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Crystallization and preliminary X-ray structural analysis of nucleoside triphosphate hydrolases from *Neospora caninum* and *Toxoplasma gondii*

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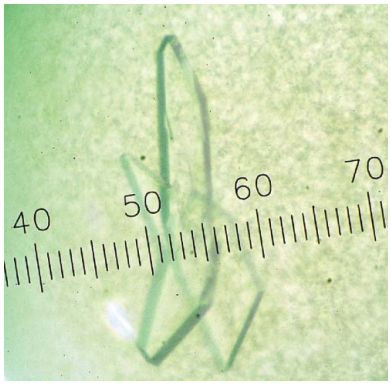


The nucleoside triphosphate hydrolases that are produced by *Neospora caninum* (NcNTPase) and *Toxoplasma gondii* (TgNTPase-I) have a different physiological function from the ubiquitous ecto-ATPases. The recombinant enzymes were crystallized at 293 K using polyethylene glycol 3350 as a precipitant and X-ray diffraction data sets were collected for NcNTPase (to 2.8 Å resolution) and TgNTPase-I (to 3.1 Å resolution) at 100 K using synchrotron radiation. The crystals of NcNTPase and TgNTPase-I belonged to the orthorhombic space group $I222$ (unit-cell parameters a = 93.6, b = 140.8, c = 301.1 Å) and the monoclinic space group $P2_1$ (unit-cell parameters a = 87.1, b = 123.5, c = 120.2 Å, β = 96.6°), respectively, with two NcNTPase (V_M = 3.7 Å^3 Da^{-1}) and four TgNTPase-I (V_M = 2.7 Å^3 Da^{-1}) molecules per asymmetric unit. SAD phasing trials using a data set (λ = 0.97904 Å) collected from a crystal of selenomethionylated NcNTPase gave an initial electron-density map of sufficient quality to build a molecular model of NcNTPase.

1. Introduction

Toxoplasma gondii is an obligate intracellular protozoan parasite that commonly infects many warm-blooded animals, including humans (Dubey, 1988). Toxoplasmosis is a significant problem among patients with AIDS, organ-transplant recipients, pregnant women who are infected during the early stage of gestation and individuals with ocular toxoplasmosis (Remington *et al.*, 1995). *Neospora caninum*, a protozoan of the phylum Apicomplexa closely related to *T. gondii*, is recognized as a major cause of disease in dogs (Reichel *et al.*, 2007) and abortions in cattle (Dubey *et al.*, 2007). *N. caninum* is a relatively newly recognized parasite (Dubey *et al.*, 1988) that is morphologically similar (Lindsay *et al.*, 1993; Speer & Dubey, 1989) and phylogenetically close (Marsh *et al.*, 1995) to *T. gondii*. Despite their structural and phylogenetic similarities, polyclonal antisera against *T. gondii* show limited cross-reactivity against *N. caninum* (Dubey, 1988) and many monoclonal antibodies against *T. gondii* antigens do not cross-react with *N. caninum* trophozoites (Howe & Sibley, 1997).

Reflecting their close evolutionary relationship, these two parasites share a novel nucleoside triphosphate hydrolase (NTPase; EC 3.6.1.15) that differs from the ubiquitous common ecto-ATPases (Asai *et al.*, 1995, 1998). The most striking feature of NTPase is that the enzyme is produced in large quantities (about 2–8% of the total protein in the tachyzoite cells of *T. gondii*) as a dormant enzyme in the tachyzoite stage, where it accumulates in the dense secretory granules (Asai & Kim, 1987; Asai *et al.*, 1983; Nakaar *et al.*, 1998). After secretion from the tachyzoite cells into the parasitophorous vacuolar space, NTPase seems to be activated by a dithiol compound or an unknown dithiol–disulfide oxidoreductase within the parasitophorous vacuole (Weiss & Kim, 2007). *In vitro*, NTPase can be activated by dithiols such as dithiothreitol (Asai *et al.*, 1983, 1998). Although the physiological function of NTPase has not been fully

elucidated, a study by Silverman *et al.* (1998) suggested that the NTPase is involved in controlling tachyzoite egress from host cells.

It has been found that type I strains of *T. gondii*, which are acutely virulent in mice, contain two isoforms of NTPase (Asai *et al.*, 1995): the type I enzyme (TgNTPase-I) preferentially hydrolyzes triphosphate nucleosides, whereas the type II enzyme (TgNTPase-II; 97% amino-acid sequence identity to TgNTPase-I) hydrolyzes both triphosphate and diphosphate nucleosides at approximately equal rates. TgNTPase-I appears to only be present in the type I virulent strains, while TgNTPase-II is universally present in all *T. gondii* strains (Asai *et al.*, 1995). *N. caninum*, which is less pathogenic in mice and has not been reported to infect humans (Dubey & Lindsay, 1996), also produces a dormat NTPase (NcNTPase) that is similar to TgNTPase-I (70% amino-acid sequence identity; Asai *et al.*, 1998). These three NTPases belong to the Gda1/CD39 (Pfam 01150) conserved family of ecto-ATPases with apyrase activity.

In the present study, we report the purification, crystallization and preliminary X-ray structural analysis of NcNTPase and TgNTPase-I.

2. Materials and methods

2.1. Expression and purification of recombinant NcNTPase and TgNTPase-I

The NcNTPase and TgNTPase-I genes (GenBank accession No. AB525222 and UniProt Q27893, respectively) have previously been cloned (Asai *et al.*, 1995, 1998). The plasmids encoding NcNTPase (602 amino-acid residues) and TgNTPase-I (603 amino-acid residues) were introduced into *Escherichia coli* strain BL21 and the recombinant enzymes were expressed and purified as described below. All purification steps were conducted at 277 K unless otherwise noted.

The transformant was grown at 310 K in 1000 ml Luria–Bertani medium containing 50 µg ml^{-1} ampicillin until the absorbance at 600 nm (A_{600}) reached about 0.6. Expression of recombinant NTPase was induced with 0.2 m*M* isopropyl β-D-1-thiogalactopyranoside (IPTG) at 310 K for 2.5 h. The cell pellet (about 3 g wet weight) harvested by centrifugation at 5000*g* for 10 min was suspended in 30 ml lysis buffer (50 m*M* Tris–HCl pH 8.0, 1 m*M* EDTA, 100 m*M* NaCl). After disruption of the cells by sonication, the lysate was centrifuged at 25 000*g* for 20 min. The precipitate containing the inclusion bodies of the expressed NTPase was suspended in 100 ml washing buffer [50 m*M* Tris–HCl pH 8.0, 1 m*M* EDTA, 100 m*M* NaCl and 4%(*w*/*v*) Thesit detergent (Sigma–Aldrich, St Louis, Missouri, USA)] and incubated at room temperature for 30 min with shaking. After centrifugation at 25 000*g* for 20 min, this procedure was repeated. The precipitate containing the inclusion bodies (about 1 g wet weight) was then washed twice with Milli-Q water to remove Thesit and any water-soluble material and dissolved (1 mg ml^{-1}) in unfolding buffer (50 m*M* glycine–NaOH pH 10.8, 8 *M* urea, 10 m*M* glycine ethyl ester, 1 m*M* EDTA). After standing for 1 h at 310 K, the unfolded NTPase was renatured by dialysis against ten volumes of folding buffer (50 m*M* glycine–NaOH pH 10.8, 10 m*M* glycine ethyl ester, 1 m*M* EDTA, 1 m*M* reduced glutathione, 0.6 m*M* oxidized glutathione) for 4 h and finally against ten volumes of desalting buffer (50 m*M* Tris–HCl pH 7.5, 50 m*M* potassium acetate) for 4 h with three exchanges of the buffer.

After centrifugation at 145 000*g* for 30 min, the supernatant was filtrated through a 0.45 µm pore-size filter and loaded onto a Toyopearl DEAE-650S (Tosoh, Tokyo, Japan) column (1.6 × 20 cm) pre-equilibrated with 20 m*M* Tris–HCl pH 8.5. The column was washed with the same buffer until the A_{280} was 0 and was eluted with a 800 ml linear gradient of 0–500 m*M* KCl at a flow rate of 1 ml min^{-1}. Fractions containing NTPase were pooled and desalted by dialysis against 20 m*M* Tris–HCl pH 8.5 and further purified using a Source 15Q (GE Healthcare, Little Chalfont, England) column (1.0 × 10 cm) according to the same procedure as described for the Toyopearl DEAE-650S column chromatography. The active peak fractions were pooled and the buffer was exchanged to 20 m*M* Tris–HCl pH 7.5 containing 200 m*M* KCl by repeated dilution and concentration with a centrifugal concentrator tube (Amicon Ultra-4 Ultracel-10K). The concentrated NTPase solution was applied onto a Superdex 200 pg (GE Healthcare) column (1.6 × 60 cm) and eluted at a flow rate of 1 ml min^{-1}. The buffer of the pooled NTPase fractions was exchanged for 20 m*M* Tris–HCl pH 7.5 and the purified NTPase was concentrated to about 10 mg ml^{-1} with a centrifugal concentrator tube (Amicon Ultra-4 Ultracel-10K).

Selenomethionylated NcNTPase was also overexpressed in *E. coli* strain BL21. The transformant was grown at 310 K in 1000 ml M9 minimal medium (Sambrook *et al.*, 1989) supplemented with 1 m*M* $MgSO_4$, 50 µ*M* $MnCl_2$, 3.3 µ*M* $FeCl_3$, 76 µ*M* thiamine, 82 µ*M* biotin, 75 µ*M* adenosine, 71 µ*M* guanosine, 82 µ*M* cytidine, 83 µ*M* thymidine and 50 µg ml^{-1} ampicillin. When the A_{600} reached about 0.3, 100 mg lysine, 100 mg phenylalanine, 100 mg threonine, 50 mg isoleucine, 50 mg leucine, 50 mg valine and 60 mg selenomethionine were added to the culture. The expression, renaturation and purification of the selenomethionylated NcNTPase were carried out as described above.

2.2. Assay of NTPase activity

The enzymatic activity of NTPase was assayed as described previously (Asai *et al.*, 1995, 2002) with minor modifications. To the reaction reagent (100 µl) consisting of 50 m*M* HEPES–KOH pH 7.5, 1 m*M* ATP, 6 m*M* magnesium acetate and 5 m*M* dithiothreitol, 0.5–2.0 µg NTPase was added. After standing for 10 min at 310 K, the reaction was terminated by the addition of 50 µl 0.1 *M* HCl; 40 µl 2.5% ammonium molybdate dissolved in 5 *N* H_2SO_4 and 10 µl 15.8 mg ml^{-1} Fiske & Subbarow Reducer (Sigma–Aldrich) were then added. The inorganic orthophosphate liberated by the ATP hydrolysis was analyzed colorimetrically by measuring the A_{710}.

2.3. Crystallization

Crystallization experiments were performed in CrystalClear Strips (Hampton Research, Riverside, California, USA) using the sitting-drop vapour-diffusion technique. In the initial screening for crystallization conditions, a 0.5 µl droplet containing about 10 mg ml^{-1} NTPase dissolved in 20 m*M* Tris–HCl pH 7.5 was mixed with an equal volume of reservoir solution and the droplet was allowed to equilibrate against 100 µl reservoir solution. Initial crystallization conditions for NcNTPase and TgNTPase-I were screened at 277 and 293 K using commercially available screening kits from Hampton Research (Crystal Screen, Crystal Screen 2, Crystal Screen Lite, MembFac and Natrix) and Emerald BioStructures (Wizard I, Wizard II, Cryo I and Cryo II). Of the 436 conditions screened, both NcNTPase and TgNTPase-I could be crystallized at 293 K using Crystal Screen Lite conditions No. 22 [15%(*w*/*v*) PEG 4000, 0.1 *M* Tris–HCl pH 8.5 and 0.2 *M* sodium acetate] and No. 17 [15%(*w*/*v*) PEG 4000, 0.1 *M* Tris–HCl pH 8.5 and 0.2 *M* lithium sulfate], MembFac condition No. 44 [12%(*w*/*v*) PEG 6000, 0.1 *M* Tris–HCl pH 8.5 and 0.1 *M* diammonium phosphate] and Crystal Screen 2 condition No. 30 [10%(*w*/*v*) PEG 6000, 0.1 *M* Na HEPES pH 7.5 and 5%(*v*/*v*) 2-methyl-2,4-pentanediol]. The conditions were then optimized at 277 and 293 K by varying the PEG concentration (4–20%) and the buffer pH (6.0–9.0) using PEG 3350, PEG 6000 and PEG 8000 as precipitants. The effects of adding salts (0.0–400 m*M* lithium

sulfate and diammonium phosphate) and organic solvents [0.0–15%(*v*/*v*) 2-methyl-2,4-pentanediol and glycerol] were also examined. The best crystals of NcNTPase were grown at 293 K using a reservoir solution consisting of 8–10%(*w*/*v*) PEG 3350, 0.1 *M* Tris–HCl pH 7.4, 50 m*M* diammonium phosphate and 2%(*v*/*v*) glycerol. Selenomethionylated NcNTPase was also crystallized using the same reservoir solution. Crystals of TgNTPase-I (0.5 × 0.2 × 0.05 mm) were obtained at 293 K from reservoir solution consisting of 10–12%(*w*/*v*) PEG 3350, 0.1 *M* Tris–HCl pH 7.4, 2%(*v*/*v*) glycerol.

2.4. Data collection and processing

X-ray diffraction experiments were performed under cryocooled conditions at 100 K. A crystal mounted in a nylon loop was transferred into and soaked briefly in reservoir solution supplemented with 20%(*v*/*v*) glycerol and then rapidly flash-cooled in a cold nitrogen stream (100 K). X-ray diffraction experiments were carried out on the BL44XU beamline (equipped with a Bruker DIP-6040 detector or a Rayonix CCD MX225HE detector) and the BL41XU beamline (equipped with a Rayonix CCD MX225HE detector) at SPring-8, Harima, Japan and the BL17A beamline (equipped with an ADSC Quantum 270 detector) at Photon Factory (PF), Tsukuba, Japan. X-ray diffraction patterns were recorded with an oscillation angle of 1°, an exposure time of 1 s per frame and a crystal-to-detector distance of 150 mm and were processed with the *HKL*-2000 software package (Otwinowski & Minor, 1997).

3. Results and discussion

After renaturation, purified recombinant NcNTPase (11 mg), selenomethionylated NcNTPase (5 mg) and TgNTPase-I (5 mg) could be obtained from 1000 ml culture using DEAE-650S, Source 15Q and Superdex 200 pg column chromatography (Fig. 1). The yields of purified protein from the inclusion bodies were about 0.5–1.1%. The recombinant enzymes showed specific activities that were comparable with those purified from the parasites (Asai *et al.*, 1998). The molecular weights estimated by gel filtration (240–270 kDa) suggested that the enzymes exist as homotetramers (66 kDa × 4) in solution.

The optimized reservoir solution for the crystallization of NcNTPase [8–10%(*w*/*v*) PEG 3350, 0.1 *M* Tris–HCl pH 7.4, 50 m*M* diammonium phosphate, 2%(*v*/*v*) glycerol] yielded crystals with maximum dimensions of 0.3 × 0.2 × 0.1 mm at 293 K in 5–6 d (Fig. 2*a*). However, this condition produced a sticky precipitate that adhered to the crystals in the sitting drops, which made it difficult to scoop up a crystal with a nylon loop. The formation of the sticky precipitate could be avoided by diluting the sitting drop twice with water before equilibration against the reservoir solution. Selenomethionylated NcNTPase was also crystallized under the same conditions. Crystals of TgNTPase-I grew to 0.5 × 0.2 × 0.05 mm at 293 K from reservoir solution consisting of 10–12%(*w*/*v*) PEG 3350, 0.1 *M* Tris–HCl pH 7.4 and 2%(*v*/*v*) glycerol within 10 d (Fig. 2*b*). Analyses of the symmetry and systematic absences in the recorded diffraction patterns indicated that the crystals of NcNTPase belonged to the orthorhombic space group *I*222 (unit-cell parameters a = 93.6, b = 140.8, c = 301.1 Å). Assuming the presence of two NcNTPase molecules in the asymmetric unit, the calculated V_M value (Matthews, 1968) is 3.7 Å^3 Da^{-1}, which corresponds to a solvent content of 67%. The crystals of TgNTPase-I belonged to the monoclinic space group $P2_1$ (unit-cell parameters a = 87.1, b = 123.5, c = 120.2 Å, β = 96.6°)

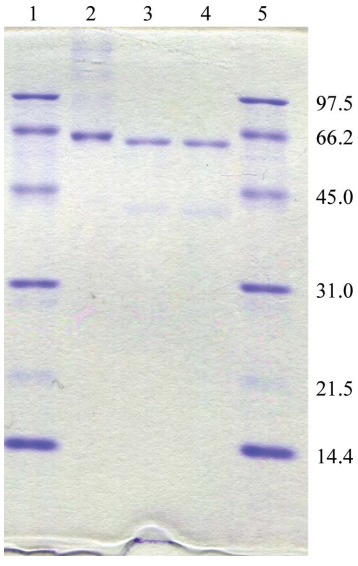
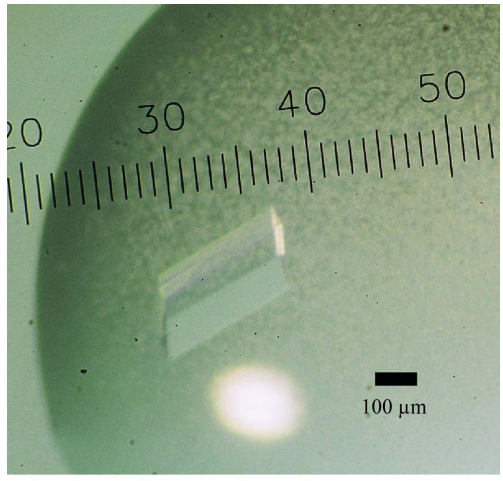
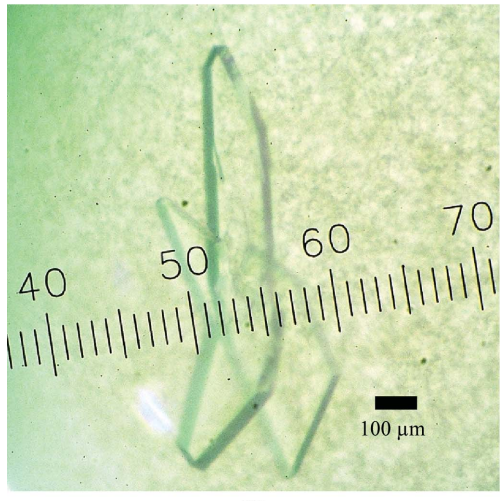


Figure 1
A Coomassie Brilliant Blue R250-stained 12.5% SDS–PAGE gel showing the purity of purified TgNTPase-I (lane 2), NcNTPase (lane 3) and selenomethionylated NcNTPase (lane 4). Lanes 1 and 5 contain molecular-weight markers (kDa).



(*a*)



(*b*)

Figure 2
Crystals of (*a*) NcNTPase and (*b*) TgNTPase-I obtained by sitting-drop vapour diffusion using PEG 3350 as a precipitant.

Table 1
Statistics of data collection and processing.

Values in parentheses are for the highest resolution shell.

	NcNTPase		TgNTPase-I
	Native	SeMet derivative	Native
X-ray source	BL41XU, SPring-8	BL44XU, SPring-8	BL17A, PF
Wavelength (Å)	1.0000	0.97904	0.9800
Space group	*I*222	*I*222	$P2_1$
Unit-cell parameters			
a (Å)	93.6	93.9	87.1
b (Å)	140.8	143.6	123.5
c (Å)	301.1	300.8	120.2
β (°)			96.6
Solvent content† (%)	68	68	50
Resolution range (Å)	50.0–2.8 (2.90–2.80)	50.0–3.6 (3.66–3.60)	50.0–3.1 (3.21–3.10)
No. of reflections	265966	383160	79915
Unique reflections	49909	23449	45499
Completeness (%)	93.5 (94.9)	100.0 (100.0)	88.6 (91.0)
$R_{\rm merge}$‡ (%)	6.0 (55.2)	12.6 (76.5)	7.6 (48.1)
Mean $I/\sigma(I)$	17.2 (3.9)	11.3 (5.6)	8.6 (1.3)

† Assuming the presence of two (NcNTPase) and four (TgNTPase-I) molecules in the asymmetric unit. ‡ $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the intensity of the *i*th observation of reflection *hkl*.

with a $V_{\rm M}$ of 2.7 Å^3 Da^{-1} and a solvent content of 50% when the presence of four TgNTPase-I molecules in the asymmetric unit was assumed. The statistics of data collection are shown in Table 1.

The initial phase angles of NcNTPase were determined at 3.6 Å resolution by the SAD method using a X-ray diffraction data set (λ = 0.97904 Å) collected from a selenomethionylated NcNTPase crystal. The program *SOLVE* (Terwilliger & Berendzen, 1999) was used to locate and refine 23 Se sites from the 28 selenomethionine residues (FOM = 0.404). The phases were extended to 3.1 Å resolution by the solvent-flattening method using the program *RESOLVE* (Terwilliger, 2000). A solvent content of 67% was assumed (FOM = 0.709). An initial model composed of 630 amino-acid residues from the two NcNTPase molecules in the asymmetric unit (602 × 2 residues) was generated by *RESOLVE*. Next, model building was continued using *Buccaneer* (Cowtan, 2006). After eight cycles of calculation, 1194 residues could be incorporated into the model. At present, the refinement of the NcNTPase structure at 2.8 Å resolution has been completed (*R* factor = 0.228 and $R_{\rm free}$ = 0.265) and the coordinates and structure factors have been deposited in the Protein Data Bank (PDB code 3agr). An attempt to solve the structure of TgNTPase-I using molecular replacement with *MOLREP* (Vagin & Teplyakov, 1997) as implemented within the *CCP*4 package (Collaborative Computational Project, Number 4, 1994) was carried out using the refined NcNTPase model (70% sequence identity). X-ray diffraction data in the resolution range 8.0–4.0 Å were used. The best solution showed a correlation coefficient of 0.640 and an *R* factor of 46.2%. Subsequent rigid-body refinement of the model gave an *R* factor of 43.2%. The refinement of the tetrameric structure of TgNTPase-I at 3.1 Å resolution is in progress. In parallel with the refinement, we are now attempting to purify and crystallize a mutant NcNTPase which shows triphosphate nucleoside hydrolysis activity without activation by dithiols. Details of the structures will be published elsewhere.

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