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# Thermostable NAD(P)H-dependent carbonyl reductase from a hyperthermophile *Aeropyrum pernix* K1: crystallization and preliminary X-ray analysis

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## 1 Introduction

We recently determined the crystal structure of a CR from chicken fatty liver in complex with NADPH and a substrate analogue, ethylene glycol (PDB entry, 3WXB; yoneda *et al*., 2012, Fukuda *et al*., 2015). Structural analysis indicated the presence of a unique substrate-binding site in the chicken fatty liver CR that is not observed in conventional CRs. Moreover, this CR exhibits broad substrate specificity. Interestingly, we found that the enzyme catalyzes the reduction of ethyl 4-chloro-3-oxobutanoate (COBE) to produce ethyl(*S*)-4-chloro-3-hydroxybutanoate [(*S*)-CHBE] (unpublished data), which is a chiral pharmaceutical intermediate with industrial applications. On the other hand, the chicken fatty liver CR is not stable enough to catalyze the conversion reaction: the half-life of the enzyme at 323 K is 10 min. Therefore, we focused on the identification of a more stable and active CR from thermophiles that may be used for (*S*)-CHBE production. Within the genomic sequence of the hyperthermophilic archaeon *Aeropyrum pernix* K1 (optimum growth temperature is approximately 368 K), we found a gene (APE_2503.1) whose predicted amino acid sequence exhibits 28% identity with that of chicken fatty liver CR. Moreover, we successfully expressed this gene in *Escherichia coli* and purified the enzyme. This enzyme exhibited higher stability than the chicken fatty liver CR. In this paper, we describe the purification, crystallization, and preliminary X-ray analysis of the stable *A. pernix* CR.

## 2 Experiment

The purified enzyme was concentrated by ultrafiltration to 11 mg $mL^{-1}$ for crystallization trials, and the buffer was changed to 10 m*M* Tris-HCl buffer (pH 8.0) containing 200 m*M* NaCl. Initial screening for crystallization was carried out with CrystalScreen and CrystalScreen 2 (Hampton Research, Aliso Viejo, CA, USA) at 293 K by using the sitting-drop vapor diffusion method, in which a 1 μL drop of protein solution containing 0.2 m*M* NADPH was mixed with an equal volume of reservoir solution and equilibrated against 0.1 mL of reservoir solution in Compact Clover Crystallization Plates (Emerald Biosystems, USA).

The NAD(P)H-dependent CR crystal was flash-cooled in liquid nitrogen at 100 K. The crystal was cryoprotected with reservoir solution supplemented with 30% (*v*/*v*) ethylene glycol; the water in the buffer (2.0 *M* NaCl, 100 m*M* acetate buffer [pH 4.5]) was replaced with the cryoprotectant. Diffraction data were collected at 2.09 Å resolution by using monochromated radiation of wavelength 1.0 Å and a PILATUS detector system on the AR-NE3A beamline at the Photon Factory, Tsukuba, Japan. The oscillation angle per image was set to 1°. The crystal-to-detector distance was 257.3 mm. The data were processed with *HKL*-2000.

## 3 Results and Discussion

We found that the enzyme could be readily purified from the crude cell extract in two simple steps, heat treatment and $Co^{2+}$-charged TALON$^{TM}$ resin column chromatography. Approximately 11.2 mg of purified enzyme was obtained from 1 L of *E. coli* culture. The enzyme showed a single band on SDS-PAGE, and the molecular mass of the subunit was determined to be approximately 27 kDa (Fig. 1).

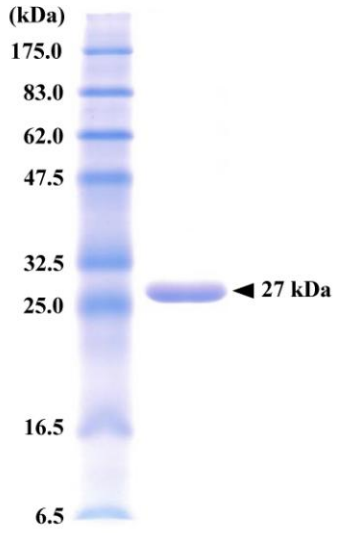
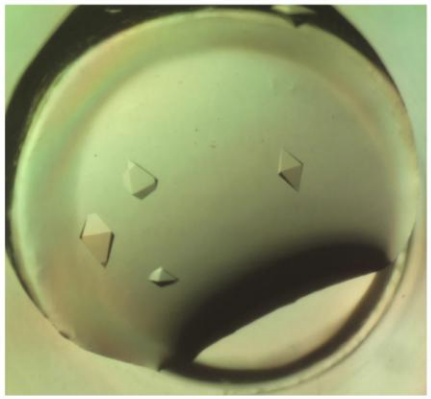
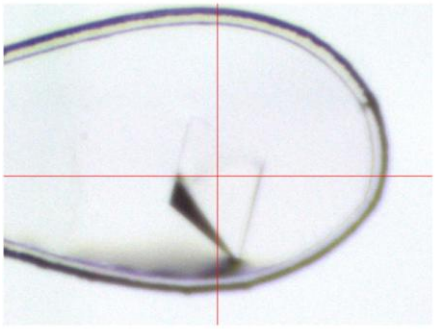



Fig. 1: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of recombinant *Aeropyrum pernix* carbonyl reductase (CR). Left lane, marker proteins; right lane, purified *A. pernix* CR after passing through a $Co^{2+}$ affinity column.

Initially, small crystals grew in reagent No. 9 (2.0 M NaCl, 100 mM acetate buffer [pH 4.6]) of CrystalScreen 2. This precipitant solution was taken as the starting point and was optimized by varying the NaCl concentration; a few diffraction-quality crystals (maximum dimensions of 0.2 × 0.2 × 0.1 mm; Fig. 2) were obtained within one week in a reservoir solution composed of 1.8-2.0 M NaCl in 100 mM acetate buffer (pH 4.5).



(*a*)



(*b*)

Fig. 2: Tetragonal crystals of *A. pernix* CR. (*a*) *A. pernix* CR/NADPH binary complex after 7 days of crystallization by the sitting-drop vapor-diffusion method at 293 K. The maximum dimensions of the crystal were 0.2 × 0.2 × 0.1 mm. (*b*) The *A. pernix* CR crystal was mounted on a nylon loop (0.7 mm diameter) and was flash-cooled in liquid nitrogen.

The *A. pernix* CR crystal diffract at 2.09 Å resolution, and belonged to the tetragonal space group $P4_12_12$ or its enantiomorph $P4_32_12$ (Fig. 3). A summary of the data-collection and processing statistics is presented in Table 1. Assuming two protein molecules in the asymmetric unit, the crystal volume per enzyme mass ($V_M$) and the solvent content were calculated to be 3.47 Å$^3$ Da$^{-1}$ and 64.5%, respectively, which are within the frequently observed ranges for protein crystals.

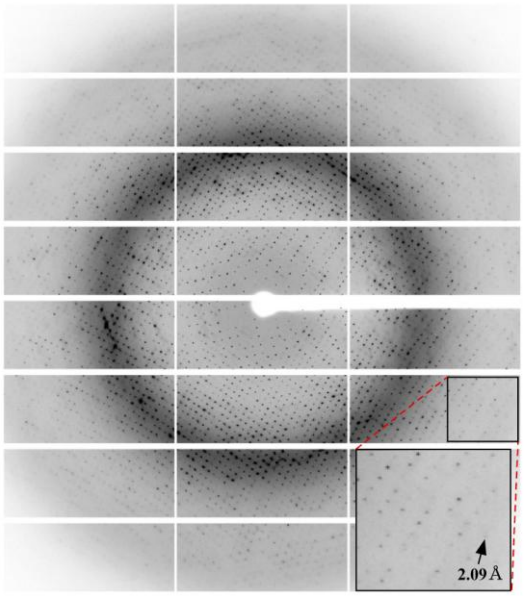



Fig. 3: X-ray diffraction pattern of a native *A. pernix* CR crystal. The resolution is ~1.98 Å at the edge. The high-resolution area is enlarged (insert).

Table 1: Data-collection and processing statistics for *A. pernix* CR. Values in parentheses are for the highest resolution shell.

| | |
|---|---|
| Beam line | AR-NE3A |
| Detector | PILATUS 2M-F |
| Wavelength (Å) | 1.0000 |
| Rotation range per frame (˚) | 1 |
| Total rotation range (˚) | 270 |
| Exposure per frame (s) | 2 |
| Crystal-to-detector distance (mm) | 257.3 |
| Temperature (K) | 100 |
| Space group | $P4_12_12/P4_32_12$ |
| Unit cell parameters (Å) | $a = b = 91.6$, $c = 177.6$ |
| Resolution range (Å) | 40.71-2.09 (2.13-2.09) |
| No. measured reflections | 721295 |
| No. unique reflections | 45349 |
| Multiplicity | 15.9 (15.4) |
| Completeness (%) | 99.7 (97.3) |
| $R_{merge}$ † | 0.064 (0.336) |
| $\langle I/\sigma(I)\rangle$ | 10.0 (6.6) |

† $R_{merge} = \sum_{hkl} \sum_i \left| I_i(hkl) - \langle I(hkl)\rangle \right| / \sum_{hkl} \sum_i I_i(hkl)$.

References

[1] K. Yoneda *et al.*, *Acta Crystallographica Section F.* (2012) **F68**, 1568-1570.

[2] Y. Fukuda *et al.*, *FEBS J.* (2015) **282**, 3918-3928.

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