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Expression, purification, crystallization and preliminary crystallographic analysis of a deblocking aminopeptidase from *Pyrococcus horikoshii*

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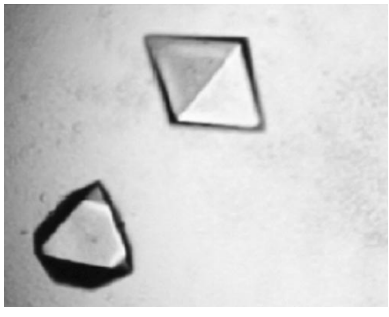

The deblocking aminopeptidase (DAP) of *Pyrococcus horikoshii* is a hyperthermophilic exoprotease that cleaves the N-terminal amino acid of peptide substrates with a putative deblocking activity for acylated peptides. DAP has been found to be homologous to a tetrahedral aminopeptidase from the halophilic *Haloarcula marismortui*. The latter enzyme is a dodecameric complex and has been revealed to be a self-compartmentalized protease whose central cavity harbouring the catalytic site is accessible through several channels of different size, unlike all other known proteolytic complexes. Three paralogues of DAP have been identified in *P. horikoshii*, with about 40% identity between them. Each of them has been overexpressed in *Escherichia coli*, purified and crystallized in the native and selenomethionine-substituted states. The results indicate that they form two kinds of assemblies, of 12 and of 24 subunits, with a molecular weight of ∼400 and ∼800 kDa, respectively. Crystals of the different variants of DAP and in their different oligomeric states diffract up to a resolution of 3 Å.

1. Introduction

Pyrococcus horikoshii OT3 is a hyperthermophilic archaeon isolated from a deep-sea hydrothermal vent in the Okinawa trough (Gonzalez *et al.*, 1998), the complete genome of which has been sequenced (Kawarabayasi *et al.*, 1998). This archaeon is able to grow at temperatures of between 361 and 377 K, with an optimal growth temperature of 371 K. The enzymes produced by this hyperthermophilic organism are extremely heat-resistant and may therefore be utilized in various industrial fields such as depollution, food, biomedical engineering *etc*.

A new aminopeptidase from *P. horikoshii* has been identified recently by Ando and coworkers (gene PH0519; Ando *et al.*, 1999). They have shown that this enzyme has an aminopeptidase activity and cleaves the N-terminal amino acid from a variety of peptide substrates and that it also has a putative acylamino-acid-releasing (deblocking) activity for acyl (blocked) peptides. This enzyme, therefore called deblocking aminopeptidase (DAP), has been revealed to be homologous to a novel large protease complex called tetrahedral aminopeptidase (TET) isolated from the salt-loving archaeon *Haloarcula marismortui* (Franzetti *et al.*, 2002). Electron microscopy showed TET to be a dodecameric complex with a tetrahedral shape, the molecular weight of the monomer being 42 kDa. However, the real originality of TET lies in its architecture, as it has a central cavity that is accessible through four narrow channels (<17 Å) and through four wider channels (21 Å wide), whereas all the proteolytic complexes described so far have a single central channel with only two openings. The existence and the dimensions of these channels suggest that TET, like the proteasome, is self-compartmentalized, with the active sites situated in the inner cavity (Franzetti *et al.*, 2002). Self-compartmentalization is a strategy developed by prokaryotic cells to control protein degradation by confining the proteolytic action to sites that are only accessible to proteins displaying some sort of degradation signal and thus to prevent unwanted destruction of proteins (Baumeister *et al.*, 1998).

In common with TET, DAP is a metallo-aminopeptidase, classified by the peptidase database MEROPS (http://merops.sanger.ac.uk/) into family M42. Unlike many zinc-dependent aminopeptidases, it has

been shown that DAP requires cobalt ions for activity (Onoe *et al.*, 2002). Very few proteases from hyperthermophilic microorganisms have been characterized biochemically so far and proteolysis in these organisms is poorly understood despite the availability of complete genome sequences (Ward *et al.*, 2002). Only three other peptidases from *P. horikoshii* have been studied (Du *et al.*, 2000; Ishikawa *et al.*, 1998; Sokabe *et al.*, 2002). We have therefore undertaken a structural study of DAP in order to better understand its proteolytic function.

A close analysis of the 1.74 Mbp genome of this archaeon revealed the presence of a set of three homologous genes of DAP: PH0519, PH1527 and PH1821, the latter two both being on the same large operon FRV. Accordingly, the corresponding proteins were called DAP1, DAP2 and DAP3. They share 37% identity between DAP1 and DAP2, 36% between DAP1 and DAP3 and 49% between DAP2 and DAP3. They possess 332, 353 and 354 residues, respectively.

Here, we present the expression, purification and crystallization conditions of these three proteins and show our preliminary results on some of the crystals obtained.

2. Protein expression and purification

We used *Escherichia coli* strain BL21 (DE3) (Novagen) for expression of recombinant DAP1 and strain BL21 pLys S (DE3) (Novagen) for expression of recombinant DAP2 and DAP3. The cells were grown in 2YT medium (1% yeast extract, 1.6% tryptone and 0.5% NaCl) containing ampicillin (100 µg ml^{-1}) for both strains and also chloramphenicol (34 µg ml^{-1}) in the case of BL21 pLys S (DE3). After incubation with shaking at 310 K until the A_{600} reached 0.6–1.0, induction was carried out by adding isopropyl β-D-thiogalactopyranoside to a final concentration of 0.05 m*M* and shaking for 4 h at 310 K.

The induced cells were harvested by centrifugation. The pellets were then maintained at 193 K overnight. Complete disruption of the cells was then achieved using a French press after thawing and suspension in 50 m*M* Tris pH 8.0, 150 m*M* NaCl supplemented with DNase I and RNase. The crude extract was heated at 358 K for 30 min. The lysate was clarified by centrifugation at 25 000*g* for 30 min. The supernatant was loaded onto a HiTrap Q column (Amersham Pharmacia Biotech) and eluted with a linear NaCl gradient (0–1.5 *M* in the same buffer). The fractions containing protein of similar weight (37–39 kDa) according to SDS–PAGE were collected. The protein solutions were then concentrated using an Amicon cell (Millipore) with a molecular-weight cutoff of 30 kDa. The protein was then loaded onto a Superdex 200 Hiload 26/60 column (Amersham Pharmacia Biotech). In the case of DAP1, three elution peaks corresponding to approximate molecular weights of >1000 kDa (pool 1), 800 kDa (pool 2) and 400 kDa (pool 3) were observed. For DAP2 and DAP3, only the first (pool 1) and the last elution peaks (pool 3) were observed (Fig. 1). According to SDS–PAGE and N-terminal sequencing, all the fractions contained DAP. This suggests that DAP exists in different oligomerization states. The first peak corresponds to soluble aggregates and was not kept. The two other fractions were kept at 278 K after concentration to about 10 mg ml^{-1}.

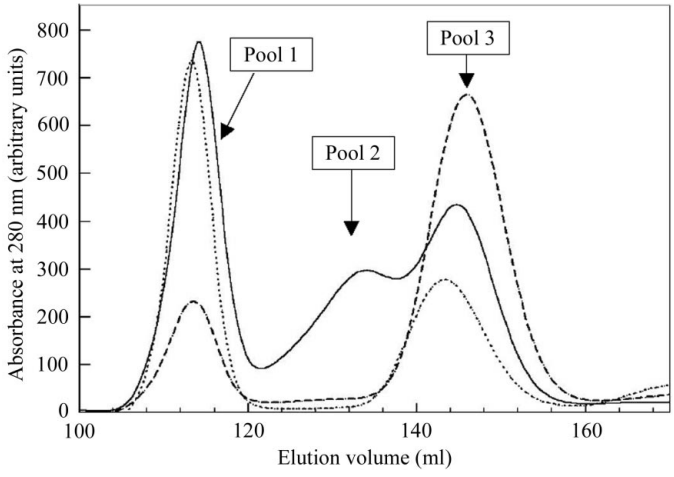

Figure 1
Elution profiles of DAP1 (continuous line), DAP2 (dashed line) and DAP3 (dotted line) obtained during gel-filtration chromatography; different pools of DAP are marked by the arrows.

The protein concentration was measured using the Biorad protein-assay reagent (Bio-Rad).

3. Preparation of selenomethionine derivative

As DAP1, DAP2 and DAP3 contain five, eight and 13 methionines, respectively, SeMet derivatives of each DAP were prepared. The same host cells were grown in M9 minimal medium supplemented with the corresponding antibiotics. Before induction, amino acids repressing methionine biosynthesis were added together with L-SeMet. Cell-harvesting and purification protocols were the same as described above. Mass-spectrometry analysis indicated that 100% selenomethionine incorporation had been obtained.

4. Crystallization

Initial crystallization screenings were performed with a Cartesian nanodrop-dispensing robot using the sitting-drop method. These screenings use three different sparse matrixes: MDL I and II (Molecular Dimension), Stura (Stura *et al.*, 1992) and Wizard. A scale-up in classical 24-well plates using the hanging-drop vapour-diffusion method (McPherson, 1999) resulted in crystals suitable for data collection.

Interestingly, pool 3 of each of the three paralogues of DAP always led to cubic crystals (Figs. 2*b*, 2*d* and 2*f*). However, elongated crystals of DAP1 were also observed (Fig. 2*c*). Pool 2, which was only obtained for DAP1, also yielded plate-like crystals (Fig. 2*a*). The drops consisted of 2 µl protein solution (10 mg ml^{-1} in 50 m*M* Tris pH 8.0 and 150 m*M* NaCl) and 2 µl precipitant. The crystallization conditions were as follows.

(*a*) DAP1 pool 2: 21%(*w*/*v*) PEG 5K, 0.1 *M* HEPES pH 7.5 (no diffraction).

(*b*) DAP1 pool 3: 1.4 *M* $(NH_4)_2SO_4$, 0.1 *M* CH_3CO_2Na pH 5.4, 1 m*M* $CoCl_2$ ($F4_132$, $a = b = c = 221.9$ Å, resolution 3.0 Å).

(*c*) DAP1 pool 3: 40%(*v*/*v*) MPD, 0.1 *M* Tris pH 8.5, 250 m*M* NaCl (primitive orthorhombic, unit-cell parameters 212.9, 222.0, 236.0, resolution = 3.8 Å).

(*d*) DAP2 pool 3: 43%(*v*/*v*) MPD, 0.1 *M* Tris pH 8.5 (face-centered cubic, resolution > 6.0 Å).

(*e*) DAP2 pool 3: 41%(*v*/*v*) MPD, 0.1 *M* Tris pH 8.5, 0.15 *M* $(NH_4)H_2PO_4$ (not tested).

(*f*) DAP3 pool 3: 50%(*v*/*v*) MPD, 0.1 *M* Tris pH 8.5, 0.1 *M* $(NH_4)H_2PO_4$ (face-centered cubic, resolution > 8.0 Å).

The crystals obtained with conditions (*a*), (*b*), (*c*), (*d*), (*e*) and (*f*) are shown in Figs. 2(*a*), 2(*b*), 2(*c*), 2(*d*), 2(*e*) and 2(*f*), respectively.

5. Preliminary X-ray diffraction results and discussion

All preliminary diffraction experiments on the DAP crystals were performed using synchrotron radiation on beamlines ID14-EH1,

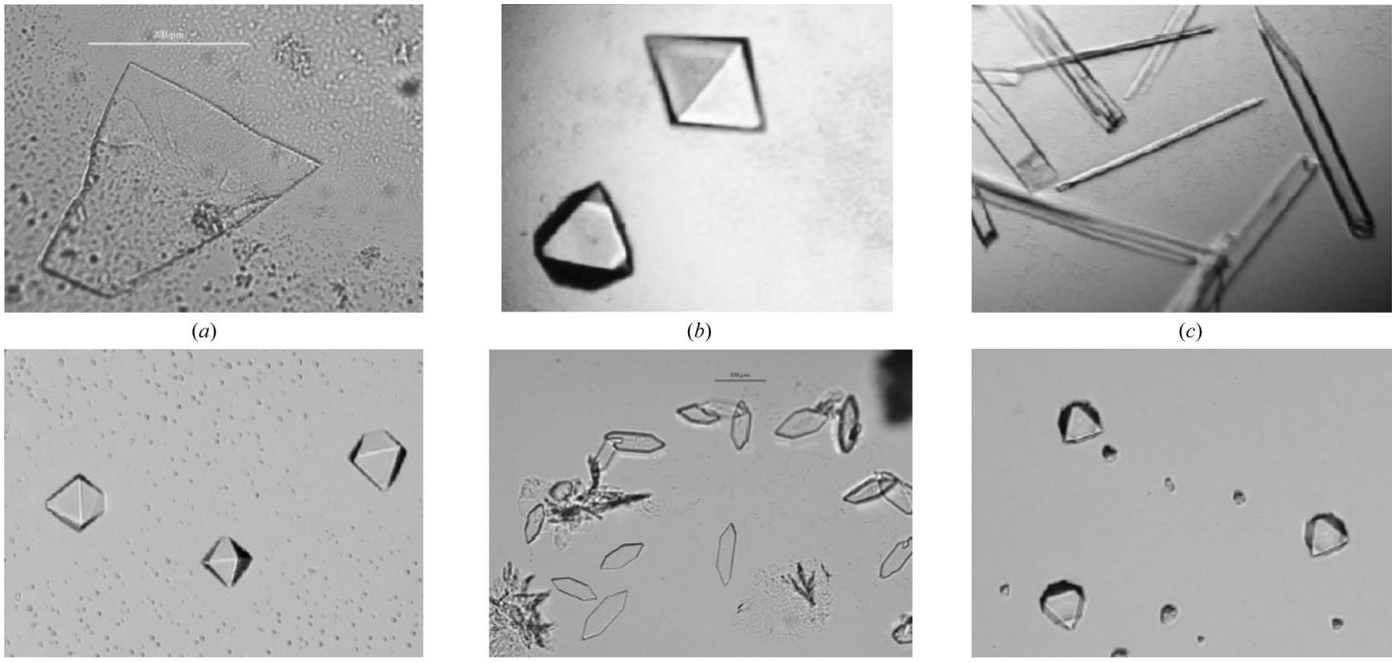

Figure 2
Crystals of DAP1, DAP2 and DAP3 obtained for the different pools and tested. (*a*) DAP1 pool 2; (*b*) and (*c*) DAP1 pool 3; (*d*) and (*e*) DAP2 pool 3; (*f*) DAP3 pool 3.

Table 1
Crystal parameter and data-collection statistic of DAP1 pool 3 (beamline ID14-EH4).

Values in parentheses refer to the highest resolution shell.

Space group	$F4_132$
Unit-cell parameter (Å)	221.9
Resolution range (Å)	28.9–3.085 (3.25–3.085)
Reflections measured	190062 (11681)
Unique reflections	8652 (1273)
Completeness (%)	95.2 (75.8)
R_{sym}† (%)	7.49 (22.12)
$\langle I/\sigma(I)\rangle$	31.25 (4.98)

† $R_{sym} = \sum_h \sum_i |I_{hi} - \langle I_h\rangle| / \sum_h \sum_i I_{hi}$.

ID14-EH2, ID14-EH4, ID29 and BM14 at ESRF, Grenoble, France. Crystals were cryoprotected with 25%(*v*/*v*) glycerol and flash-cooled in a nitrogen cold stream at 100 K (Oxford Cryosystems). A number of data sets (>10) were collected from cubic crystals of native DAP, seleniomethionine and heavy-atom derivatives. All data processing was performed using either *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997) or *MOSFLM* and *SCALA* (Evans, 1993; Leslie *et al.*, 1986). The program *SOLVE* (Terwilliger & Berendzen, 1999) was used to evaluate heavy-atom derivatives. Crystals of the several forms of DAP diffracted poorly, only up to 3.15 Å resolution in the best cases. Only a few images were taken of crystals that diffracted with resolution lower than 5 Å. The best results were obtained with crystals of DAP1 pool 3 that belong to space group $F4_132$ with unit-cell parameters $a = b = c = 221.9$ Å (Table 1). The unit-cell dimensions, together with the results of gel filtration and the homology of DAP with *H. marismortui* TET, suggest that pool 3 of DAP1, DAP2 and DAP3 correspond to dodecamers of about 400 kDa, while DAP1 pool 2 corresponds to an assembly of 24 subunits of about 800 kDa. MAD phasing was attempted, without success, owing to the low resolution reached. Meanwhile, the three-dimensional structure of the homologous peptidase/glucanase YsdC from *Bacillus subtilis* has become available in the Protein Data Bank in the context of structural genomics research (PDB code 1vhe). This protein shares 36.3% sequence identity with DAP1. The structure of DAP1 pool 3 has been solved by molecular replacement using the three-dimensional structure of the monomer of the *B. subtilis* peptidase/glucanase with the program *PHASER* (Read, 2001; Storoni *et al.*, 2004). Briefly, the fast rotation function returned ten potential peaks that were input into the fast translation-function calculations. Rotation peak number 4 gave a single molecular-replacement solution with a *Z* value of 9.62, an LLG value of 35.93 and proper packing inside the $F4_132$ cell. The asymmetric unit contains one monomer, leading to a solvent content of nearly 80% (V_M = 5.7 Å^3 Da^{-1}). A manuscript describing the structure is in preparation. We are also working to obtain better diffracting crystals in order to obtain higher resolution data, in the hope of solving the structure of DAP2 and DAP3.

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