrophobic ring is the substrate binding site of AH.

**FIG. 4.** (A) Active-site structure of acetylene hydratase from *P. acetylenicus* indicating the positions of residues Asp13 and Lys48; W is shown in cyan, and H$_2$O is in red. The radii of the spheres correspond to the covalence radii of the atoms according to http://www.periodensystem.info/. (B) Active-site structure of acetylene hydratase from *P. acetylenicus* indicating the position of residue Ile142; W is shown in cyan, and H$_2$O is in red. The surface of the hydrophobic ring formed by three isoleucine and three tryptophan residues, with one acetylene molecule (in gray) placed into the pocket, is shown in the top figure. In the bottom figure, distances between acetylene and catalytic residues Ile142, Asp13, and the water ligand are shown. The radii of the spheres correspond to the covalence radii of the atoms according to http://www.periodensystem.info/.
Ethylene, in addition to acetylene, was also tested as a substrate for the heterologously expressed AH and variants, and as in the case of wild-type AH ethylene, was not accepted as a substrate.

**DISCUSSION**

The strictly anaerobic bacterium *P. acetylenicus* can grow with acetylene as a single carbon and energy source. The first step in the fermenting pathway is the transformation of acetylene to acetaldehyde, which is consecutively converted to acetate and ethanol. It appears that *P. acetylenicus* conserves only the free energy available in the acetate kinase reaction and not the amount of free energy available from hydration of acetylene (equation 1). Earlier, it was speculated that the conversion of acetylene to acetaldehyde might represent a bifunction of an unspecific hydratase enzyme which mainly acts in the natural environment in detoxification of acetylenic compounds, nitriles, or cyanides (24).

Catalysis of AH is rather peculiar in the sense that two complex metal sites and a strong reductant are required for the addition of one molecule of water to the C≡C bond. Notably, there do exist iron-sulfur proteins that catalyze hydration reactions, with aconitase being among the first discovered examples (2).

**Heterologous expression of AH.** Acetylene hydratase has been found exclusively in the soluble fraction of *P. acetylenicus* (24). Following the procedures originally described for the heterologous expression of *Rhodobacter sphaeroides* DMSO reductase (8), the first attempts were carried out with three strains of *E. coli* under different experimental conditions including the variation of the copper concentration (19). By this means, only insoluble protein was obtained (32). Upon anaerobic cultivation of *E. coli* Rosetta (DE3), with glycerol as a carbon source and sodium fumarate as an electron acceptor, soluble *E. coli* AH could be isolated and purified to homogeneity. The protein was active but exhibited a low content of molybdopterin, iron, and tungsten (Table 1). Notably, the wild-type enzyme purified from *P. acetylenicus* (as isolated under the exclusion of dioxygen) was also always low in molybdopterin and tungsten (1.3 mol of Moco and 0.5 mol of W per mol of AH), whereas the iron content usually reached the theoretical value of 4 mol of Fe per mol of AH (18, 24). In crystalline AH, however, the sites of the two MGD ligands were fully occupied, in contrast to the occupancy of the tungsten site, which remained low (≈40%). Thus, at a resolution of 1.10 to 1.26 Å, the MGD sites with and without tungsten could be clearly differentiated (28; also G. Scifert, P. M. H. Kronec, and O. Einsle, submitted for publication).

Extensive studies on the maturation of Moco-containing enzymes had revealed a family of chaperones that facilitated the incorporation of the cofactor during protein biosynthesis and prevented the export of periplasmic enzymes before its proper insertion (11, 22, 23, 25). Complementation studies indicated that these chaperones were highly specific for their partner and could not complement the absence of another chaperone (11). Amino acid sequence alignments showed that such an N-terminal chaperone binding site was missing in the *P. acetylenicus* AH gene. In order to improve the insertion of cofactors and metals, the first 108 bp of the NarG gene or the first 117 bp of the TorA gene were successfully fused to the AH gene. This operation not only increased the yield of protein in the case of the NarG-AH enzyme but also helped to increase the content of molybdopterin, tungsten, and iron accompanied by a significant increase in activity (Table 1). Earlier, a protocol had been published for the heterologous expression of *R. sphaeroides* DMSO reductase. In contrast to AH, this enzyme carried an N-terminal chaperone binding site which had been removed prior to the expression in *E. coli*. In addition, the binding site TorA had been fused to the DMSOR gene which, in contrast to the experiments with TorA-AH, led to a significant decrease in activity (8).

At this point, the different steps leading to the maturation of the soluble enzyme AH in both *P. acetylenicus* and *E. coli* are not understood and will require further investigations. Obviously, fusion of the AH gene with the N-terminal chaperone binding site of the *E. coli* nitrate reductase NarG improved the quality of the protein. This finding suggests that the main function of the chaperone is to keep the protein unfolded for a longer time period and therefore extend the time frame for cofactor assembly and metal insertion during biosynthesis of AH. To get more information about this issue, experiments with radioactive metal isotopes are planned (29).

**Substrate specificity.** As the hydration of acetylene depends on tungsten and as most tungsten enzymes described to date have been purified from strictly anaerobic, thermophilic, or extremely thermophilic bacteria, one might speculate that metabolism of acetylene represents an early form of life (7). However, *P. acetylenicus* is a mesophilic organism, and the temperature optimum of AH has been determined to be 55°C. Furthermore, a molybdemenum-dependent active form of AH could be obtained from *P. acetylenicus* cultivated on molybdate (2 µM) in the presence of nanomolar concentrations of tungstate (18), as reported for DMSOR from *Rhodobacter capsulatus* (31). Attempts to replace tungsten with vanadium, however, have thus far failed (1).

AH is highly specific toward its substrate acetylene as no other substrates have been found to date. Our search included ethylene and derivatives of acetylene (propargyl alcohol and acetylene mono- and dicarboxylic acid), cyanide, nitriles, and isonitriles. In summary, a possible physiological function of AH beyond the conversion of acetylene to acetaldehyde cannot be defined at present.

**Active-site access, active-site architecture, and reaction mechanism.** In the structures of proteins of the DMSO reductase family available to date, access to the active center is provided through a funnel-like entrance whose position is conserved in enzymes such as DMSO and TMAO reductases, as well as in formate and nitrate reductases. In AH, however, this entire region has been completely rearranged. The substrate acetylene must approach the tungsten site from a different angle through a funnel close to the N-terminal domain that harbors the [4Fe-4S] cluster (28). Above the tungsten ion, the substrate funnel ends in a ring of six hydrophobic residues (three Ile and three Trp) which form the substrate cavity. Through shape complementarity, the residues of the hydrophobic ring are a key determinant for the enzyme’s substrate specificity (Fig. 4B). Numerous attempts to pressurize crystals of AH with acetylene, ethylene, carbon monoxide, nitric oxide, or dinitrogen, as well as soaking of crystals of AH with different
compounds including the inhibitor propargyl alcohol, have failed thus far to produce a crystalline substrate complex of AH. With xenon gas, one Xe atom could be trapped in the funnel but not, however, in bonding distance to the tungsten center (27, 32).

The formation of acetaldehyde is accomplished by activation of a water molecule bound to a W(IV) ion interacting with residue Asp13 and the [4Fe-4S] cluster, one of whose ligands, Cys12, is an immediate neighbor of Asp13. Usually, the [4Fe-4S] cluster is involved in electron transfer in enzymes of the DMSOR family, as Lys48 is considered to be essential for electron transfer from the iron-sulfur cluster to the Q-MGD ligand (5). In AH, however, the active site is found at a different side of the tungsten ion, closer to the [4Fe-4S] cluster. Furthermore, in the structure of AH, the conserved water molecule is missing (27), which has been assumed to be a crucial component of the electron transfer pathway (reference 14 and Fig. 5 therein). Thus, the tungsten center remains in the W(IV) state during catalysis, and electron transfer does not occur. Instead, a significant increase in pKₐ for Asp13 is caused by the desolvation of this residue, and the [4Fe-4S] cluster appears to push electrons toward Asp13 and thus helps to increase its proton affinity (28). The increased specific activity of AH under reducing conditions is in part explained by this finding, as the shift in pKₐ and the degree of activation of the water ligand will be stronger in a cluster in the reduced [4Fe-4S]⁺² state (28). Recent redox titrations of the iron-sulfur cluster in P. acetylenicus AH gave a midpoint potential E₅₀ of −410 mV ± 20 mV (Nernst coefficient n = 1), and the enzyme activity depended on the potential of the solution, with 50% of the catalytically active W(IV) state, its replacement by alanine did not affect the acetylene hydratase activity of this enzyme (27, 32).

Second, as catalysis does not include an electron transfer between the two metal centers, replacing residue Lys48 with alanine did not affect the acetylene hydratase activity of this variant as expected (Table 1). Cys46 (3.27 Å) ligates the [4Fe-4S] cluster, and Q-MGD (2.94 Å) are the closest neighbors of Lys48 (Fig. 4A). Replacement of the positively charged Lys48 residue with the neutral alanine is expected to influence the reduction potential of the [4Fe-4S] cluster; however, its value will still be quite negative (4).

Third, in view of the strongly reduced activity of the I142A variant, a more detailed picture of the mode of substrate bind-


