Acta Crystallographica Section F
Structural Biology and Crystallization Communications
ISSN 1744-3091

Crystallization and preliminary X-ray analysis of the tungsten-dependent acetylene hydratase from *Pelobacter acetylenicus*

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Received 24 January 2005
Accepted 2 February 2005
Online 12 February 2005

Acetylene hydratase is a tungsten-containing hydroxylase that converts acetylene to acetaldehyde in a unique reaction that requires a strong reductant. The subsequent disproportionation of acetaldehyde yields acetate and ethanol. Crystals of the tungsten/iron–sulfur protein acetylene hydratase from *Pelobacter acetylenicus* strain WoAcy 1 (DSM 3246) were grown by the vapour-diffusion method in an N_2/H_2 atmosphere using polyethylene glycol as precipitant. Growth of crystals suitable for X-ray analysis strictly depended on the presence of Ti^{III} citrate or dithionite as reducing agents.

1. Introduction

To date, acetylene is the only hydrocarbon known to be metabolized in the same manner in the absence and presence of molecular oxygen (Schink, 1985). Acetylene hydratase from *Pelobacter acetylenicus* converts acetylene to acetaldehyde, a reaction distinct from the conversion of acetylene to ethylene by nitrogenase (Burgess & Lowe, 1996). The enzyme was purified as a monomer of 85 kDa as determined by MALDI–MS. The N-terminus of the protein shows a typical binding motif for an iron–sulfur cluster of the type Cys-X_2-Cys-X_3-Cys (Rosner & Schink, 1995). 4.4 ± 0.4 mol Fe and 0.5 ± 0.1 mol W (ICP/MS), 3.9 ± 0.4 mol acid labile sulfur and 1.3 ± 0.1 mol molybdopterin guanine dinucleotide were found per mole of enzyme, while selenium was absent. The specific activity of the enzyme peaks between pH 6.0 and 7.0, with a temperature optimum at 323 K. Although the addition of water to the C≡C triple bond (1) does not change the oxidation level of the product, a strong reductant such as Ti^{III} citrate or dithionite is required to observe activity (Meckenstock *et al.*, 1999),

$$HC{\equiv}CH + H_2O \rightarrow [H_2C{=}C(OH)H] \rightarrow CH_3CHO. \quad (1)$$

According to spectroscopic data, acetylene hydratase contains a single [4Fe–4S] cluster with a midpoint redox potential of −410 ± 20 mV. Enzyme activity also depends on the redox state of the solution, with 50% maximum activity at −340 ± 20 mV. Acetylene hydratase is oxygen-sensitive; when purified under air the [4Fe–4S] cluster degrades to a [3Fe–4S] cluster as demonstrated by EPR spectroscopy (Meckenstock *et al.*, 1999).

2. Methods

2.1. Protein preparation

P. acetylenicus strain WoAcy 1 (DSM 3246) was grown anaerobically in bicarbonate-buffered freshwater mineral medium reduced with sodium sulfide (Abt, 2001). The enzyme was purified under the exclusion of dioxygen in an N_2/H_2 atmosphere. Cells were broken by incubation with lysozyme and subsequent centrifugation at 10 000*g*; 2.3 *M* of ammonium sulfate was then added and after a further centrifugation step at 10 000*g* the pellet was discarded. The supernatant was brought to 3.2 *M* in ammonium sulfate and centrifuged at 10 000*g*. In this step, acetylene hydratase was found in the pellet and was desalted by dialysis. A subsequent chromatography step was carried out on a Q-Sepharose anion-exchange column followed by a

Table 1
Purification of acetylene hydratase from 25 g (wet weight) *P. acetylenicus* cells grown in a tungstate-supplemented freshwater medium.

Activity was measured at 293 K; 1 U = 1 µmol min^{-1} acetylene; AS = ammonium sulfate.

	Protein (mg)	Activity (U)	Specific activity (U mg^{-1})	Yield (protein) (%)	Yield (activity) (%)	Enrichment factor
Crude extract	1712	616	0.36	100	100	1
3.2 *M* AS	332	395	1.19	19	64	3.3
Q-Sepharose	86	374	4.35	5	61	12.1
Superdex 75	14	150	10.7	0.8	24	29.7

final gel-filtration step on a Superdex 75 size-exclusion chromatography column.

2.2. Crystallographic analysis

Diffraction experiments were carried out on beamline BW6 at DESY, Hamburg, Germany. Images were indexed, integrated and scaled using the *HKL* suite of programs (Otwinowski & Minor, 1996). For molecular replacement and the calculation of Patterson maps, programs from the *CCP*4 package (Collaborative Computational Project, Number 4, 1994) were used.

3. Results and discussion

3.1. Protein purification

Acetylene hydratase was purified to electrophoretic homogeneity and high specific activity (Fig. 1). The purification is summarized in Table 1.

3.2. Crystallization

Crystals of *P. acetylenicus* acetylene hydratase were obtained by sitting-drop vapour diffusion directly from Hampton Crystal Screen I condition 36 (Hampton Research, Laguna Niguel, USA) under an N_2/H_2 (95%/5%) atmosphere at 293 K. Crystals grew within three weeks from a 10 mg ml^{-1} protein solution in 5 m*M* HEPES–NaOH pH 7.5 reduced by addition of Ti^{III} citrate or sodium dithionite to a final concentration of 3 m*M*. 2 µl of this solution were mixed with 2 µl of the precipitant solution in a Cryschem sitting-drop plate (Hampton Research, Laguna Niguel, USA) using 300 µl of the precipitant solution as a reservoir. 20% methylpentanediol was added as a cryoprotectant and the crystals were flash-cooled in a nitrogen stream (Oxford Cryosystems). Data were collected from a flash-cooled crystal at 100 K at beamline BW6 of the Deutsches Elektronensynchrotron (DESY, Hamburg, Germany).

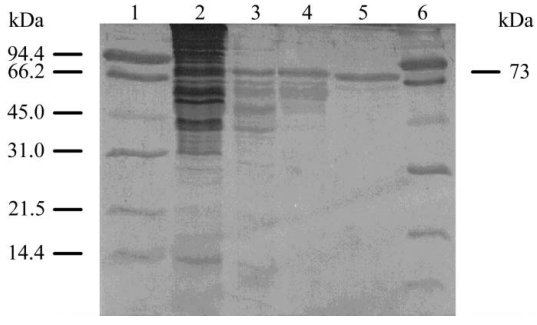

Figure 1
SDS–PAGE analysis of the purification steps of acetylene hydratase. Lanes 1 and 6, molecular-weight markers. Lane 2, crude extract after ultrafiltration (10 µg protein). Lane 3, precipitation of protein with 3.2 *M* $(NH_4)_2SO_4$ (7 µg). Lane 4, acetylene hydratase after anion-exchange chromatography, Q-Sepharose (4 µg). Lane 5, acetylene hydratase after Superdex 75 chromatography (2.5 µg).

Table 2
Data-collection statistics.

Values in parentheses are for the outer shell.

Wavelength (Å)	1.0601
Resolution limits (Å)	50.0–2.3
Unique reflections	33 221
Completeness (%)	99.8 (99.8)
Observation redundancy	3.9
R_{sym}	0.108 (0.368)
$I/\sigma(I)$	11.5 (2.3)
Space group	*C*2
Unit-cell parameters (Å, °)	a = 120.7, b = 70.5, c = 106.6, $\alpha = \gamma$ = 90.0, β = 123.8
Monomers per AU	1

3.3. Data collection

Although crystals of acetylene hydratase were small in size (200 × 50 × 20 µm), data could be collected to a resolution of 2.3 Å (Table 2). For synchrotron data collection, a wavelength of 1.06 Å was chosen, close to the intensity maximum of the synchrotron radiation. At this wavelength, tungsten exhibits a considerable anomalous signal owing to the close proximity of the element's *L* edge.

The crystal belonged to space group *C*2, with unit-cell parameters a = 121.2, b = 70.7, c = 106.8 Å, $\alpha = \gamma$ = 90.0, β = 124.3°. Assuming a molecular weight of 85 kDa and the presence of one monomer per asymmetric unit, the resulting Matthews coefficient was 2.22 Å^3 Da^{-1}, corresponding to a solvent content of 44.5%.

3.4. Molecular replacement

Based on sequence homologies of the available structures of molybdenum/tungsten hydroxylases, the structure of the tungsten-containing formate dehydrogenase from *Desulfovibrio gigas* (Raaijmakers *et al.*, 2002) was chosen as a starting point for the construction of a model for molecular replacement. Only the first 570 amino acids of this structure were used and all non-identical residues were mutated to alanine. All temperature factors were uniformly set to 20 Å^2 and two loop regions in the model that were absent in the acetylene hydratase sequence were removed. Molecular replacement was carried out to a resolution of 3.5 Å using the program *MOLREP* (Collaborative Computational Project, Number 4, 1994), yielding a solution with a correlation coefficient of 0.11 at an *R* value of 0.564. This solution produced a sensible packing of molecules, but the derived electron-density maps were not of sufficient quality to allow model building.

3.5. Anomalous signal

In a v = 0 Harker section of an anomalous difference Patterson map, a prominent peak consistent at all maximum resolution limits observed was found at fractional coordinates u = 0.02, w = 0.53 (Fig. 2*a*). Choosing an appropriate symmetry equivalent, these coordinates after orthogonalization transform into x = 44.8, z = 21.5 Å, which corresponds exactly to the coordinates obtained through molecular replacement (Fig. 2*b*). It is therefore intended to calculate SAD phases using the anomalous signal of tungsten and subsequently combine these with phases obtained from a further improved molecular-replacement model.

Synchrotron data were collected on beamline BW6 at Deutsches Elektronensynchrotron (DESY), Hamburg. The authors wish to

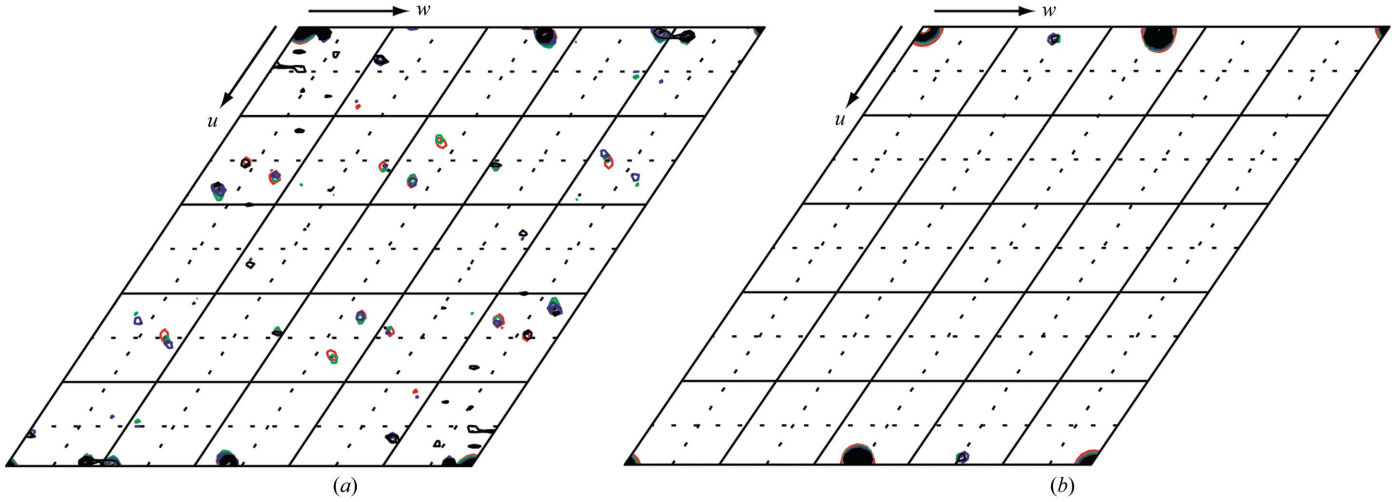

Figure 2
(*a*) The $v = 0$ Harker section of an anomalous difference Patterson map for the *C*2 cell of acetylene hydratase crystals. The map was calculated at four resolution levels: 5.5 Å (red), 4.5 Å (green), 3.5 Å (blue) and 2.5 Å (black). (*b*) The same Harker section calculated using the metal positions from the obtained molecular-replacement solution with a model derived from the structure of formate dehydrogenase (Raaijmakers *et al.*, 2002).

thank Gleb Bourenkov and Hans D. Bartunik for assistance during data collection.

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