Acta Crystallographica Section D
**Biological Crystallography**
ISSN 0907-4449

# Cloning, preliminary characterization and crystallization of nucleoside hydrolases from *Caenorhabditis elegans* and *Campylobacter jejuni*

**Wim Versées,* Els Van Holsbeke, Stefan De Vos, Klaas Decanniere, Ingrid Zegers and Jan Steyaert**

Department of Ultrastructure, Vrije Universiteit Brussel, Vlaams Interuniversitair Instituut voor Biotechnologie (VIB), Pleinlaan 2, 1050 Brussels, Belgium

Correspondence e-mail: wversees@vub.ac.be

Received 17 February 2003
Accepted 27 March 2003

The nucleoside hydrolases (NHs) are a family of nucleoside-modifying enzymes. They play an important role in the purine-salvage pathway of many pathogenic organisms which are unable to synthesize purines *de novo*. Although well characterized in protozoan parasites, their precise function and mechanism remain unclear in other species. For the first time, NHs from *Caenorhabditis elegans* and *Campylobacter jejuni*, which are representatives of mesozoa and bacteria, respectively, have been cloned and purified. Steady-state kinetics indicate a different substrate-specificity profile to previously described hydrolases. Native diffraction data sets were collected from crystals of NH from each organism. The hexagonal crystals (space group $P6_222$ or $P6_422$) of NH from *C. elegans* diffracted to a resolution of 2.8 Å, while the data set from the orthorhombic crystals (space group $I222$ or $I2_12_12_1$) of NH from *C. jejuni* could be processed to 1.7 Å resolution. The unit-cell parameters were $a = b = 102.23$, $c = 117.27$ Å in the former case and $a = 101.13$, $b = 100.13$, $c = 81.37$ Å in the latter.

## 1. Introduction

The nucleoside hydrolases or *N*-ribohydrolases (NHs; EC 3.2.2.1) are key enzymes in the purine-salvage pathway of many pathogenic organisms (Berens *et al.*, 1995). They catalyse the hydrolysis of the *N*-glycosidic bond between the anomeric C atom of ribose and the base in common nucleosides. Although NHs are ubiquitous in protozoa, their appearance is not just restricted to this family. Extensive comparative analysis of known genome sequences reveals the widespread distribution of homologues in various bacteria, plants, insects and helminth parasites. Neither the encoding genes nor nucleoside hydrolase activity have ever been observed in mammalian cells or tissues (Hammond & Gutteridge, 1984).

At present, only some exemplary NHs from parasitic protozoa have been focused on. High-resolution structures combined with classical biochemical experiments have clearly depicted their function and mechanism (Versées *et al.*, 2002; Degano *et al.*, 1998; Shi *et al.*, 1999). Since protozoan parasites rely entirely upon salvage enzymes to obtain vital purine bases from the mammalian host, these enzymes are considered as very attractive targets for drug development. In particular, highly potent nucleoside hydrolase inhibitors could be very effective against protozoan infections.

While parasitic protozoa are purine auxotrophic, most other microorganisms possess a *de novo* biosynthetic pathway for purines. As a consequence, the exact function and mechanism of 'salvage' *N*-ribohydrolases in these organisms remain rather obscure. In this study, the NHs of *Caenorhabditis elegans* and *Campylobacter jejuni* have been selected as representative NHs of non-protozoan parasites. *C. elegans* embodies a multicellular eukaryote, whereas *C. jejuni* is a member of the bacterial world. Their amino-acid sequence identity to known protozoan NHs does not exceed 30%. NHs of both species were cloned, expressed and purified to homogeneity. Successful crystallization procedures led to collection of two native data sets. For the first time, attempts to reveal the function, activity and crystal structures of these particular non-protozoan NHs are reported.

## 2. Materials and methods

### 2.1. Cloning, bacterial expression and protein purification

Full-length clones corresponding to the NH ORF were generated by PCR amplification from a cDNA library of *C. elegans* (λACT-RB1) and genomic DNA from *C. jejuni* (NCTC11168, ATCC 700819), respectively. Both DNA fragments were cloned into a pQE-30 (Qiagen) expression plasmid vector, coding for an N-terminal His-tagged NH, and transformed in *Escherichia coli* strain WK6. After inoculation (100-fold dilution) in TB medium, expression of NH was initiated after 4 h incubation at 310 K by the addition of IPTG (0.5 m*M*). Growth was continued overnight at 301 K. Cells were harvested by

centrifugation and disrupted by a French press cell in 20 m*M* Tris pH 7.5 containing 10 m*M* imidazole and 1 *M* NaCl. The soluble fraction after centrifugation was loaded onto a 5 ml Ni–NTA column (Qiagen). After extensive washing with 20 m*M* Tris pH 7.5, 10 m*M* imidazole and 1 *M* NaCl, proteins were eluted using 20 m*M* Tris pH 7.5, 1 *M* imidazole and 1 *M* NaCl. The relevant fractions were pooled and run through a preparative Superdex-200 gel-filtration column (Amersham Bioscience) in 20 m*M* Tris pH 7.0, 150 m*M* NaCl, 1 m*M* $CaCl_2$.

### 2.2. Molecular mass and kinetic parameters

Kinetic parameters of NH from *C. elegans* (Table 1) and *C. jejuni* were determined as described previously (Versées *et al.*, 2001). The molecular mass of both enzymes was determined in a gel-filtration experiment on a Superdex-200 HR column (Amersham Bioscience). The oligomerization state of the proteins in the crystals was further confirmed by native gel electrophoresis (NuPAGE 7% Tris–acetate gel; Invitrogen).

### 2.3. Crystallization

NH crystals of *C. elegans* and *C. jejuni*, respectively, were obtained by application of the hanging-drop vapour-diffusion method. For *C. jejuni* NH, initial crystallization trials were carried out at room temperature using a variety of screening kits (Crystal Screen 2, Crystal Screen Cryo and Grid Screen sodium malonate; Hampton Research). Useful crystals (final protein concentration of 3.5 mg ml$^{-1}$) appeared in 1.9 *M* sodium malonate pH 7.0 (condition D3 of Grid Screen sodium malonate) after a few days.

NH crystals from *C. elegans* grew under the same conditions used for obtaining crystals of NH from *Trypanosoma vivax* (Versées *et al.*, 2001): a protein solution (at 12 mg ml$^{-1}$) was diluted with mother liquor (100 m*M* Tris pH 8.5 with 1.6 *M* ammonium sulfate) in a 1:1 ratio.

### 2.4. Data collection and processing

Prior to data collection, crystals from *C. elegans* NH were transferred into a cryoprotectant containing 100 m*M* Tris pH 8.5, 1.6 *M* ammonium sulfate and 20%(*w*/*v*) glycerol. The cryosolution for *C. jejuni* NH crystals consisted of the appropriate mother liquor (1.9 *M* sodium malonate pH 7.0) and 28%(*w*/*v*) glycerol. Both data sets were collected at 100 K on beamline BW7A of the EMBL Outstation at DESY (Hamburg, Germany) using a MAR (Hamburg, Germany) CCD detector.

The data sets were indexed, integrated, scaled and merged using the *DENZO*/*HKL* software package (Otwinowski & Minor, 1997). Intensities were converted to structure factors using *TRUNCATE* (French & Wilson, 1978). Relevant statistics for all the data sets are summarized in Table 2.

## 3. Results

### 3.1. *C. elegans* NH

The nucleoside hydrolase of the nematode *C. elegans* has been expressed and purified from *E. coli* using a simple two-step purification protocol. Yields ranged from 90 to 140 mg l$^{-1}$. The enzyme appeared as a homotetramer, while the molecular weight per monomer amounts to approximately 38 kDa.

The steady-state kinetic parameters of the *C. elegans* NH for the common nucleosides and one nucleoside analogue were determined at pH 7.0 (308 K) and are summarized in Table 1. On the basis of their substrate specificity, the NHs have thus far been divided into three subclasses: the base-aspecific inosine-uridine preferring NHs (IU-NH; Parkin *et al.*, 1991), the purine-specific inosine-adenosine-guanosine preferring NHs (IAG-NH; Versées *et al.*, 2001; Parkin, 1996) and the 6-oxo-purine-specific inosine-guanosine preferring NHs (IG-NH; Estupiñán & Schramm, 1994). The $k_{cat}/K_M$ ratios of *C. elegans* NH indicate that the enzyme is more specific towards the naturally occurring purine nucleosides than towards the pyrimidine nucleosides, owing to both a faster turnover and a higher substrate affinity (lower $K_M$) for the former. However, compared with well established IAG-NHs, this preference for purines is between one and two orders of magnitude less explicit. The extraordinary high affinity for the substrate analogue *p*-nitrophenylriboside (p-NPR) is also remarkable and differs from conventional IAG-NH behaviour. Hence, no strong evidence can be found in the substrate-activity profile of the *C. elegans* NH to classify it unequivocally into one of the three subclasses of the NH family.

*C. elegans* NH crystals that diffracted to 2.8 Å were obtained from hanging-drop vapour-diffusion experiments using ammonium sulfate as precipitant. The crystals belong to the hexagonal crystal system, with unit-cell parameters $a = b = 102.23$, $c = 117.27$ Å. At this stage of the study, no distinction can be made between the space group $P6_222$ or $P6_422$. The unit-cell volume is consistent with one monomer per asymmetric unit, giving a Matthews coefficient of 2.30 Å$^3$ Da$^{-1}$ (Matthews, 1968) and a calculated solvent content of 40%. Using synchrotron radiation, a data set 99.6% complete to 2.8 Å resolution with an $R_{merge}$ of 4.3% could be collected.

**Table 1**
Kinetic constants for the nucleoside hydrolase from *C. elegans*.

| Substrate | $k_{cat}$ (s$^{-1}$) | $K_M$ (μ*M*) | $k_{cat}/K_M$ (*M*$^{-1}$ s$^{-1}$) |
|---|---|---|---|
| Inosine | 0.79 ± 0.04 | 295 ± 44 | 2680 ± 422 |
| Adenosine | 0.78 ± 0.05 | 93 ± 16 | 8390 ± 1540 |
| Guanosine | 0.84 ± 0.09 | 164 ± 56 | 5120 ± 1830 |
| Cytidine | 0.50 ± 0.03 | 10004 ± 1320 | 50 ± 7 |
| Uridine | 0.0022 ± 0.0002 | 2843 ± 813 | 0.8 ± 0.2 |
| Xanthosine | 0.029 ± 0.002 | 1038 ± 274 | 28 ± 8 |
| p-NPR | 0.119 ± 0.003 | 0.9 ± 0.1 | $1.3 \times 10^5 \pm 2 \times 10^4$ |

**Table 2**
Diffraction data of NHs from *C. elegans* and *C. jejuni*.

Values in parentheses are for the highest resolution shell.

| | *C. elegans* | *C. jejuni* |
|---|---|---|
| Space group | $P6_222$ or $P6_422$ | $I222$ or $I2_12_12_1$ |
| Unit-cell parameters | | |
| *a* (Å) | 102.23 | 101.13 |
| *b* (Å) | 102.23 | 100.13 |
| *c* (Å) | 117.27 | 81.37 |
| α (°) | 90 | 90 |
| β (°) | 90 | 90 |
| γ (°) | 120 | 90 |
| Matthews coefficient (Å$^3$ Da$^{-1}$) | 2.30 | 2.65 |
| Solvent content (%) | 40.0 | 54.3 |
| Unit-cell volume (Å$^3$) | 1061374.8 | 823972.8 |
| No. molecules per unit cell | 12 | 8 |
| No. molecules per AU | 1 | 1 |
| No. unique reflections | 9327 | 45122 |
| No. observed reflections | 78023 | 264057 |
| Criterion for observed reflections | All | All |
| Wavelength (Å) | 0.976 | 0.979 |
| Resolution range | 20.0–2.80 (2.90–2.80) | 25.0–1.7 (1.76–1.70) |
| Completeness (%) | 99.6 (100.0) | 98.5 (95.7) |
| $R_{merge}$ | 0.043 (0.218) | 0.061 (0.752) |
| $\langle I/\sigma(I)\rangle$ | 42.69 (6.50) | 13.73 (2.20) |

### 3.2. *C. jejuni* NH

Expression and purification of *C. jejuni* NH was carried out in the same fashion as for *C. elegans* NH. The NH ORF was cloned in a pQE-30 vector (Qiagen), which allowed easy purification on an Ni–NTA column, followed by gel filtration. The production levels of the enzyme were quite similar to the *C. elegans* NHs and reached the

100 mg $l^{-1}$ scale. The protein behaves as a dimer or tetramer of 39 kDa subunits.

Despite an almost 25% amino-acid sequence identity with the NHs of *Crithidia fasciculata* and *Trypanosoma brucei brucei*, which are strong IU- and IAG-preferring NHs, respectively, no activity towards the natural nucleosides nor towards common nucleoside analogues could be measured for this enzyme. At this point, it remains unclear what the real function of NHs in this particular prokaryote might be. Since most prokaryotes are capable of synthesizing their purines *de novo*, the NHs might have evolved to salvage less common nucleoside analogues.

Crystallization efforts succeeded and we were able to collect a data set from crystals that diffracted to a resolution of 1.7 Å. These crystals belong to space group $I222$ or $I2_12_12_1$, with unit-cell parameters $a = 101.13$, $b = 100.13$, $c = 81.37$ Å. The solvent content (54.3%) is somewhat higher than for ordinary soluble proteins (43%; Matthews, 1968). A $V_M$ of 2.65 Å$^3$ Da$^{-1}$ is in agreement with one monomer in the asymmetric unit. Since the protein behaves as a dimer or tetramer in solution, this strongly favours $I222$ over $I2_12_12_1$ as the appropriate space group. 264 057 reflections (45 122 unique) were used for an overall completeness of 98.5% (95.7% in the outer shell). $R_{merge}$ amounts to 6.1% and the average $I/\sigma(I)$ is 13.73.

This work was supported by the Vlaams Interuniversitair Instituut voor Biotechnologie (VIB) and by the Nationaal Fonds voor Wetenschappelijk Onderzoek-Vlaanderen (FWO). We acknowledge the use of beamline BW7A at EMBL-Hamburg.

## References

Berens, R. L., Krug, E. D. & Marr, J. J. (1995). *Biochemistry and Molecular Biology of Parasites*, edited by J. J. Marr & M. Müller, pp. 89–117. New York: Academic Press.

Degano, M., Almo, S. C., Sacchetini, J. C. & Schramm, V. L. (1998). *Biochemistry*, **37**, 6277–6285.

Estupiñán, B. & Schramm, V. L. (1994). *J. Biol. Chem.* **269**, 23068–23073.

French, S. & Wilson, K. (1978). *Acta Cryst.* A**34**, 517–525.

Hammond, D. J. & Gutteridge, W. E. (1984). *Mol. Biochem. Parasitol.* **13**, 243–261.

Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.

Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.

Parkin, D. W. (1996). *J. Biol. Chem.* **271**, 21713–21719.

Parkin, D. W., Horenstein, B. A., Abdulah, D. R., Estupiñán, B. & Schramm, V. L. (1991). *J. Biol. Chem.* **266**, 20658–20665.

Shi, W., Schramm, V. L. & Almo, S. C. (1999). *J. Biol. Chem.* **274**, 21114–21120.

Versées, W., Decanniere, K., Van Holsbeke, E., Devroede, N. & Steyaert, J. (2002). *J. Biol. Chem.* **277**, 15938–15946.

Versées, W., Decanniere, K., Pellé, R., Depoorter, J., Brosens, E., Parkin, D. W. & Steyaert, J. (2001). *J. Mol. Biol.* **307**, 1363–1379.