Crystallization and preliminary X-ray diffraction studies of cyanuric acid hydrolase from *Azorhizobium caulinodans*

Seunghye Cho, Ke Shi,
Lawrence P. Wackett and
Hideki Aihara*

Department of Biochemistry, Molecular Biology
and Biophysics, University of Minnesota,
Minneapolis, MN 55455, USA

Correspondence e-mail: aihara001@umn.edu

Received 29 April 2013
Accepted 19 June 2013

Cyanuric acid is synthesized industrially and forms during the microbial metabolism of *s*-triazine herbicides. Cyanuric acid is metabolized by some microorganisms *via* cyanuric acid hydrolase (CAH), which opens the *s*-triazine ring as a prelude to further metabolism. CAH is a member of the rare cyanuric acid hydrolase/barbiturase family. Here, the crystallization and preliminary X-ray diffraction analysis of CAH from *Azorhizobium caulinodans* are reported. CAH was cocrystallized with barbituric acid, a close analog of cyanuric acid that is a tight-binding competitive inhibitor. Crystals suitable for X-ray diffraction experiments were grown in conditions containing PEG 8K or magnesium sulfate as precipitants. An X-ray diffraction data set was collected from CAH–barbituric acid crystals to 2.7 Å resolution. The crystals were found to belong to space group $I4_122$, with unit-cell parameters $a = b = 237.9$, $c = 105.3$ Å, $\alpha = \beta = \gamma = 90^\circ$.

1. Introduction

Cyanuric acid is found in nature, but it occurs principally as a result of industrial synthetic processes. It is produced on the order of 160 000 tonnes annually, largely as a precursor in the synthesis of disinfectants, dyes and pesticides (Huthmacher & Most, 2005).

Cyanuric acid is problematic in swimming pools, where it accumulates because of the decomposition of chlorinated isocyanuric acids that are added for water disinfection. Additionally, in recent pet food and milk poisonings, the presence of cyanuric acid in combination with melamine was shown to cause insoluble crystals to form in the kidneys, leading to renal failure (Dobson *et al.*, 2008). Cyanuric acid also forms inside cells during the microbial metabolism of melamine (Jutzi *et al.*, 1982) and *s*-triazine herbicides (Martinez *et al.*, 2001). Certain bacteria further metabolize cyanuric acid by hydrolytically opening the *s*-triazine ring and subsequent amide-bond hydrolysis reactions (Cheng *et al.*, 2005).

The ring-opening reaction, the first step in the metabolism of cyanuric acid, is catalyzed in microbes by a homologous set of enzymes known as cyanuric acid hydrolases (CAHs; Karns, 1999; Fruchey *et al.*, 2003). CAH catalyzes the addition of water to a C–N bond in cyanuric acid to produce carboxybiuret (Fig. 1). Carboxybiuret subsequently undergoes spontaneous decarboxylation to yield biuret, the substrate for the next enzyme in the metabolic pathway (Seffernick *et al.*, 2012; Cameron *et al.*, 2011). CAH enzymes are related to the enzyme barbiturase that functions in purine catabolism (Soong *et al.*, 2001), catalyzing a similar hydrolytic ring-opening reaction. However, barbituric acid is not a substrate for CAH but was rather observed to be a tight-binding competitive inhibitor (Karns, 1999). The cyanuric acid hydrolase/barbiturase family has recently been analyzed by bioinformatics, gene-cloning and protein-characterization studies, which allowed the identification of new genes that belong to this protein family (Seffernick *et al.*, 2012). Still, despite the analysis of thousands of genomes and the GenBank database, only 41 members of this family were identified and they could not be linked by sequence to any known protein superfamily. The absence of any homologs outside of the 41 proteins identified by extensive bioinformatics analysis suggested that this protein family might represent a unique fold. In this context, we conducted X-ray crystallographic studies to obtain the three-dimensional structure of a

Figure 1
Ring-opening reaction of cyanuric acid by cyanuric acid hydrolase.

cyanuric acid hydrolase and describe here the crystallization and preliminary X-ray diffraction studies of the CAH of AZC_3892 from *Azorhizobium caulinodans* (NCBI reference sequence YP_001526808.1).

2. Materials and methods

2.1. Protein expression and purification

An *Nde*I–*Hind*III fragment encoding CAH from *A. caulinodans* was subcloned into the *Nde*I–*Hind*III sites of the pET28b+ vector (Novagen, Madison, Wisconsin, USA). The resulting expression vector was introduced into *Escherichia coli* strain BL21(DE3). The transformed strain was grown at 310 K in LB medium (10 g Bacto tryptone, 5 g yeast extract, 10 g NaCl per litre) supplemented with 50 $\mu\text{g ml}^{-1}$ kanamycin. Cells were grown until the optical density of the culture at 600 nm reached 0.6. The expression of CAH was induced with isopropyl β -D-1-thiogalactopyranoside (IPTG) at a final concentration of 0.5 mM and the cell culture was continued for 30 h at 298 K.

The bacterial cells were harvested by centrifugation (6400g, 10 min, 277 K) and resuspended in binding buffer (50 mM Tris–HCl pH 7.0, 200 mM NaCl, 5 mM imidazole, 1 mM β -mercaptoethanol) with protease inhibitors (Roche). A microfluidizer was used for cell disruption and the cell lysate was centrifuged at 63 988g for 40 min at 277 K. The supernatant was loaded onto a column packed with Ni-NTA resin (Qiagen) that had been equilibrated with three column volumes of the binding buffer. Unbound proteins were washed away with ten column volumes of washing buffer (50 mM Tris–HCl pH 7.0, 200 mM NaCl, 10 mM imidazole, 1 mM β -mercaptoethanol). The

target protein was eluted with two column volumes of elution buffer (50 mM Tris–HCl pH 7.0, 200 mM NaCl, 200 mM imidazole, 1 mM β -mercaptoethanol). The eluted protein was concentrated by ultra-filtration and applied onto a Superdex 200 10/300 GL gel-filtration column (GE Healthcare) equilibrated with a running buffer consisting of 20 mM Tris–HCl pH 7.0, 200 mM NaCl, 5 mM dithiothreitol (DTT). The gel-filtration column had previously been calibrated using a gel-filtration standard (Bio-Rad). The purity and size of the protein were confirmed using SDS–PAGE. The protein concentration was determined by UV absorption at 280 nm with an extinction coefficient of $1.33 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ using a NanoDrop 8000 UV–Vis spectrophotometer (Thermo Scientific) and the purified protein was stored at 277 K. The yield of purified CAH was 28 mg l^{-1} .

To form a CAH–inhibitor complex, a 10 mM barbituric acid solution was made in 20 mM Tris–HCl pH 7.0, 200 mM NaCl, 5 mM DTT. The barbituric acid solution was added to purified CAH at a molar ratio of 5:1 (inhibitor:protein) and incubated at 277 K for 14 h. Either the unliganded CAH or the CAH–inhibitor complex was concentrated to 27 mg ml^{-1} for crystallization. Selenomethionine-substituted CAH was expressed using the methionine-biosynthesis inhibition method as described previously (Van Duyne *et al.*, 1993) and was purified as described above for the native CAH.

2.2. Crystallization

Crystallization screening of CAH or the CAH–inhibitor complex was performed by the sitting-drop vapor-diffusion method using a Rigaku CrystalMation system in the Nanolitre Crystallization Facility at the University of Minnesota. Conditions from the following screening kits were tested at 293 K: Crystal Screen HT, Index Screen, PEGRx HT, PEG/Ion and SaltRx (Hampton Research) and JCSG+ and PACT (Qiagen). Each drop was prepared by mixing 100 nl protein or protein–inhibitor complex solution with 100 nl reservoir solution. Within 3 d of incubation, small crystals were observed for the CAH–inhibitor complex, but not for unliganded CAH, in drops that contained magnesium sulfate and Tris–HCl pH 7.0 or PEG 8K

Figure 2
An optimized crystal of the CAH–barbituric acid complex obtained from the crystallization condition containing 1.7 M magnesium sulfate and 0.1 M Tris–HCl pH 7.0. The dimensions of the crystal were approximately $0.3 \times 0.3 \times 0.1 \text{ mm}$.

Figure 3
Diffraction image from the CAH–barbituric acid complex crystal.

Table 1

Data-collection statistics for CAH.

Values in parentheses are for the outermost resolution shell.

Space group	$I4_122$
Unit-cell parameters ($\text{\AA}$, $^\circ$)	$a = b = 237.9$, $c = 105.3$, $\alpha = \beta = \gamma = 90.0$
Wavelength ($\text{\AA}$)	0.97864
Detector	NOIR-1
Resolution range ($\text{\AA}$)	50.00–2.70 (2.75–2.70)
$R_{\text{merge}}^\dagger$	0.088 (0.566)
Completeness (%)	98.4 (94.7)
Multiplicity	9.9 (8.2)
$\langle I/\sigma(I) \rangle$	20.3 (2.20)
Total No. of reflections	403575 (15752)
No. of unique reflections	40849 (1921)

$^\dagger R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $I_i(hkl)$ is the i th observed intensity of individual reflection hkl and $\langle I(hkl) \rangle$ is the average intensity of individual reflection hkl .

and HEPES–NaOH pH 7.5. Optimizations for improving the sizes of these crystals were conducted by varying the precipitant concentration, buffer pH and protein concentration with the sitting-drop vapor-diffusion method. After optimization, crystals suitable for X-ray diffraction experiments were obtained with the following two conditions: (i) 1.0–1.7 *M* magnesium sulfate, 0.1 *M* Tris–HCl pH 7.0–7.5 and (ii) 5% PEG 8K, 0.1 *M* HEPES–NaOH pH 7.5. Crystals obtained in the optimized conditions (Fig. 2) grew to maximum size within 3 d in drops prepared by mixing 100 nl protein solution and 100 nl reservoir solution. The initial diffraction experiments were conducted at 100 K using a Rigaku R-Axis IV X-ray diffractometer.

2.3. Data collection and processing

The X-ray diffraction data were collected on beamline 4.2.2 of the Advanced Light Source, Berkeley, California, USA. The oscillation angle for each diffraction image (Fig. 3) was 0.5° . The crystals were briefly soaked in the cryoprotectant (reservoir solution containing 25% glycerol) and flash-cooled in liquid nitrogen before X-ray irradiation. The diffraction data were indexed, integrated and scaled with *HKL-2000* (Otwinowski & Minor, 1997). We collected a complete data set from a selenomethionine-substituted CAH–barbituric acid complex crystal at the *K* absorption edge of selenium for single-wavelength anomalous dispersion (SAD) phasing (Table 1).

3. Results and discussion

CAH was successfully expressed in *E. coli* strain BL21 (DE3) and purified to homogeneity. After affinity chromatography using a six-histidine tag attached to the N-terminus of the protein, CAH was further purified by size-exclusion chromatography. The specific activity of CAH purified using this protocol with cyanuric acid as the substrate was $7.9 \mu\text{mol min}^{-1} \text{mg}^{-1}$ as determined using the Berthelot reaction for ammonia and coupling with the enzyme biuret hydrolase (Patton & Crouch, 1977; Weatherburn, 1967). We found that the molecular mass of CAH as estimated by size-exclusion chromatography was 160 kDa, suggesting that CAH exists as a tetramer. The size of the CAH monomer as determined by SDS–PAGE (15%) was consistent with the expected molecular weight of 36 040 Da for the native protein plus the N-terminal His-tag sequence (MGSSHHHHHHSSGLVPRGSH).

In the initial crystallization screening, the unliganded form of CAH did not yield any crystals. Hence, a competitive inhibitor, barbituric acid, was added to the purified protein to stabilize the enzyme and thereby facilitate crystallization. The bound competitive inhibitor is

also helpful in discerning the active site within the CAH structure. Crystals of the inhibitor-bound CAH were observed within 3 d in conditions containing magnesium sulfate, PEG 8K or PEG 10K as precipitants. On a rotating-anode home X-ray source, the CAH–barbituric acid complex crystals grown under PEG 8K and magnesium sulfate conditions showed good diffraction images (extending to $\sim 3.0 \text{\AA}$ resolution), while crystals grown with PEG 10K did not

Figure 4

Self-rotation functions showing the presence of threefold rotational symmetry in the CAH–barbituric acid complex crystal, calculated at $3.0 \text{\AA}$ resolution using the program *MOLREP* (Vagin & Teplyaev, 2010): (a) $\kappa = 120^\circ$, (b) $\kappa = 180^\circ$ section. The integration radius was $29 \text{\AA}$. The x and y axes are parallel to the a and b axes of the crystal, respectively.

diffract well (diffraction extending to ~ 8.0 Å resolution). Because no homologous protein structure was available for CAH, we pursued structure determination by selenomethionine SAD phasing. Selenomethionine-derivative crystals could be produced in the magnesium sulfate condition, but not in the PEG-containing conditions. Therefore, an Se SAD data set extending to 2.7 Å resolution was collected on a crystal obtained from the crystallization condition containing 1.7 M magnesium sulfate and 0.1 M Tris-HCl pH 7.0.

The CAH-inhibitor complex crystals belonged to space group *I*4₁22, with unit-cell parameters $a = b = 237.9$, $c = 105.3$ Å, $\alpha = \beta = \gamma = 90^\circ$. The self-rotation function showed strong peaks on the $\kappa = 120^\circ$ section, indicating that there might be three CAH-inhibitor complexes in the crystallographic asymmetric unit related by noncrystallographic threefold symmetry or that pseudo-threefold symmetry may exist within the CAH monomer structure (Fig. 4). Assuming that two or three CAH monomers are in the asymmetric unit, the solvent content would be 73.5 or 60.2%, respectively.

The structure of the CAH-barbituric acid complex has been successfully determined by the selenomethionine SAD phasing method using PHENIX (Adams *et al.*, 2010), giving figures of merit of 0.347 and 0.690 before and after density modification, respectively, based on 29 Se sites found out of a possible 30. Pseudo-threefold internal symmetry is evident within the CAH monomer, consistent with the self-rotation function results.

Notably, while this manuscript was under review, the crystal structure was reported of AtzD, another cyanuric acid hydrolase/barbiturase family member from *Pseudomonas* sp. strain ADP which shares 51% sequence identity with the *Azorhizobium* CAH crystallized in this study (Peat *et al.*, 2013). Structural analyses of the CAH-barbituric acid complex, including detailed structural comparison between these two enzymes, will be published elsewhere.

We thank Jay Nix at beamline 4.2.2 of the Advanced Light Source for his support during data collection. The Advanced Light Source is

supported by the Director, Office of Science, Office of Basic Energy Sciences of the US Department of Energy under Contract No. DE-AC02-05CH11231. We also thank Jennifer Seffernick for valuable discussions. This work was partly supported by a grant from Lonza and from the Synthetic Ecology Initiative of the BioTechnology Institute at the University of Minnesota.

References

- Adams, P. D. *et al.* (2010). *Acta Cryst.* **D66**, 213–221.
- Cameron, S. M., Durchschein, K., Richman, J. E., Sadowsky, M. J. & Wackett, L. P. (2011). *ACS Catal.* **1**, 1075–1082.
- Cheng, G., Shapir, N., Sadowsky, M. J. & Wackett, L. P. (2005). *Appl. Environ. Microbiol.* **71**, 4437–4445.
- Dobson, R. L., Motlagh, S., Quijano, M., Cambron, R. T., Baker, T. R., Pullen, A. M., Regg, B. T., Bigalow-Kern, A. S., Vennard, T., Fix, A., Reimschuessel, R., Overmann, G., Shan, Y. & Daston, G. P. (2008). *Toxicol. Sci.* **106**, 251–262.
- Fruchey, I., Shapir, N., Sadowsky, M. J. & Wackett, L. P. (2003). *Appl. Environ. Microbiol.* **69**, 3653–3657.
- Huthmacher, K. & Most, D. (2005). *Ullmann's Encyclopedia of Industrial Chemistry*. Weinheim: Wiley-VCH. doi:10.1002/14356007.a08_191.
- Jutzi, K., Cook, A. M. & Hütter, R. (1982). *Biochem. J.* **208**, 679–684.
- Karns, J. S. (1999). *Appl. Environ. Microbiol.* **65**, 3512–3517.
- Martinez, B., Tomkins, J., Wackett, L. P., Wing, R. & Sadowsky, M. J. (2001). *J. Bacteriol.* **183**, 5684–5697.
- Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.
- Patton, C. J. & Crouch, S. R. (1977). *Anal. Chem.* **49**, 464–469.
- Peat, T. S., Balotra, S., Wilding, M., French, N. G., Briggs, L. J., Panjikar, S., Cowieson, N., Newman, J. & Scott, C. (2013). *Mol. Microbiol.* **88**, 1149–1163.
- Seffernick, J. L., Erickson, J. S., Cameron, S. M., Cho, S., Dodge, A. G., Richman, J. E., Sadowsky, M. J. & Wackett, L. P. (2012). *J. Bacteriol.* **194**, 4579–4588.
- Soong, C. L., Ogawa, J. & Shimizu, S. (2001). *Biochem. Biophys. Res. Commun.* **286**, 222–226.
- Vagin, A. & Teplyakov, A. (2010). *Acta Cryst.* **D66**, 22–25.
- Van Duyn, G. D., Standaert, R. F., Karplus, P. A., Schreiber, S. L. & Clardy, J. (1993). *J. Mol. Biol.* **229**, 105–124.
- Weatherburn, M. W. (1967). *Anal. Chem.* **39**, 971–974.