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# Crystallization and preliminary X-ray diffraction studies of a novel alcohol dehydrogenase from the hyperthermophilic archaeon *Aeropyrum pernix*

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A novel alcohol dehydrogenase enzyme has been cloned from the hyperthermophilic archaeon *Aeropyrum pernix* and overexpressed in *Escherichia coli*. This zinc-containing enzyme has been crystallized by the sitting-drop vapour-diffusion method using PEG 600 as precipitant. The crystals diffract to 1.5 Å resolution and belong to the orthorhombic space group $P2_12_12$, with unit-cell parameters $a$ = 100.7, $b$ = 103.2, $c$ = 67.5 Å. The asymmetric unit contains two enzyme monomers. Two synchrotron data sets have been collected: one at a wavelength near the absorption edge of zinc and one at a remote wavelength. Three strong zinc-ion positions were visible in the anomalous Patterson map. Two additional weaker zinc ions have been identified by anomalous Fourier synthesis.

## 1. Introduction

The alcohol dehydrogenases (ADHs; EC 1.1.1.1) belong to the oxidoreductase family, a class of enzymes responsible for the catalysis of all biological oxidation–reduction reactions. Alcohol dehydrogenases catalyse the cofactor-dependent interconversion of alcohols from the corresponding aldehyde or ketone. The enzymes are widely distributed and are present throughout the three domains of life: archaea, bacteria and eukarya (Rella *et al.*, 1987; Reid & Fewson, 1994; Von Wartburg *et al.*, 1964).

The ADHs may be separated into three distinct classes based on molecular size (Reid & Fewson, 1994). The type I or medium-chain ADHs form the largest and most extensively studied group. With approximately 370 residues per chain, this class includes the well characterized horse liver ADH (Eklund *et al.*, 1976). Enzymes of this class may be dimeric or tetrameric and all contain bound zinc ions. In general, bacterial type I ADHs are tetramers, while those from higher eukaryotes are dimeric enzymes. Type II ADHs are classified as short chain, having approximately 250 residues per chain, and do not normally contain metals (Persson *et al.*, 1991). This group includes the ADH found in *Drosophila melanogaster* (Thatcher, 1980). Type III or long-chain ADHs contain over 380 but often as many as 900 amino-acid residues. Enzymes of this class are frequently activated by iron. Represented by ADH II from *Zymomonas mobilus* (Scopes, 1983), these form the least characterized class and show little sequence homology to type I or type II ADHs.

*Aeropyrum pernix*, the first strictly aerobic hyperthermophilic archaeon to be identified, was isolated from a coastal solfataric vent at Kodakara-Jima Island, Japan (Sako *et al.*, 1993). The organism is heterotrophic and grows optimally at 363–368 K and pH 7.0. The complete genome sequence of *A. pernix* has been determined and published (Kawarabayasi *et al.*, 1999). A putative ADH sequence was identified from the genome sequence. The enzyme has subsequently been shown to exhibit ADH activity and has been cloned, overexpressed and biochemically characterized (Guy *et al.*, manuscript in preparation). The subunit molecular weight calculated from the gene sequence is 39.57 kDa, which agrees with the apparent molecular weight of 39 kDa observed on SDS–PAGE. The elution profile of the enzyme from gel-filtration chromatography indicates the native state of the enzyme to be tetrameric.

Crystal structures are available for a number of eukaryotic and prokaryotic type I ADHs. Horse liver ADH was the first to be studied by X-ray crystallography (Eklund *et al.*, 1976); more recently, an atomic resolution structure has been obtained (Meijers *et al.*, 2001). Only two ADHs have been crystallized from thermophiles. The NADP(H)-dependent ADH from the thermophilic bacterium *Thermoanaerobium brockii* was crystallized in its holoenzyme form, with the structure solved to 2.5 Å resolution (Korkhin *et al.*, 1998). The X-ray structure of a tetrameric NAD(H)-dependent ADH from the hyperthermophilic archaeon *Sulfolobus solfataricus* has been very recently solved to 1.85 Å resolution in its apo form (Esposito *et al.*, 2002). Here, we present the crystallization of a novel type I ADH from the archaeon *A. pernix* in the presence of its

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cofactor NADH in a form suitable for high-resolution X-ray studies and the preliminary X-ray data analysis.

## 2. Materials and methods

### 2.1. Expression and purification

The gene coding for the ADH was cloned into the expression vector pTRC99 (Amann *et al.*, 1988) and overexpressed in *Escherichia coli* DH5 (Guy *et al.*, manuscript in preparation). The cells were resuspended in 20 m*M* Tris–HCl pH 7.5, 0.1 m*M* phenylmethylsulfonyl fluoride (PMSF) and disrupted by sonication on ice using a Soniprep 150 (Sanyo). Centrifugation was performed at 277 K (10 000*g*, 15 min) and the supernatant was incubated with Benzonase (Merck, Darmstadt, Germany) and 5 m*M* $MgCl_2$ at 310 K for 40 min. Protamine sulfate (1 mg ml$^{-1}$) was added and the extract incubated at 277 K for a further 30 min. The extract was heated at 348 K for 15 min to precipitate the majority of the *E. coli* proteins. After centrifugation (7500*g*, 30 min, 277 K) the supernatant was dialysed overnight against 20 m*M* Tris–HCl pH 8.5, 0.1 m*M* PMSF (buffer *A*).

The recombinant protein was purified by absorption to Fast Flow Q anion-exchange resin (Amersham Biosciences UK Ltd, Buckinghamshire, England) and was eluted with a linear gradient of 0–1 *M* NaCl in buffer *A*. Fractions containing the recombinant protein were pooled and dialysed overnight against buffer *A* containing 2 *M* KCl, then further purified using a butyl Sepharose column (HiTrap Butyl Fast Flow; Amersham Biosciences UK Ltd) eluted with a gradient from 2 to 0 *M* KCl in buffer *A*. A final purification step was carried out using gel-filtration chromatography (Superdex 200 prep grade; Amersham Biosciences UK Ltd). The gel-filtration column was eluted with buffer *A* containing 0.1 *M* NaCl. The purity of the sample was assessed by SDS–PAGE (Laemmli, 1970).

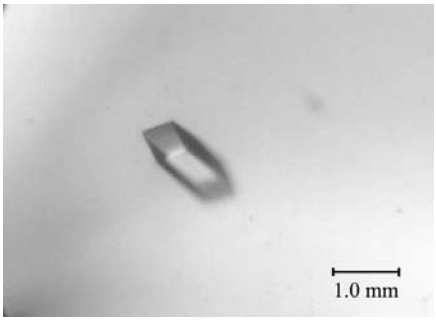


**Figure 1**
Orthorhombic crystals of *A. pernix* ADH. Maximum dimensions are approximately 0.4 × 0.4 × 1.0 mm.

Assays for ADH activity were performed by spectrophotometrically following the changes in absorbance at 340 nm arising from cofactor utilization. The standard reaction mixture contained 5 m*M* cyclohexanone and 0.25 m*M* NADH in 50 m*M* Tris–HCl (pH adjusted to 7.5 at assay temperature). The assay was performed at 338 K. 990 µl of the reaction mixture was pre-incubated for 5 min and the reaction started by the addition of 10 µl of enzyme sample. The change in absorbance at 340 nm was monitored over a period of 5 min using a Shimadzu UV-2100 double-beam spectrophotometer.

The protein concentration was determined spectrophotometrically by the method of Warburg & Christian (1941). The extinction coefficient for the ADH enzyme was estimated from the amino-acid sequence using the ExPASY database (Bairoch & Apweiler, 2000).

### 2.2. Crystallization

Crystallization was carried out using the sitting-drop vapour-diffusion method at 290 K. Prior to crystallization, the protein was concentrated to 10 mg ml$^{-1}$ in buffer *A* containing 0.1 *M* NaCl using a Vivaspin centrifugal concentrator (Vivascience Ltd, Lincoln, England) with a 10 kDa molecular-weight cutoff. 2 µl aliquots of protein solution were mixed with 2 µl reservoir solution to form the droplet. Initial crystallization trials were performed using Structure Screen kits 1 and 2 (Molecular Dimensions Ltd, Luton, England). Several potential crystallization conditions were identified using PEG as a precipitant. The most promising condition, producing small crystal clusters, was from 20% PEG 10 000, 0.1 *M* HEPES pH 7.5. This condition was optimized by variation of the pH and buffer of the reservoir. Further improvement of the crystals obtained was achieved by variation of the molecular weight and concentration of the PEG precipitant and inclusion of the cofactor NADH. The best quality crystals were obtained using a reservoir solution containing 100 m*M* PIPES pH 6.75, 13–16% PEG 600 and 0.5 m*M* NADH.

### 2.3. Crystallographic data collection

The initial data were collected in-house at both 290 and 100 K using a MAR Research 345 image plate and Cu $K\alpha$ radiation from a Siemens rotating-anode generator. High-resolution data were collected at EMBL station BW7A at the DESY Synchrotron, Hamburg using a single cryocooled crystal at 100 K and a MAR Research CCD detector. The data were collected at a remote wavelength of 1.0704 Å and at an anomalous wavelength of 1.232 Å.

**Table 1**
Processing statistics for native and anomalous data sets.

Values in parentheses are for the highest resolution shell.

| | Native | Anomalous |
|---|---|---|
| Wavelength (Å) | 1.0704 | 1.2320 |
| Oscillation range (°) | 0.4 | 0.4 |
| Resolution range (Å) | 14–1.65 (1.68–1.65) | 14–1.88 (1.91–1.88) |
| No. measured reflections | 1008062 | 597020 |
| No. unique reflections† | 168938 | 107875 |
| Completeness | 97.9 (96.2) | 97.8 (95.9) |
| $I > 3\sigma(I)$ (%) | 69.6 | 86.3 |
| $\langle I\rangle/\sigma\langle I\rangle$ | 28.8 | 37.1 |
| $R_{sym}$‡ (%) | 5.4 (35.4) | 4.5 (21.0) |

† $I^+$ and $I^-$ were scaled separately. ‡ $R_{sym} = \sum|I - \langle I\rangle|/\sum I$, where $I$ is the intensity of reflection.

The cryogenic liquor contained 35% PEG 600 in 100 m*M* PIPES pH 6.75, 0.1 *M* NaCl, 0.5 m*M* NADH. To avoid crystals being damaged by osmotic shock arising from the sharp increase in PEG concentration, large crystals were soaked for 1 min in mother liquor as above but with 25% PEG 600 prior to transfer into the cryogenic liquor.

## 3. Results and discussion

Single orthorhombic crystals with maximum dimensions of 0.4 × 0.4 × 1 mm (Fig. 1) grew from 0.1 *M* PIPES pH 6.75 containing 13–15% PEG 600 and 0.5 m*M* NADH in approximately four weeks. The crystals diffracted to better than 1.5 Å using synchrotron radiation and belonged to the orthorhombic space group $P2_12_12$, with unit-cell parameters $a$ = 100.7, $b$ = 103.2, $c$ = 67.5 Å. Data were collected to 1.65 Å resolution at the zinc remote wavelength and to 1.88 Å resolution at an anomalous wavelength. The data were processed using the programs *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997). A summary of the X-ray data statistics is shown in Table 1. A self-rotation function (Fig. 2) suggests that the ADH tetramer has 222 point-group symmetry and that one of the molecular dyads coincides with the crystallographic rotational twofold axis. The solvent content of the crystals, which contain a dimer in the asymmetric unit, has been estimated at 44%; $V_M$ = 2.2 Å$^3$ Da$^{-1}$ (Matthews, 1968).

Three zinc ions were clearly visible in the anomalous Patterson synthesis map (Fig. 3). Their positions were found using data collected at a wavelength of 1.232 Å

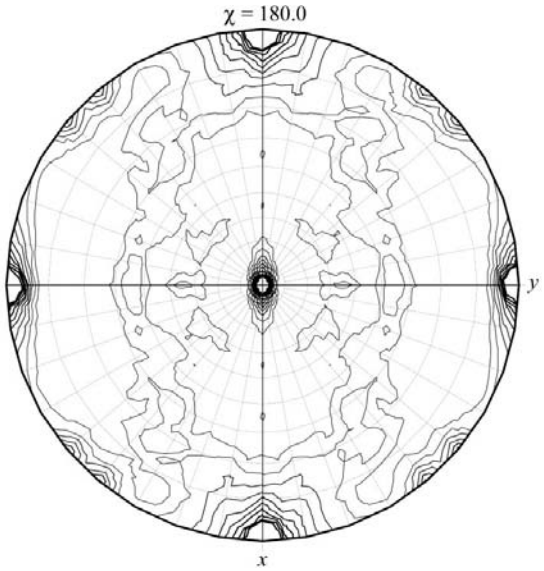


**Figure 2**
$\kappa = 180^\circ$ section of the self-rotation function of the *A. pernix* ADH data calculated with an integration radius of 30 Å at 14–3 Å resolution using the program *MOLREP* (Vagin & Teplyakov, 1997). Two molecular dyads of the ADH tetramer are visible at polar angles ($\omega$, $\varphi$) of (90, 40°) and (90, 130°), while the third molecular dyad coincides with the crystallographic $z$ axis.

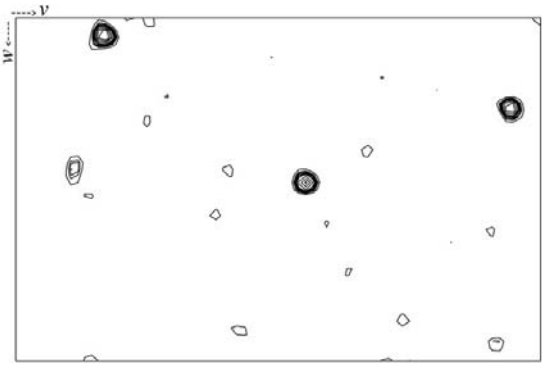


**Figure 3**
$u = 0.5$ section of the anomalous Patterson map calculated at 14.0–1.9 Å resolution.

between 14 and 1.9 Å resolution by the Patterson interpretation routine of *SHELX*90 (Sheldrick, 1991). Two of these sites were consistent with the molecular symmetry of *A. pernix* ADH, which allowed determination of the NCS symmetry operations. Heavy-atom refinement and phasing to 1.88 Å resolution was performed using *MLPHARE* (Collaborative Computational Project, Number 4, 1994). This resulted in phases with a figure of merit of 0.284. The twofold NCS averaging was performed using the program *DM* (Cowtan, 1994).

Phased molecular replacement based on the spherically averaged phased translation function (Vagin & Isupov, 2001) was implemented in the program *MOLREP* (Vagin & Teplyakov, 1997). This allowed successful positioning of the human ADH $\beta$1 isozyme (Niederhut *et al.*, 2001; PDB code 1hsz), which was used as a model, into the averaged electron-density map. The rebuilding of this model using the *A. pernix* ADH sequence is currently in progress. The refinement of the partial model using the averaged phases is being performed using the program *REFMAC*5 (Murshudov *et al.*, 1997). The current crystallographic $R$ factor is 0.168 and $R_{\rm free}$ is 0.203.

Two additional weaker zinc sites were found in the difference anomalous Fourier synthesis using the averaged phases. These both obey NCS symmetry and correspond to structural zinc ions, which appear to have partial occupancy. Two of the strong zinc sites described previously are catalytic zinc ions. The third strong zinc site is found on the interface between tetramers within the crystal lattice and is probably an artefact of crystallization.

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