Acta Cryst. (1997). **D53**, 798–801

Crystallization and preliminary X-ray analysis of methionine aminopeptidase from the hyperthermophilic bacterium *Pyrococcus furiosus*

TAHIR H. TAHIROV,[a] HIDEYUKI OKI,[a] TOMITAKE TSUKIHARA,[a]* KYOKO OGASAHARA,[a] YUKIKO IZU,[b] SUSUMU TSUNASAWA,[b] IKUNOSHIN KATO[b] AND KATSUHIDE YUTANI[a] *at* [a]*Institute for Protein Research, Osaka University, 3-2 Yamadaoka, Suita, Osaka 565, Japan, and* [b]*Biomedical Group, Takara Shuzo Co., Ltd, Seta 3-4-1, Otsu, Shiga, 520-21, Japan.*
E-mail: tsuki@protein.osaka-u.ac.jp

(*Received 11 February 1997; accepted 5 May 1997*)

Abstract

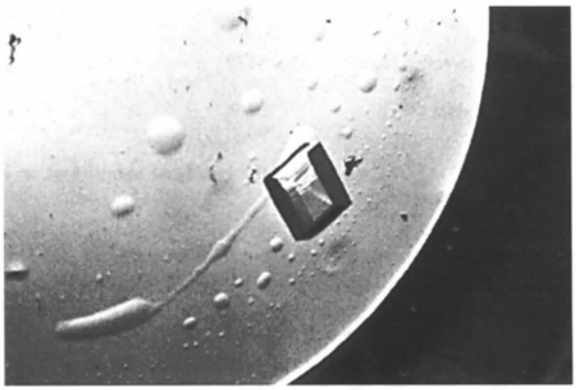
Methionine aminopeptidase (MAP) from *Pyrococcus furiosus* (Pfu) has been crystallized in four different forms (*A*, *B*, *C* and *D*). Form *A* crystals belong to space group $P2_1$ with unit-cell dimensions a = 54.18, b = 85.72, c = 72.84 Å, β = 108.34°. Forms *B*, *C* and *D* belong to space group $P6_{2(4)}$ with unit-cell dimensions a = 139.1, c = 63.7 Å for form *B*, a = 198.6, c = 243.8 Å for form *C*, and a = 111.0, c = 125.0 Å for form *D*. Forms *A* and *D* diffract to 2.9 Å, form *B* diffracts to 3.5 Å, and form *C* crystals diffract to 4.5 Å. Form *A* contains two molecules of MAP-Pfu per asymmetric unit. The binuclear metal center positions and a non-crystallographic twofold symmetry matrix has been determined for the form *A* crystals.

1. Introduction

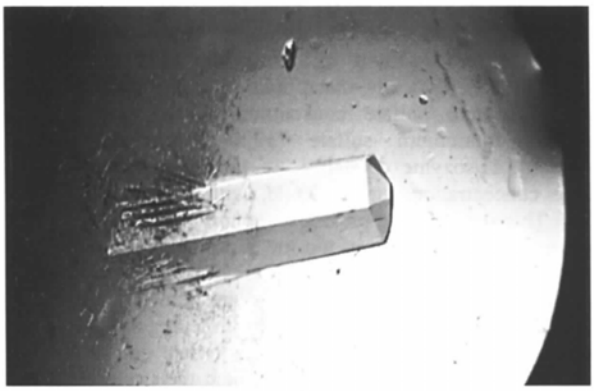
The entire family of the cobalt-dependent methionine aminopeptidases is represented by two types: procaryotic-type (type 1) and human-type (type 2) (Afrin *et al.*, 1995). In comparison with type 1 MAP, type 2 MAP has a long insertion with 63 residues in the catalytic domain and a variable N-terminal extension. Only the three-dimensional structure for type 1 MAP from *Escherichia coli* (MAP-Ec) has been reported (Roderick & Matthews, 1993). The two halves of the molecule are related by internal pseudo-twofold symmetry. A central antiparallel β-sheet with two pairs of α-helices on the periphery are the core secondary-structure elements of the molecule. A related fold is also found in creatinase (Hoeffken *et al.*, 1988). Amino-acid sequence comparisons suggest that several other non-cobalt-dependent enzymes, such as prolidase, aminopeptidase P and agropine synthase, might adopt a similar fold to MAP-Ec (Bazan, Weaver, Roderick, Huber & Matthews, 1994). In MAP-Ec, the two cobalt ions are located in approximately in the center of molecule at the bottom of active-site cleft. The cobalt ions are coordinated by two Asp, two Glu and one His residues. In order to elucidate the catalytic mechanism of MAP, find the difference between type 1 and type 2 MAPs, and as a separate task to study the protein thermostability, we started the three-dimensional structure determination of MAP derived from the hyperthermophilic bacterium *Pyrococcus furiosus* growing at 373 K. Herein we report four different crystal forms of MAP-Pfu which are useful for further X-ray structure determination.

2. Crystallization

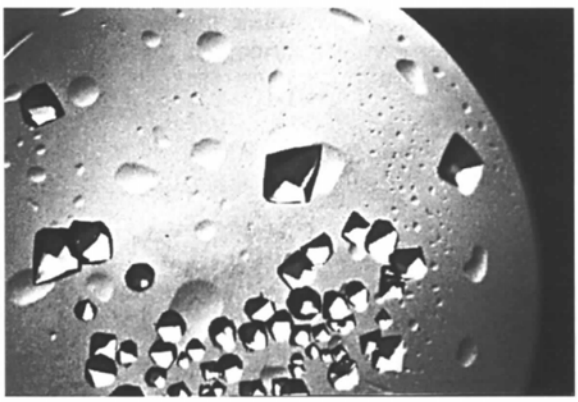
The gene of MAP from *P. furiosus* was cloned into *E. coli* strain JM109 (Tsunasawa *et al.*, in preparation). Cells of *E. coli* strain JM109/pMap8 harboring the MAP gene from *P. furiosus* were routinely grown in 15 l of LB medium supplemented with ampicilin of 100 mg l^{-1} at 310 K for 20 h with shaking. MAP



(*a*)



(*b*)



(*c*)

Fig. 1. Photomicrographs for crystalline MAP-Pfu for (*a*) form *A*, (*b*) form *B* and (*c*) form *C* or *D*.

ISSN 0907-4449

Table 1. *Crystal parameters and data-collection statistics*

Crystal type	*A*	*B*	*C*	*D*
Crystal parameters				
a (Å)	54.18	139.1	198.6	111.0
b (Å)	85.72	139.1	198.6	111.0
c (Å)	72.84	63.7	243.8	125.0
β (°)	108.34			
Space group	$P2_1$	$P6_{2(4)}$	$P6_{2(4)}$	$P6_{2(4)}$
Data collection				
Device	R-AXIS IIC	R-AXIS IIC	DIP2030	R-AXIS IIC
No. of crystals	1	1	1	1
Resolution (Å)	50–2.9	50–3.5	50–4.5	50–2.9
No. of observations	33741	59765	221036	91220
No. of unique reflections ($I \geq \sigma_I$)	11428	7571	29760	17502
R_{merge} (%)*	6.4/23.4	11.9/26.5	13.1/31.4	7.2/24.5
Completeness (%)	81.4/55.6	84.3/64.9	91.3/82.3	90.0/62.7

* $R_{merge} = \sum |I_j - \langle I_j \rangle| / \sum \langle I_j \rangle$, where I_j is the intensity of reflection j and $\langle I_j \rangle$ is the average intensity for reflection j. R_{merge} and completeness are presented for all data and for data in the last resolution shell.

extracted from the cells was purified using DEAE–Sephacel columns, gel filtration (Superdex TM20026/60), and SP Sepharose10/16 columns (Ogasahara *et al.*, in preparation). Purified MAP showed a single band on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE). The crystallization trials were conducted at temperatures of 277 and 293 K by the sitting-drop variant of the vapour-diffusion method. Sets of screening conditions described by Jancarik & Kim (1991), and by Cudney, Patel, Weisgraber, Newhouse & McPherson (1994) were used for the initial screening. Drops of mixed 1 µl of protein solution (10 mg ml^{-1} MAP-Pfu and 2 m*M* $CoCl_2$ in 20 m*M* potassium acetate buffer at pH 4.5) and 1 µl of reservoir solution, were equilibrated against 0.5 ml of reservoir solution. Well shaped crystals with three different morphologies (Fig. 1) were grown within 1–3 d from various reservoir solutions. To obtain larger crystals the drop volume was increased 3–5 times and the protein/reservoir solution ratio in the drops was varied. Crystals in the form of parallelepipeds (form *A*) and of approximate dimensions 0.6 × 0.4 × 0.2 mm (Fig. 1*a*) were grown at 293 K by equilibrating against a reservoir containing 20% ethanol in 0.1 *M* tris buffer at pH 8.5. Crystals in the form of prisms with a hexagonal cross-section (form *B*) were grown at 293 K to a size of 0.8 × 0.3 × 0.3 mm (Fig. 1*b*) by equilibrating against a 2.0 *M* sodium chloride in a 0.1 *M* sodium acetete buffer at pH 4.6. Slightly flattened prisms of form *B* were also grown using 10% PEG 6000 and 2.0 *M* sodium chloride. An addition of 1–3% dioxane to the latter reservoir changed the shape of prisms from flattened-out to elongated. Crystals of a third morphology, hexagonal bipyramids (form *C*, Fig. 1*c*) were ubiquitous at 293 K. They often crystallized in the same drop as form *A* or *B* but disappeared after several days. Relatively larger (up to 1.0 × 0.7 × 0.7 mm) and more stable crystals of form *C* were also grown from 25% ethylene glycol. Crystals grown at 277 K using a reservoir containing 0.6% 2-propanol and 1.6% PEG 4000 in 0.1 *M* sodium hepes buffer at pH 7.5 have exactly the same morphology as crystals of form *C*. However, X-ray examination shows that these crystals have different cell constants so we assigned them as form *D*.

We made several attempts to suppress the high crystal growth rate in order to increase the resolution. Addition of 9% glycerol to the reservoir solution gave the best result. The growth time of form *A* crystals was reduced from 24 h up to 5 d and their stability increased. Unfortunately, the maximum resolution of X-ray diffraction did not improve.

In the case of MAP-Ec crystallization (Roderick & Matthews, 1988) preparation of protein samples with additional methionine significantly improved the stability and quality of crystals, and increased the resolution from 2.4 to 2 Å. In our case, with the use of additional methionine we observed the opposite effect on the stability of the crystals. For example, form *A* crystals are relatively stable and can be kept at least 1 month without changes in color and diffraction quality. Addition of 30 m*M* L-methionine to the protein solution produced colorless crystals in 3–5 d that gradually turned violet in color. The color change was accompanied by a reduction in diffraction quality. Presently, a search of other conditions in which to grow crystals suitable for high-resolution experiments is in progress. Our main strategy is to modify the crystal screen conditions in which the small crystalline precipitants were observed.

3. Characterization of crystals

Diffraction data sets from the crystals of form *A*, *B* and *D* were collected on a Rigaku R-AXIS II imaging-plate using a graphite-monochromated Cu $K\alpha$ radiation from a Rigaku RU-200 rotating anode operated at 50 kV and 100 mA. The data set for crystals of form *C* was collected on Mac Science DIP2030 imaging plate using a nickel-filtered double-mirror focused X-rays from a Rigaku RU-200 rotating anode operated at 50 kV and 100 mA. All intensity data were indexed and integrated with *DENZO* and scaled by *SCALEPACK* (Otwinowski, 1993). The diffraction-data processing statistics are summarized in Table 1. Form *A* crystals diffract to resolution of 2.9 Å. The crystals are monoclinic with space group $P2_1$. The molecular weight of MAP-Pfu molecule is 32 740 Da, so the value of V_m calculated for two molecules in asymmetric unit is 2.45 Å^3 Da^{-1} which is corresponds to a solvent content of 49.8%. All three remaining crystal forms of *B*, *C* and *D* belong to the hexagonal crystal system with space group $P6_2$ or $P6_4$. Form *B* crystals diffract to 3.5 Å resolution and start to decay rapidly after 30 h of exposure. V_m values for two and three molecules in the asymmetric unit are 2.72 and 1.81 Å^3 Da^{-1}, respectively. Both V_m values are within the normal range of 1.6–3.6 Å^3 Da^{-1} for water-soluble protein crystals (Matthews, 1968) and correspond to solvent contents of 54.7 and 32.1%, respectively. Form *D* crystals diffract to 2.9 Å resolution. The unit-cell constants of form *D* are roughly equal to half of the form *C* parameters along each axis (Table 1). The solvent content calculation shows that two or three MAP-Pfu molecules can be located in the asymmetric unit with corresponding V_m values of 3.4 or 2.26 Å^3 Da^{-1} and solvent contents of 64.8 or 45.7%, respectively. Form *C* crystals diffract to 4.5 Å. Because of the larger unit-cell volume it is difficult to predict the number of protein molecules in the asymmetric unit of form *C*. If we assume some relevance of molecular packing in forms *C* and *D*, then there should be eight times the number of MAP-Pfu molecules in the asymmetric unit of form *D* in form *C*, *i.e.* 16 or 24, with corresponding V_m values of 2.65 or 1.77 Å^3 Da^{-1} and solvent contents of 53.6 or 30.4%, respectively.

4. Positions of the binuclear metal centers in form *A* crystals

The amino-acid sequences of MAP-Ec and MAP-Pfu are identical in only in 76 positions, *i.e.* 29% of the MAP-Ec sequence. Nevertheless, all five cobalt-ligated residues of MAP-Ec are strictly conserved in MAP-Pfu and with one exception in other reported MAPs (Afrin *et al.*, 1995), thus indicating that the active-site geometry of MAPs is very similar. In MAP-Ec, the cobalt ions are separated by 2.9 Å (Roderick & Matthews, 1993). At low resolution the neighboring cobalt ions contribute to a single peak in the anomalous $|F^+ - F^-|$ Patterson map, in effect twice amplifying the signal from the imaginary part of the Co^{2+} anomalous scattering. An interpretable anomalous Patterson map (Fig. 2) was calculated for the form *A* crystals of MAP-Pfu using 1798 out of the 2361 Friedel pairs (R_{ano} = 3.5%) in the resolution range 50–5 Å. The Friedel pairs were rejected if $F^+ \leq 38\sigma(F^+)$, $F^- \leq 38\sigma(F^-)$, or $|F^+ - F^-| \geq 5$. Most of the reflections were rejected on first or second criterion and ten reflections were rejected on the third. The map calculations were performed with the *FFTBIG* program from the *CCP*4 suite, version 2.14 (Collaborative Computational Project, Number 4, 1994). The positions of two Co—Co centers were determined from the Patterson vectors ($x = 0.361$, $y = 0.5$, $z = 0.476$ and $x = 0.234$, $y = 0.38$, $z = 0.057$),

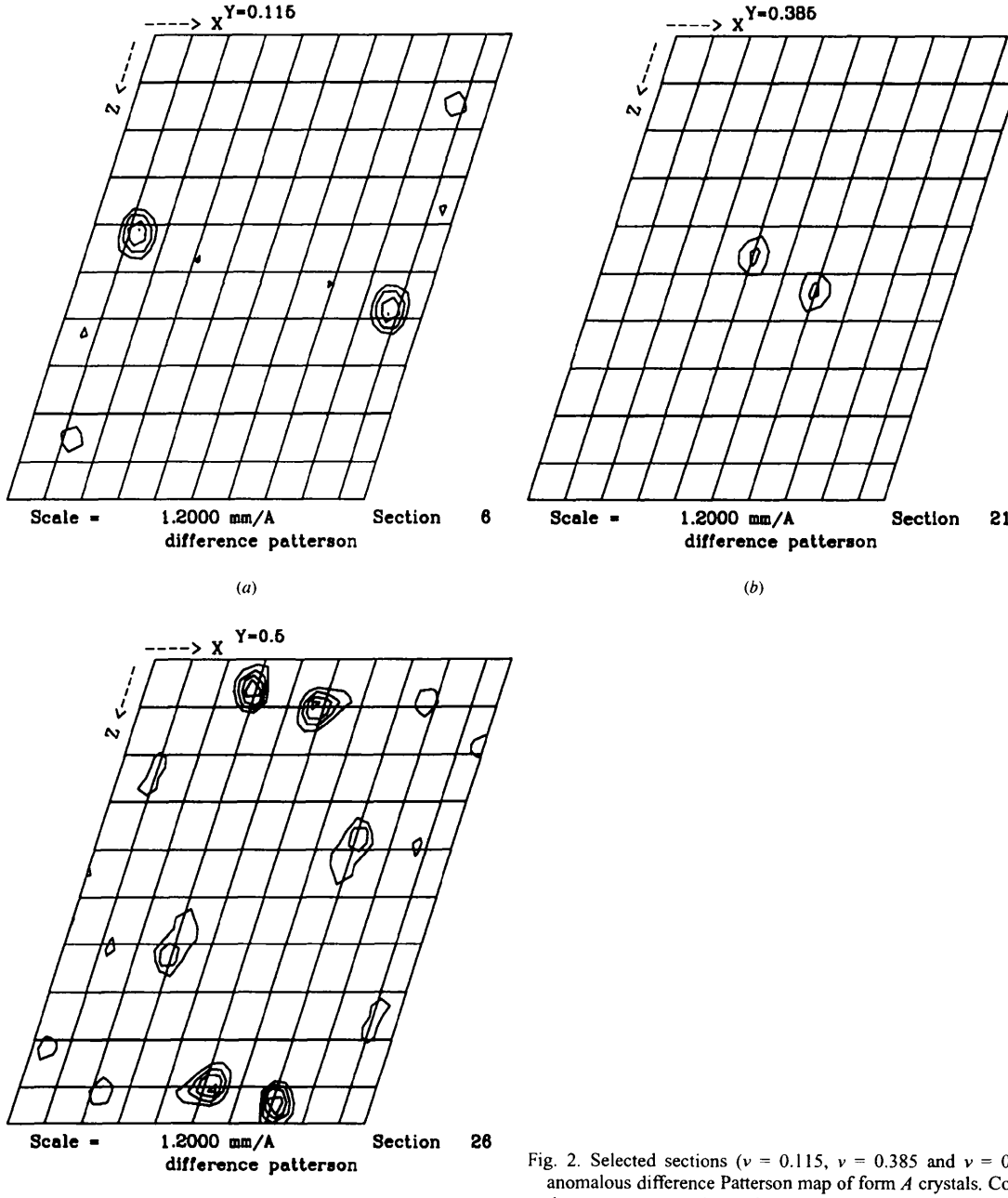


Fig. 2. Selected sections (v = 0.115, v = 0.385 and v = 0.5) of the anomalous difference Patterson map of form *A* crystals. Contours are drawn every σ starting at 2σ.

which agrees with the prediction of the solvent content calculations that there are two MAP molecules in the asymmetric unit.

5. Non-crystallographic twofold symmetry axis in form *A* crystals

A self-rotation search for non-crystallographic twofold symmetry axis was performed by the real-space Patterson search method implemented in *X-PLOR* (Brünger, 1992) in the form *A* crystals. Reflections in the resolution range from 15 to 4 Å were included for Patterson map calculations. The 4000 highest peaks with coordinates between 8 and 20 Å relative to the origin were selected from the rotated Patterson map for comparison with the stationary Patterson map using an angular grid interval of 3.5°. The calculations revealed a non-crystallographic twofold symmetry element with spherical polar angles at $\psi = 21.18$, $\varphi = 112.94$ and $\kappa = 180°$. These spherical angles in combination with previously determined positions of binuclear metal centers enable us to calculate a matrix for non-crystallographic twofold symmetry transformation, which furthermore will be used for calculations of masks and in density averaging.

This work was supported in part by the 'Research for Future' Program from the Japan Society for the Promotion of Science (TT) and a Grant-in-Aid for Special Research from the ministry of Education, Science and Culture of Japan (KY).

References

Afrin, S. M., Kendall, R. L., Hall, L., Weaver, L. H., Stewart, A. E., Matthews, B. W. & Bradshaw, R. A. (1995). *Proc. Natl Acad. Sci. USA*, **92**, 7714–7718.

Bazan, J. F., Weaver, L. H., Roderick, S. L., Huber, R. & Matthews, B. W. (1994). *Proc. Natl Acad. Sci. USA*, **91**, 2473–2477.

Brünger, A. T. (1992). *X-PLOR Version* 3.1 *Manual*. Yale University, New Haven, Connecticut, USA.

Collaborative Computational Project, Number 4 (1994). *Acta Cryst.* D**51**, 760–763.

Cudney, B., Patel, S., Weisgraber, K., Newhouse, Y. & McPherson, A. (1994). *Acta Cryst.* D**50**, 414–423.

Jancarik, J. & Kim, S.-H. (1991). *J. Appl. Cryst.* **24**, 409–411.

Hoeffken, H. W., Knof, S. H., Bartlett, P. A., Huber, R., Moellering, H. & Schumacher, G. (1988). *J. Mol. Biol.* **204**, 417–433.

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

Otwinowski, Z. (1993). *DENZO. An Oscillation Data Processing Program for Macromolecular Crystallography*. Yale University, Connecticut, USA.

Roderick, S. L. & Matthews, B. W. (1988). *J. Biol. Chem.* **263**, 16531–16531.

Roderick, S. L. & Matthews, B. W. (1993). *Biochemistry*, **32**, 3907–3912.