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Expression, purification, crystallization and preliminary crystallographic analysis of a stand-alone RAM domain with hydrolytic activity from the hyperthermophile *Pyrococcus furiosus*

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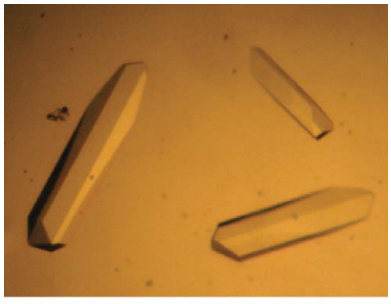

The RAM domain is one of several ligand-binding modules present in prokaryotes that are presumed to regulate the transcription of specific genes. To date, no hydrolytic activity has been reported for such modules. Curiously, a stand-alone RAM domain in *Pyrococcus furiosus* was isolated during a screen for hydrolytic activity against chromogenic esters. The gene encoding this protein was cloned and expressed in *Escherichia coli* and crystallized after a single purification step. X-ray diffraction data from the crystals were obtained to a resolution of 2.8 Å using a conventional X-ray source. The cocrystallization of the recombinant protein with 1,2-epoxy-3-(4-nitrophenoxy)propane (EPNP) and phenylmethylsulfonyl fluoride (PMSF) produced crystals that yielded data to 2.2 and 2.8 Å, respectively, using synchrotron radiation. Both the untreated and EPNP-treated crystals crystallize isomorphously in space group *C*2 and contain three dimers in the asymmetric unit. The PMSF-treated crystals also belong to this space group and have almost identical packing density, but show dramatically different unit-cell parameters.

1. Introduction

Pyrococcus furiosus is an obligate anaerobe that grows optimally at 373 K and neutral pH (Fiala & Stetter, 1986). Its genome has been completely sequenced and contains 1.908 Mbp encoding approximately 2000 proteins (Maeder *et al.*, 1999). About 80% of these are significantly expressed when the archaeon is grown on either maltose or peptides (Schut *et al.*, 2003), with hydrolases comprising less than 10% of them. Most of the purified hydrolytic enzymes are involved in carbohydrate metabolism and protein degradation, including glycosylases [*e.g.* α-amylase (Laderman *et al.*, 1993), β-glucosidase and β-mannosidase (Bauer *et al.*, 1996)] and peptidases [*e.g.* pyrolysin (Voorhorst *et al.*, 1996) and metallocarboxypeptidase (Cheng *et al.*, 1999)]. Carboxylesterases, which are often associated with fatty-acid biosynthesis and lipid degradation, are apparently lacking in *P. furiosus* cell-free extracts (Cornec *et al.*, 1998). Their absence may arise from the unique make up of archaeal lipids, which have ether linkages between isoprenoid alcohol and glycerol instead of ester bonds (Nishihara *et al.*, 2000). However, carboxylesterases have been isolated from other hyperthermophilic archaea such as *Pyrobaculum calidifontis* VA1 (Hotta *et al.*, 2002), *Aeropyrum pernix* K1 (Gao *et al.*, 2003) and *Archaeoglobus fulgidus* (Mandrich *et al.*, 2004). A 'shotgun' experiment of cloning and expressing a *P. furiosus* gene in *Escherichia coli* yielded a thermostable esterase with activity towards 4-methylumbelliferyl acetate (Ikeda & Clark, 1998). Owing to extensive plasmid loss from the host cells when grown in liquid culture, no further characterization of the protein was made.

The presence of esterolytic activity in *P. furiosus* was investigated using various assays. As a result, a protein of 8.6 kDa was purified from the cell-free extract of starch-grown cells based on its activity against a chromogenic substrate, the *N*-CBZ-L-tyrosine *p*-nitrophenyl ester. N-terminal amino-acid sequence determination and comparison with the *P. furiosus* genome database identified the protein as Q8U228 (gene locus name PF1022), tentatively annotated as a transcriptional regulatory protein of the AsnC family (EBI; http://www.ebi.ac.uk/genomes/AE009950.html).

Table 1
X-ray data-collection statistics.

Values in parentheses refer to the highest resolution shell.

	Recombinant RAM-1	Recombinant RAM-2	EPNP–RAM	PMSF–RAM
Source	Rotating anode	DESY/ EMBL X13	DESY/ EMBL X13	DESY/ EMBL X13
Wavelength (Å)	1.5418	0.8034	0.8067	0.8067
Temperature	Ambient	100 K	100 K	100 K
Resolution (Å)	2.8	2.3	2.2	2.75
Space group	*C*2	$P2_12_12$	*C*2	*C*2
Unit-cell parameters				
a (Å)	145.36	46.52	143.70	119.62
b (Å)	47.60	110.34	46.38	92.32
c (Å)	93.08	92.72	92.17	46.42
α (°)	90	90	90	90
β (°)	129.81	90	129.86	112.79
γ (°)	90	90	90	90
V_M (Å^3 Da^{-1})	2.3	2.5	2.3	2.3
Solvent content (%)	45.7	49.0	45.9	45.9
Unique reflections	11178	21364	23988	12244
Redundancy	2.7	6.5	3.7	3.9
Completeness (%)	91.4 (92.9)	99.7 (99.6)	98.1 (88.9)	99.7 (100)
R_{sym}† (%)	6.3 (43.7)	4.7 (49.0)	4.4 (42.2)	4.2 (47.6)
Average $I/\sigma(I)$	15.1 (2.2)	34.3 (3.4)	25.3 (2.8)	29.3 (3.2)

† $R_{sym} = \sum_h \sum_i |I(h,i) - \langle I(h)\rangle| / \sum_h \sum_i I(h,i)$, where $I(h,i)$ is the intensity of the ith measurement of reflection h and $\langle I(h)\rangle$ is the average value over multiple measurements.

Transcriptional regulatory proteins of the Lrp/AsnC family are widely distributed among prokaryotes. Most of these consist of two domains: a DNA-binding N-terminal domain with a helix–turn–helix motif, attached by a hinge to a C-terminal domain with an α/β-fold (Brinkman *et al.*, 2003). The C-terminal half is referred to as a RAM (regulation of amino-acid metabolism) domain because of its involvement in regulating the metabolism of amino acids through allosteric interactions with a ligand (Ettema *et al.*, 2002). Since Q8U228 lacks the N-terminal DNA-binding module, it is classified as a stand-alone RAM domain. So far, only one other stand-alone RAM domain has been crystallized, DM1 from *Pyrococcus horikoshii* OT3 (Kudo *et al.*, 2001), but no reports have been made of its hydrolytic activity. It is difficult to explain the hydrolytic activity of Q8U228 from *P. furiosus* based on its amino-acid sequence, as it contains no readily identifiable signature sequences. For example, it contains no histidine or cysteine residues, while EPR spectra confirm the absence of metals such as Mn^{2+} or Co^{2+} that could be involved in this activity. It is therefore anticipated that elucidation of the three-dimensional structure of Q8U228, particularly in complex with inhibitors of its hydrolytic activity, will be crucial in delineating its mode of action.

To obtain substantial amounts of the *P. furiosus* stand-alone RAM domain for biochemical and structural studies, the PF1022 gene was cloned and expressed in *E. coli*. Here, we report the expression, crystallization and preliminary crystallographic studies for this intriguing hydrolase.

2. Methods and results

2.1. Protein expression, purification and crystallization

E. coli strain BL21(DE3) (Stratagene) transformed with pET24dEm3 [pET24d (Novagen) with the PF1022 gene] was cultured in LB medium containing 50 µg l^{-1} chloramphenicol and 20 µg l^{-1} kanamycin at 310 K. Protein expression was induced with 100 µ*M* isopropyl β-D-thiogalactopyranoside (IPTG). The cells were harvested after overnight growth and the pellets were suspended in 50 m*M* Tris–HCl buffer pH 8.0 containing 10%(*v*/*v*) glycerol. The mixture was passed twice through a cell-disruption unit (Basic Z model, Constant Systems Ltd) at 135 MPa before incubation at 310 K for 30 min with a few micrograms of DNase and RNase. The homogenate was centrifuged at 45 000 rev min^{-1} and 277 K for 1 h. The supernatant was loaded onto a Q-Sepharose Fast Flow column (Pharmacia) equilibrated with 50 m*M* Tris–HCl buffer pH 8.0 and then eluted with an NaCl gradient (0–0.5 *M*). The hydrolytic activities of the fractions were tested using *N*-CBZ-L-tyrosine *p*-nitrophenyl ester. The active fractions were pooled and concentrated by ultrafiltration using a YM-3 (3 kDa cutoff) Amicon membrane. An SDS–PAGE gel (8–25% gradient, Amersham Bioscience) of the concentrated sample shows one band of approximately 8.6 kDa in weight. Using electrospray mass spectrometry, the molecular weight was determined to be 8587 ± 0.13 kDa. This is consistent with the expected molecular weight derived for the protein encoded by the gene PF1022.

Crystal Screen from Hampton Research was used to identify crystallization leads for the purified RAM domain using the hanging-drop method. The droplets contained 2 µl protein solution (5 mg ml^{-1} in 50 m*M* Tris–HCl pH 8.0) and 2 µl reservoir solution and were equilibrated by vapour diffusion at 293 K over reservoirs containing 500 µl of the candidate crystallization condition. Within 3 d, crystals (Fig. 1) formed in two out of 48 conditions. The components of the reservoir solutions were 28% PEG 400, 0.1 *M* HEPES pH 7.5, 0.2 *M* $CaCl_2$ (Fig. 1*a*) and 30% PEG 400, 0.1 *M* HEPES pH 7.5, 0.2 *M* $MgCl_2$ (Fig. 1*b*). Crystals were optimized using the components of the first solution (solution 14 from Crystal Screen I, Hampton Research) owing to their larger size and stability. It was

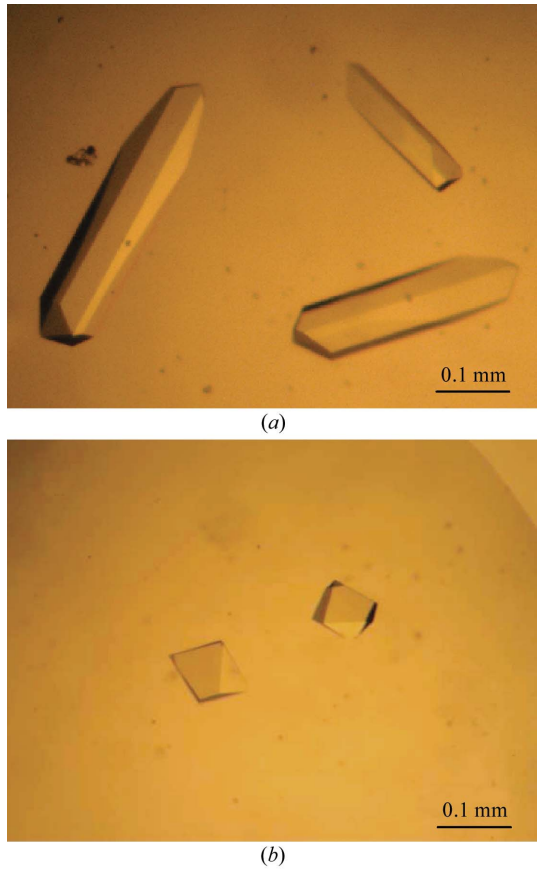

Figure 1
P. furiosus stand-alone RAM domain crystals from (*a*) 28% PEG 400, 0.1 *M* HEPES pH 7.5, 0.2 *M* $CaCl_2$ and (*b*) 30% PEG 400, 0.1 *M* HEPES pH 7.5, 0.2 *M* $MgCl_2$.

found that crystals could readily be reproduced using a range of conditions (26–32% PEG 400 and pH 6.5–7.5). Furthermore, the crystal size and quality were observed to depend critically on the pH and to a lesser extent on the PEG concentration. Eventually, it was concluded that the best condition was in fact that identified in the screen. Using this condition, cocrystallization of the RAM domain was attempted with two protease inhibitors, 1,2-epoxy-3-(4-nitrophenoxy)propane (EPNP; 10 m*M*), which is an aspartic protease inhibitor, and phenylmethylsulfonyl fluoride (PMSF; 4 m*M*), which is a serine protease inhibitor. This approach produced crystals of similar morphology to the untreated form of the protein within the same period of time but with markedly larger size.

2.2. X-ray data collection and processing

Crystals of the recombinant stand-alone RAM domain and its complexes with EPNP and PMSF mounted in quartz capillaries directly from their droplets diffracted X-rays to 2.8 Å at ambient temperature using in-house X-ray facilities (FR591 rotating-anode generator, Bruker–Nonius). It quickly became apparent that two crystal forms existed in the crystallization droplets but that they were morphologically indistinguishable. One could be indexed as *c*-centred monoclinic and the other as primitive orthorhombic (recombinant RAM-1 and RAM-2; Table 1). Both forms diffracted quite anisotropically, indicating a significant amount of internal disorder. From tests on a number of crystals, it appears that the orthorhombic form was significantly more abundant than the *c*-centred monoclinic form. There was also no obvious correlation of the two forms with the parameters of the crystallization conditions. To prepare the crystals for data collection under cryogenic conditions (100 K), they were flash-cooled by plunging them directly from their native drops into liquid nitrogen. A series of cryocooling conditions using a variety of cryoprotecting reagents such as glycerol, sucrose, PEG 400 and Paratone indicated that only crystals flash-cooled through direct plunging into liquid nitrogen from the native drops produced diffraction of acceptable quality. As expected, the cryocooled crystals exhibited increased diffraction anisotropy. Data processing was carried out using the programs *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997). Intensities were converted into structure-factor amplitudes using the program *TRUNCATE* from the *CCP*4 suite (Collaborative Computational Project, Number 4, 1994).

Analysis of the crystal-packing densities (Matthews, 1968) for the two forms clearly revealed the possibility of two to four dimers of RAM in the *c*-centred monoclinic form and two dimers in the primitive orthorhombic form. However, the self-rotation functions for each crystal form were noisy and thus did not help in identifying any clear non-crystallographic symmetry (NCS) relationships. Moreover, the native Patterson synthesis for the primitive orthorhombic form at 4 and 6 Å resolution contained a large non-origin peak at $u = 23.26$, $v = 46.36$, $w = 55.17$, with a peak height of about 60% of the origin peak. This strongly suggested the presence of translational NCS and signalled caution in the ensuing efforts to determine the structure by molecular replacement.

Based on a sequence alignment of the stand-alone RAM domain from *P. furiosus* with the C-terminal domain of the protein FL11 from *P. horikoshii* OT3 (Koike *et al.*, 2004; PDB code 1r17), a search model was constructed in which all non-conserved residues were mutated to alanine. This resulted in a marginal search model containing about 30% of the scattering mass of the target molecule. The first attempts to determine the structure by molecular replacement using maximum-likelihood methods implemented in *PHASER* v. 1.2 (Storoni *et al.*, 2004) focused on the primitive orthorhombic forms of untreated crystals owing to the presence of good-quality synchrotron data (recombinant RAM-2; Table 1) and the more favourable number of molecules in the asymmetric unit of the crystal. Using data set 'recombinant RAM-2', it was indeed possible to place two dimers of the search model in the asymmetric unit of the crystal. However, the model resisted crystallographic refinement, with *R* factors around 50%, which would otherwise have suggested its validity. Reprocessing of the data in a primitive monoclinic lattice to examine the possibility of pseudo-symmetry, followed by new attempts to determine the structure, also led to the same dead end. It was therefore decided to shift efforts to the *c*-centred monoclinic form (recombinant RAM-1; Table 1) for which it was possible to find a solution for three dimers in the crystal asymmetric unit. Eventually, this was also possible for the data from the RAM domain cocrystallized with EPNP (EPNP–RAM) and with PMSF (PMSF–RAM). The models behaved well in preliminary rounds of crystallographic refinement, with *R* factors dropping rapidly to about 43%. Furthermore, initial electron-density maps revealed several unique structural features of the recombinant stand-alone RAM domain and in the case of EPNP–RAM and PMSF–RAM revealed plausible density for bound inhibitor molecules. Model building and refinement of the untreated and inhibitor-treated forms of the domain in the *c*-centred monoclinic form are under way.

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