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Purification and Characterization of Acetylene Hydratase of *Pelobacter acetylenicus*, a Tungsten Iron-Sulfur Protein

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Acetylene hydratase of the mesophilic fermenting bacterium *Pelobacter acetylenicus* catalyzes the hydration of acetylene to acetaldehyde. Growth of *P. acetylenicus* with acetylene and specific acetylene hydratase activity depended on tungstate or, to a lower degree, molybdate supply in the medium. The specific enzyme activity in cell extract was highest after growth in the presence of tungstate. Enzyme activity was stable even after prolonged storage of the cell extract or of the purified protein under air. However, enzyme activity could be measured only in the presence of a strong reducing agent such as titanium(III) citrate or dithionite. The enzyme was purified 240-fold by ammonium sulfate precipitation, anion-exchange chromatography, size exclusion chromatography, and a second anion-exchange chromatography step, with a yield of 36%. The protein was a monomer with an apparent molecular mass of 73 kDa, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The isoelectric point was at pH 4.2. Per mol of enzyme, 4.8 mol of iron, 3.9 mol of acid-labile sulfur, and 0.4 mol of tungsten, but no molybdenum, were detected. The K_m for acetylene as assayed in a coupled photometric test with yeast alcohol dehydrogenase and NADH was 14 μM, and the V_{max} was 69 μmol · min^{-1} · mg of protein^{-1}. The optimum temperature for activity was 50°C, and the apparent pH optimum was 6.0 to 6.5. The N-terminal amino acid sequence gave no indication of resemblance to any enzyme protein described so far.

The strictly anaerobic fermenting bacterium *Pelobacter acetylenicus* converts the unsaturated hydrocarbon ethine (trivial name, acetylene) to acetate and ethanol via acetaldehyde as an intermediate (36). The first step of the fermentation pathway, the hydration of acetylene to acetaldehyde, is catalyzed by the enzyme acetylene hydratase. The reaction is highly exergonic: $C_2H_2 + H_2O \rightarrow CH_3CHO$ ($\Delta G^{\circ\prime} = -111.9$ kJ/mol) (36). Until recently, attempts to demonstrate this enzyme activity in cell extracts of *P. acetylenicus* have failed (27, 36). Also the aerobic acetylene-degrading bacteria *Mycobacterium lacticola*, *Nocardia rhodochrous*, *Rhodococcus* strain A1, and *Rhodococcus rhodochrous* have been reported to convert acetylene to acetaldehyde (7, 16, 20, 25); the reaction was proposed to be catalyzed by an acetylene hydratase activity. Yet acetylene hydratase activity could be demonstrated in cell extracts of *Rhodococcus* strain A1 only when the assay was performed under anoxic conditions (16). Therefore, acetylene appears to be the only hydrocarbon that is converted in the presence and absence of molecular oxygen by the same type of enzyme (36).

Tungsten-containing enzymes have been reported so far to catalyze redox reactions of a low reduction potential ($E_0' < -400$ mV), such as those involving formate dehydrogenase of *Clostridium thermoaceticum* (51) and *Clostridium formicoaceticum* (13), aldehyde ferredoxin oxidoreductase of *Pyrococcus furiosus* (30), formaldehyde ferredoxin oxidoreductase of *Thermococcus litoralis* (31), carbonic acid reductase of *C. thermoaceticum* (42) and *C. formicoaceticum* (41), and formylmethanofuran dehydrogenase of *Methanobacterium wolfei* (38) and *Methanobacterium thermoautotrophicum* (6). Formylmethanofuran dehydrogenase of *M. wolfei* and *M. thermoautotrophicum*, aldehyde ferredoxin oxidoreductase of *P. furiosus*, and formaldehyde ferredoxin oxidoreductase of *T. litoralis* have been shown to contain a tungsten pterin cofactor analogous to the molybdopterin cofactor found in most molybdenum-containing enzymes (6, 11, 24, 38). All known tungsten-containing enzymes are iron-sulfur proteins (6, 15, 30, 31, 41, 43). Most tungsten enzymes described to date have been purified from strictly anaerobic, thermophilic or extremely thermophilic bacteria, which are supposed to have been among the earliest forms of life on earth (46, 47). It has therefore been speculated that the first forms of life were not only thermophilic but also tungsten dependent (1, 2, 6). However, a tungsten-containing formate dehydrogenase has been purified also from the mesophilic bacterium *C. formicoaceticum* (13). There is evidence for tungsten-containing enzymes in other mesophilic anaerobic bacteria as well, e.g., in *Desulfovibrio gigas* and other sulfate-reducing bacteria (22), and even in aerobic bacteria (21, 26).

Here we report on a tungsten iron-sulfur protein purified from a mesophilic bacterium that catalyzes a net hydration reaction rather than a redox reaction.

MATERIALS AND METHODS

Materials. Alcohol dehydrogenase (from *Saccharomyces cerevisiae* cells) was purchased from Boehringer Mannheim (Mannheim, Germany). Tungstate (Na_2WO_4; purity, >99%) and molybdate (Na_2MoO_4; purity, >99%) were obtained from Fluka (Neu-Ulm, Germany). Tungstate contained at a maximum 0.005% molybdate. Acetylene (purity, 99.6%) and other gases were from Sauerstoffwerk (Friedrichshafen, Germany). Fast protein liquid chromatography columns were from Pharmacia (Freiburg, Germany).

Bacterial strains and growth conditions. *P. acetylenicus* WoAcyl (DSM 3246) was obtained from the Deutsche Sammlung von Mikroorganismen (Braunschweig, Germany).

P. acetylenicus was grown in 1,200-ml glass infusion bottles (Müller & Krempel, Bülach, Switzerland) filled with 1 liter of sulfide-reduced bicarbonate-buffered mineral salts medium and sealed with rubber septa or in 12-liter glass bottles filled with 10 liters of medium as described previously (36). Acetylene was added to the gas phase with gas-tight glass syringes through butyl rubber septa in the culture vessels to provide a concentration of dissolved acetylene of 7 to 10 mM. For mass cultivation of *P. acetylenicus* in 12-liter bottles, acetylene was added daily for 3 days. Other substrates were added from filter-sterilized stock solutions. Medium without tungstate or molybdate was prepared by omitting tungstate and molybdate from the selenite-tungstate solution (45) and the trace

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element solution SL 10 (45). Growth was monitored by measuring the A_{578} of the culture. Cells were harvested in the exponential growth phase at an A_{578} of 0.13 to 0.17. Small volumes were harvested by centrifugation at 13,000 × *g* for 30 min at 4°C. Cells from 10-liter cultures were concentrated with a Pellicon filter cassette system with an exclusion size of 100 kDa (Millipore, Eschborn, Germany) to 2 liters and then harvested by centrifugation as described above. The cell pellet was washed once in buffer appropriate for the particular experiment and stored at −20°C.

Purification of acetylene hydratase. All purification steps were performed under air at room temperature. For preparation of cell extract, the cell pellet was suspended in 20 mM Tris-HCl, pH 7.5, containing 60 μg of DNase I (Boehringer Mannheim) per ml and 1 mM $MgCl_2$. Cells were disrupted by three passages through a French pressure cell (Aminco, Silver Spring, Md.) at 136 MPa. Cell debris was removed by centrifugation at 30,000 × *g* for 15 min. The supernatant contained 20 mg of protein per ml. In a two-step ammonium sulfate fractionation, enzyme activity precipitated between 40 and 75% ammonium sulfate saturation. The pellet was resuspended in 20 mM Tris-HCl, pH 7.5 (buffer A), and dialyzed for 12 h against buffer A. The sample was applied to a Mono Q column (HR 10/10) equilibrated with buffer A. The column was washed (4 ml/min) with 24 ml of buffer A before an ascending linear gradient of 0 to 500 mM NaCl in buffer A was applied. Enzyme activity eluted as a single peak with 330 mM NaCl. Active fractions were combined, concentrated by ultrafiltration (YM 10 membrane; Amicon, Witten, Germany), and further purified on a Superdex 200 HiLoad column (1.6 by 60 cm) equilibrated with buffer A containing 300 mM NaCl, at a flow rate of 0.5 ml/min. In a further purification step, the active fractions were applied again to a Mono Q column (HR 10/10) equilibrated with buffer A. Enzyme activity was eluted with an ascending linear NaCl gradient as described above. The flow rate was reduced to 1 ml/min. Enzyme activity eluted as a single peak with 380 mM NaCl. Active fractions were combined, concentrated by ultrafiltration (Centriprep-10 microconcentrators; Amicon), and stored at −20°C until further use.

Determination of enzyme activities. Acetylene hydratase activity was determined under anoxic conditions in a photometric assay at 30°C. The reaction product acetaldehyde was converted to ethanol by yeast alcohol dehydrogenase, and NADH oxidation was monitored at 340 nm in glass cuvettes (light path, 1 cm) sealed with latex stoppers and evacuated and flushed with nitrogen at least six times before use. The assay mixture (1 ml) contained anoxic 50 mM potassium phosphate buffer, pH 7.0, with 2 mM titanium(III) citrate, 0.2 mM NADH, 20 U of yeast alcohol dehydrogenase, and 6 to 200 μg of protein. The protein was incubated in the assay mixture for 20 min before the reaction was started by addition of gaseous acetylene to a dissolved concentration of 20 mM. Solutions and acetylene gas were added with gas-tight syringes (Unimetrics; Macherey-Nagel, Düren, Germany).

For inhibition studies, the enzyme was preincubated in 50 mM potassium phosphate buffer, pH 7.0, with 2 mM titanium(III) citrate or dithionite for 20 min. The possibly inhibiting agent was added, and the enzyme was incubated in its presence for 5 to 20 min. Immediately before the reaction was started with acetylene, NADH and yeast alcohol dehydrogenase were added. Controls were run with 1 to 5 mM acetaldehyde as a substrate. The effect of iron-chelating agents on acetylene hydratase activity was checked for by 20-min incubation of the enzyme in phosphate buffer with dithionite in the presence of citrate (1 mM), ascorbate (1, 2, or 5 mM), tiron (1, 2, or 5 mM), ferrocene (0.2 mM), or EDTA (5 or 10 mM). With $HgCl_2$ as the possible inhibitor, the assay was performed in phosphate buffer with titanium(III) citrate. The enzyme was incubated for 5 min in the presence of 0.01, 0.1, or 0.2 mM $HgCl_2$. The influence of potassium cyanide on acetylene hydratase activity was investigated by incubating the enzyme in phosphate buffer with dithionite for 15 min in the presence of 1, 5, or 10 mM KCN. Carbon monoxide was bubbled through the assay mixture for 3 to 5 min before the reaction was started by addition of NADH, alcohol dehydrogenase, and acetylene. Nitric oxide was added to the mixture to a concentration of 3 mM, and ethylene was added to a concentration of 8 mM. After an incubation time of 5 min, NADH, alcohol dehydrogenase, and acetylene were added. Concentrations of dissolved gases were calculated according to their solubility coefficients (14). A possible conversion of ethylene to acetaldehyde was checked for in an assay mixture containing 50 mM potassium phosphate buffer, pH 7.0, with 0.12 mM titanium(III) citrate. As an electron acceptor, 0.25 mM methylene blue or 0.5 mM anthraquinone disulfonate was added, and the reaction was started by addition of 8 mM ethylene. Reduction of the electron acceptor was measured at 570 and 406 nm, respectively. Formation of acetaldehyde was checked for with yeast alcohol dehydrogenase and NADH.

Acetaldehyde dehydrogenase activity (coenzyme A acetylating) was measured anoxically in an assay modified from that of Odom and Peck (33). The assay mixture (1 ml) contained 100 mM Tris-HCl, pH 7.4, with 2.5 mM dithioerythritol, 0.5 mM coenzyme A, 20 mM acetaldehyde, 5 mM benzyl viologen, 0.2 mM dithionite, and cell extract (150 to 300 μg of protein) or purified acetylene hydratase (7 μg of protein). The reduction of benzyl viologen was monitored at 578 nm (ε for benzyl viologen = 8.6 $mM^{-1} \cdot cm^{-1}$ [29]). The reaction was started by addition of acetaldehyde.

Cell extracts were tested for cyanide hydratase activity under anoxic conditions in a discontinuous assay modified from those of Schygulla-Banek (39) and Ingvorsen et al. (23). The assay mixture (1 ml) contained 50 mM potassium phosphate buffer, pH 7.0, with 2 mM titanium(III) citrate or 1 mM dithiothreitol, cell extract (1.5 mg of protein), and 1 mM potassium cyanide. Samples (10 μl) were taken with syringes at defined time intervals. The cyanide content of the sample was determined in a colorimetric assay as described below.

Cell extracts were tested for nitrile hydratase activity in a discontinuous assay under anoxic conditions. The assay mixture (2 ml) contained 50 mM potassium phosphate buffer, pH 7.0, with 2 mM titanium(III) citrate, cell extract (0.3 mg of protein), and 5 mM acetonitrile or propionitrile. Samples (80 μl) were taken with syringes at defined time intervals. Concentrations of acetonitrile or propionitrile and of the possible reaction products acetamide, propionamide, acetate, and propionate were determined by gas chromatography as described below.

Acetylene monocarboxylate and acetylene dicarboxylate hydratase activities of cell extracts were determined in anoxic photometric assays modified from those in references 49 and 50. Oxaloacetate as a possible reaction product of acetylene dicarboxylate activity (49) was tested for with malate dehydrogenase (from porcine heart tissue) instead of lactate dehydrogenase and with 2 mM acetylene dicarboxylate as a substrate.

Analytical methods. The purified enzyme was analyzed for transition metals by inductively coupled plasma mass spectrometry (ICP-MS) with indium as the internal standard by using a VG Plasmaquad II (VG Elemental, Winsford, England). For quantitative determination of iron and molybdenum contents, the purified protein was analyzed by atomic absorption spectrometry performed with a 3030-B atomic absorption spectrometer fitted with an HGA-600 graphite furnace assembly (Perkin-Elmer, Überlingen, Germany). For determination of iron content, the protein was diluted to a concentration of 13 μg/ml with metal-free water. Subsamples (5 μl) were injected, dried for 20 s at 90°C, charred for 12 s at 1,100°C, and atomized for 1 s at 2,000°C. $A_{248.3}$ was measured. The iron content of the sample was calculated from a calibration curve of external standards (0 to 80 μg of iron per ml) according to peak height. For determination of molybdenum content, a 50-μl sample was injected, dried for 40 s at 100°C, charred for 20 s at 1,600°C, and atomized for 3 s at 2,650°C. $A_{313.3}$ was measured.

Acid-labile sulfur content was measured according to the method of Beinert (3). The assay was modified as described before (35).

Protein content was determined by the method of Bradford (8) with bovine serum albumin as the standard.

Cyanide was measured in a colorimetric assay with chloramine T and barbituric acid as described previously (17). Cyanide content determination was not influenced by components of the cell extract or by titanium(III) citrate present in the cyanide hydratase activity assay.

Acetate and propionate were analyzed by gas chromatography as previously described (34). Samples (50 μl) were acidified with 2.5 μl of 10 M formic acid before injection. Acetonitrile, propionitrile, acetamide, and propionamide were analyzed with a gas chromatograph (VEGA 6000; Carlo Erba, Milan, Italy) equipped with a flame ionization detector and a packed glass column (2 mm by 1.2 m) containing Porapak QS (80 to 100 mesh). Operating conditions of the gas chromatograph were as follows: oven temperature, 200°C; injector and detector temperature, 220°C; N_2 carrier gas at 40 ml min^{-1}. Two microliters of sample was injected.

Analysis of the N-terminal amino acid sequence. The amino acid sequence was analyzed after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and subsequent transfer of the protein onto a polyvinylidene difluoride membrane (Immobilon P; Millipore). The 73-kDa protein band was cut out of the membrane and sequenced in a type 477 A protein sequencer (Applied Biosystems, Weiterstadt, Germany). For detection of cysteine residues, the protein was denatured for 2 min at 80°C in 20 mM borate buffer, pH 8.2, containing 0.1% (wt/vol) SDS and 0.1 mM EDTA and then the cysteine residues were reduced with dithiothreitol and modified with vinylpyridine. After SDS-PAGE, the protein was transferred onto the membrane and sequenced as described above.

RESULTS

Cell extracts of *P. acetylenicus* cells grown in mineral salts medium with 7.3 mM acetylene as the sole source of carbon and energy had a specific acetylene hydratase activity of 0.75 $\mu mol \cdot min^{-1} \cdot mg\ of\ protein^{-1}$. Specific acetylene hydratase activity was 0.30 $\mu mol \cdot min^{-1} \cdot mg\ of\ protein^{-1}$ after growth with 10 mM glycerol plus 1 mM acetate and 0.10 $\mu mol \cdot min^{-1} \cdot mg\ of\ protein^{-1}$ after growth with 10 mM acetoin.

Growth of *P. acetylenicus* and specific enzyme activity depended on addition of tungstate or molybdate to the growth medium. In medium free of added tungstate and molybdate, growth was slow. After addition of 0.1 μM tungstate or 0.1 μM molybdate, logarithmic growth started immediately, whereas after addition of tungstate plus molybdate (0.1 μM each), logarithmic growth started only after a lag phase of 7 h. The highest cell density reached with both transition metals was two times higher than the highest cell density reached after addition of either tungstate or molybdate alone (Fig. 1). With

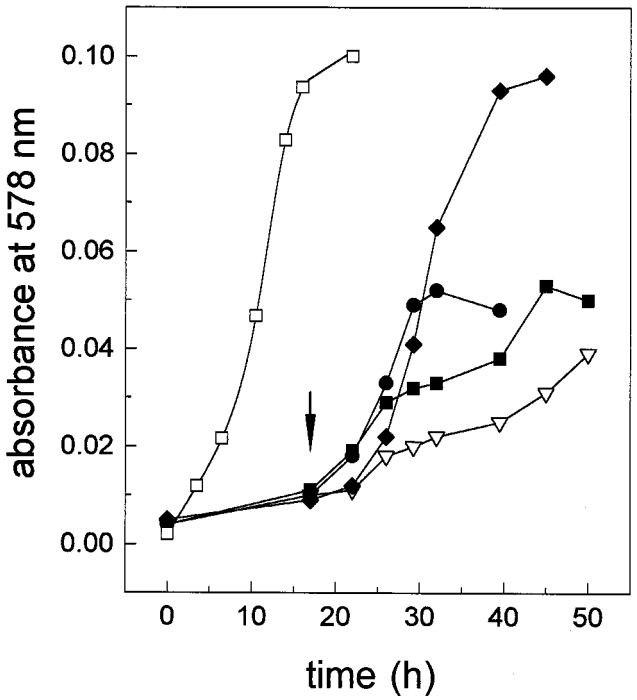

FIG. 1. Growth of *P. acetylenicus* with acetylene in mineral salts medium (36) with tungstate and molybdate omitted. After 17 h, 0.1 μM tungstate (●), 0.1 μM molybdate (■), or 0.1 μM tungstate plus 0.1 μM molybdate (◆) was added (indicated by arrow). No tungstate or molybdate was added to the control (▽). Growth of *P. acetylenicus* in standard mineral medium is depicted as well (□).

tungstate and molybdate omitted from the medium, the specific acetylene hydratase activity was low. Enzyme activity was highest after addition of tungstate alone. The presence of molybdate in addition to tungstate in the medium decreased the specific activity (Table 1).

Determination of acetylene hydratase activity. Acetylene hydratase activity could be measured only in the presence of a strong reducing agent such as titanium(III) citrate ($E_0' = -480$ mV [52]) or dithionite ($E_0' = -527$ mV [28]). No enzyme activity was detected in assays containing dithiothreitol or dithioerythritol ($E_0' = -330$ mV [28]), cysteine ($E_0' = -340$ mV [28]), sulfide ($E_0' = -243$ mV [12, 28]), or sodium ascorbate ($E_0' = +58$ mV [28]) as reducing agents. Enzyme activity was not detectable in assays free of a reducing agent; assays without a reducing agent in the presence of an oxygen-scavenging system consisting of glucose oxidase (20 U), glucose (2 mM), and catalase (0.5 U); or assays in the presence of air.

TABLE 1. Specific acetylene hydratase activity in cell extracts of *P. acetylenicus* after growth under various culture conditions[a]

Culture conditions	Sp act ($\mu mol \cdot min^{-1} \cdot mg\ of\ protein^{-1}$)
No W or Mo added	0.06
100 nM Mo	0.30
100 nM W	2.30
100 nM Mo plus 100 nM W	1.20
150 nM Mo plus 12 nM W	0.75

[a] Cells were grown with 7.3 mM acetylene as the sole source of carbon and energy with tungstate and molybdate omitted from the medium. After 17 h, tungstate, molybdate, or both (100 nM each) were added to the medium. Specific enzyme activity after growth of cells in standard mineral salts medium is listed as well. W, tungstate (Na_2WO_4); Mo, molybdate (Na_2MoO_4).

TABLE 2. Purification of acetylene hydratase of *P. acetylenicus*[a]

Fraction	Amt of protein (mg)	Activity (U)	Sp act (U/mg)	Purification (fold)	Yield (%)
Cell extract	2,950	845	0.29	1	100
$(NH_4)_2SO_4^-$ Precipitate	345	562	1.63	5.6	66
Mono Q (I)	52	511	9.85	40.0	60
Superdex 200	14	(613)	(44.70)	(154.1)	(73)
Mono Q (II)	4	306	69.20	238.7	36

[a] Cell extract was prepared from 80 g (wet mass) of cells suspended in 20 mM Tris-HCl, pH 7.5, that had been harvested from 200 liters of acetylene-grown culture by filtration as described in Materials and Methods. Cells were disrupted by three passages through a French pressure cell in the presence of DNase and $MgCl_2$. The enzyme was purified as described in Materials and Methods. One unit of enzyme activity is defined as the amount catalyzing the conversion of 1 μmol of acetylene to acetaldehyde per min under standard assay conditions. Protein content was determined according to the method of Bradford (8) with bovine serum albumin as the standard. Numbers in parentheses indicate that enzyme activity is unusually high in comparison with the other data.

Although reducing conditions in the assay were a prerequisite for detection of enzyme activity, the acetylene hydratase was quite stable in the presence of air. Cell extracts and enriched or purified enzyme that had been stored at −20°C under air for several months still showed at least 30% of the initial enzyme activity. Therefore, cell harvesting and all enzyme purification steps could be performed under air without significant loss of activity.

Purification and molecular properties. Enzyme activity was located exclusively in the soluble fraction of the cell extract. The enzyme was purified 240-fold from acetylene-grown cells by ammonium sulfate fractionation, anion-exchange chromatography, size exclusion chromatography, and a second anion-exchange chromatography step, with a yield of 36% (Table 2).

The purified enzyme was a monomer with a molecular mass of 73 kDa as determined by SDS-PAGE (Fig. 2). A molecular mass of 70 kDa was determined by gradient gel electrophoresis under nondenaturing conditions. In size exclusion chromatography on a Superose 12 column (HR 10/30) the protein eluted according to a molecular mass of 60 kDa. The isoelectric point as determined by chromatofocusing on a Mono P column (HR 5/20) was at pH 4.2. The amino acid sequence of the N terminus was Ala-Ser-Lys-Lys-His-Val-Val-Cys-Gln-Cys-Cys-Asp-Ile-Asn-Cys-Val-Val-Glu-Ala-. No sequence homology to any amino acid sequences or DNA sequences stored in the NCBI database was found.

The protein contained 0.4 mol of W per mol of enzyme (73 kDa) and 3.8 mol of Fe per mol of enzyme (73 kDa) as determined by ICP-MS. An iron content of 4.8 mol of Fe per mol of enzyme (73 kDa) was determined by atomic absorption spectrometry. No molybdenum was found in the purified protein by ICP-MS or atomic absorption spectrometry. A total of 3.85 ± 0.4 mol of acid-labile sulfur per mol of enzyme (73 kDa) was detected.

Catalytic properties. The rate of the enzyme reaction depended on substrate concentration according to Michaelis-Menten saturation kinetics. A K_m value for acetylene of 14 μM as assayed in the coupled photometric assay with yeast alcohol dehydrogenase and NADH within a concentration range of 5 to 20 mM acetylene and a V_{max} of 69 $\mu mol \cdot min^{-1} \cdot mg\ of\ protein^{-1}$ were determined. The apparent optimum pH of enzyme activity measured in the range of pH 4.0 to 9.0 in a buffer mixture consisting of 100 mM Tris, 50 mM MES (mor-

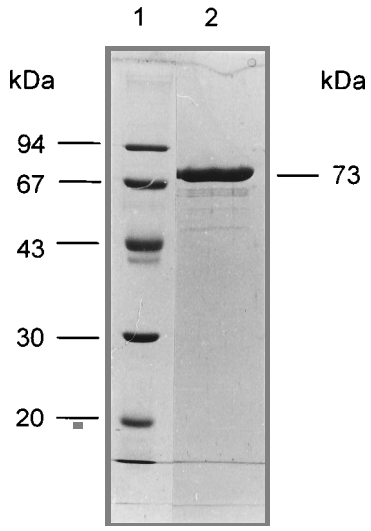

FIG. 2. SDS-PAGE of the purified acetylene hydratase of *P. acetylenicus*. Protein was denatured in SDS at 100°C and separated on a 12% slab gel (8 by 7 cm) which was subsequently stained with Coomassie brilliant blue R250. Lane 1, molecular mass standards; lane 2, 2 μg of acetylene hydratase.

pholineethanesulfonic acid), and 50 mM acetate with 2 mM titanium(III) citrate was found to be 6.0 to 6.5. The apparent optimum temperature between 22 and 80°C measured in 50 mM phosphate buffer, pH 7.0, containing 2 mM titanium(III) citrate was 50°C. Enzyme activity was not changed in the presence of the following iron-chelating agents: citrate, ascorbate, tiron, ferrocene, and EDTA. $HgCl_2$ reduced enzyme activity by 40% (0.01 mM), 80% (0.1 mM), and 98% (0.2 mM). KCN inhibited acetylene hydratase activity only at rather high concentrations: after an incubation time of 15 min, 80% of the activity could be measured in the presence of 1 mM KCN and 60% could be measured in the presence of 5 or 10 mM KCN. Carbon monoxide (0.8 mM) and nitric oxide (3 mM) reduced the enzyme activity by 90 and 100%, respectively. Addition of 8 mM ethylene to the assay mixture did not impair enzyme activity, indicating that ethylene cannot be used as a substrate by the enzyme. After addition of ethylene instead of acetylene, in the presence of methylene blue or anthraquinone disulfonate as an electron acceptor, neither reduction of the electron acceptor nor conversion of ethylene to acetaldehyde could be detected. Enzyme activity was not increased in the presence of 5 mM tungstate in the assay mixture. The purified enzyme had no aldehyde dehydrogenase (coenzyme A-acetylating) activity. In cell extracts, no activity of cyanide hydratase, nitrile hydratase, acetylene monocarboxylate hydratase, or acetylene dicarboxylate hydratase could be detected, indicating that these compounds are not converted by acetylene hydratase.

DISCUSSION

Acetylene hydratase of *P. acetylenicus* catalyzes the hydration of the unsaturated hydrocarbon acetylene to acetaldehyde and therefore belongs to the group of hydro-lyases (EC 4.2.1). The enzyme was shown to be a tungsten iron-sulfur protein. This was proven by detection of 0.4 mol of tungsten per mol of enzyme by ICP-MS and was further supported by growth experiments. Growth of *P. acetylenicus* and specific acetylene hydratase activity depended on the presence of tungstate or molybdate in the growth medium. After growth in the presence of 12 nM tungstate and 150 nM molybdate, only tungsten was detectable in the purified enzyme. In the presence of tungstate, additional molybdate, either in equal amounts or in 12-fold excess, resulted in two- to threefold decreases in the specific enzyme activity (Table 1). This indicates that molybdenum might exert an antagonistic effect on the tungsten-containing enzyme, similar to that described for carbonic acid reductase of *C. thermoaceticum* (43). In *M. wolfei* and *M. thermoautotrophicum*, two isoenzymes of formylmethanofuran dehydrogenase are synthesized in the presence of molybdate and tungstate, one containing molybdenum and the other containing tungsten (6, 37). Growth of *P. acetylenicus* after addition of only molybdate to the medium (Fig. 1) indicates that either molybdenum can be incorporated into the same enzyme if tungsten is not available, resulting in a lower specific activity, or a molybdenum-containing enzyme with a lower specific activity is synthesized in the absence of tungstate. The highest cell densities were reached with both tungstate and molybdate in the growth medium (Fig. 1), implying that the cells need both transition metals for optimal growth and that molybdenum is needed for functions other than acetylene hydratation.

The tungsten content of the acetylene hydratase as determined semiquantitatively by ICP-MS was rather low (0.4 mol of W per mol of protein). However, low tungsten contents have been found by other authors as well (6, 38). Obviously, the ICP-MS method tends to underestimate heavy metal contents in enzyme preparations. The possibility that tungsten was lost from the enzyme during the purification procedure cannot be ruled out either. Incubation of purified enzyme fractions with tungstate did not increase the specific enzyme activity.

As pointed out by Bertram et al. (5), all tungsten-containing enzymes known to date catalyze reactions of very low reduction potentials, e.g., those involving formate dehydrogenase (E_0' of CO_2-formate = −432 mV [40]), aldehyde ferredoxin oxidoreductase (E_0' of carbonic acid-aldehyde = −560 mV [30]), and formylmethanofuran dehydrogenase (E_0' of CO_2 plus methanofuran-formylmethanofuran = −497 mV [38]). Since the reaction catalyzed by acetylene hydratase is a hydration reaction rather than a redox reaction, it remains unclear why a strong reducing agent is necessary in the assay system. A primary reduction of acetylene to ethylene with subsequent hydration could be assumed; however, ethylene was not converted to acetaldehyde or ethanol in our assays, and it did not inhibit acetylene hydratase activity. *P. acetylenicus* did not use ethylene as a carbon and energy source, not even in the presence of a syntrophic partner organism (36).

In contrast to all tungsten-containing enzymes known so far, acetylene hydratase was quite stable in the presence of air. Formylmethanofuran dehydrogenases of *M. wolfei* and *M. thermoautotrophicum*, aldehyde ferredoxin oxidoreductase of *P. furiosus*, and formaldehyde oxidoreductase of *T. litoralis* can be purified only under strict exclusion of oxygen, because they lose activity within a few minutes to several hours under air (6, 31, 38). Sensitivity to oxygen has been described recently also for the benzyl viologen-dependent aldehyde dehydrogenase of *D. gigas*, which is supposed to contain tungsten (22).

Several of the tungsten-containing enzymes show aldehyde dehydrogenase activity (2, 22, 30, 31, 41, 44). Yet for acetylene hydratase of *P. acetylenicus*, no such activity could be demonstrated. The acetaldehyde dehydrogenase of *P. acetylenicus* is a coenzyme A-acetylating enzyme (36) and obviously a different enzyme protein.

In addition to tungsten, acetylene hydratase was shown to contain iron (4.8 mol per mol of enzyme) and acid-labile sulfur (3.9 mol per mol of enzyme), indicating that the enzyme is an iron-sulfur protein. In preliminary electron paramagnetic resonance studies, the presence of an iron-sulfur cluster could be

demonstrated. Several iron-sulfur proteins that catalyze not redox reactions but hydration reactions are known to exist (10), with aconitase being the most prominent example (4, 18). Within the N-terminal amino acid sequence of purified acetylene hydratase, the relatively large number of cysteine residues was striking. On account of their sequence, these cysteine residues could be possible binding sites for an iron-sulfur cluster (9, 10) or for a pterin cofactor (48).

In preliminary studies with 40-fold-enriched enzyme (about 50% purity as judged from SDS-PAGE) fluorescence spectra provided evidence for the existence of a pterin cofactor in acetylene hydratase (data not shown). All tungsten enzymes that have been analyzed for a pterin cofactor were shown to contain a tungsten pterin cofactor, analogous to molybdopterin cofactors (6, 24, 38). In a manner similar to that of other tungsten-containing enzymes (6, 31, 38), acetylene hydratase was inactivated by cyanide only at rather high concentrations. Cyanide as well as nitriles resembles acetylene structurally. It has been suggested that the natural function of acetylene hydratase might be the hydrolysis of toxic substances such as cyanide or nitriles (36). Cyanide hydratases and nitrile hydratases have been described for several aerobic bacteria and fungi (19, 32). However, we could demonstrate neither cyanide nor nitrile hydratase activity in cell extracts of *P. acetylenicus*. Acetylene monocarboxylate and acetylene dicarboxylate, which could represent simple model compounds for polyacetylenes (49), were not converted by cell extracts of *P. acetylenicus* either. Thus, a possible physiological function of acetylene hydratase beyond acetylene hydration cannot be defined at present.

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