Acta Crystallographica Section D
Biological Crystallography
ISSN 0907-4449

Crystallization and characterization of the prolidase from *Pyrococcus furiosus*

Katrina Willingham,[a] Megan J. Maher,[a] Amy M. Grunden,[b] Mousumi Ghosh,[c] Michael W. W. Adams,[b] Hans C. Freeman[a] and J. Mitchell Guss[a]*

[a]Department of Biochemistry, University of Sydney, NSW 2006, Australia, [b]Department of Biochemistry and Molecular Biology and Center for Metalloenzyme Studies, University of Georgia, Athens, Georgia 30602, USA, and [c]Department of Biochemistry, West Virginia University, Morgantown, West Virginia 26505, USA

Correspondence e-mail: m.guss@biochem.usyd.edu.au

Received 21 August 2000
Accepted 12 December 2000

The prolidase (proline-specific amino dipeptidase) from the hyperthermophilic archaeon *Pyrococcus furiosus* has been crystallized. The enzyme has been shown to be a homodimer and to require two Co atoms per subunit for optimum activity. Two crystal forms have been obtained under similar growth conditions. Both are monoclinic, space group $P2_1$. Form I has unit-cell parameters $a = 130.4$, $b = 97.4$, $c = 129.9$ Å, $\beta = 118.3^\circ$. Form II has a smaller unit cell, with $a = 56.5$, $b = 97.3$, $c = 70.0$ Å, $\beta = 97.1^\circ$. If the crystal density is assumed to lie near the center of the normal range then the form I crystals will have four dimers per asymmetric unit, whereas the form II crystals will have only one dimer in each asymmetric unit. Diffraction data have been recorded from native form I and form II crystals to resolutions of 3.2 and 1.95 Å, respectively.

1. Introduction

Prolidases are peptidases with specificity for Xaa-Pro dipeptides. This activity was first identified in isolates from intestinal mucosa (Bergmann & Fruton, 1937), but has since been found in mammals, archaea and bacteria (Yaron & Naider, 1993). The gene encoding the prolidase from the hyperthermophilic archaeon *P. furiosus* has been cloned and expressed in *E. coli* (Ghosh *et al.*, 1998). Prolidases are one of a family of enzymes with specificity for proline. Deficiency of the human enzyme leads to a rare genetic disorder (Endo & Matsuda, 1991). The clinical phenotypes are associated with a lack of proline for collagen synthesis. Proline-specific proteases have presumably arisen to deal with the problem that most 'general-purpose' proteases will not cleave a peptide bond before proline. Proline aminopeptidase (aminopeptidase P; AMPP) is another enzyme that cleaves the N-terminal residue from a peptide if the second residue is proline, but unlike prolidase it is not limited to dipeptide substrates. Proline aminopeptidases and prolidases are about 30% identical at the amino-acid sequence level, depending on their origin, indicating that they have similar tertiary structures and that they evolved from a common ancestor.

The original isolation of prolidase from intestinal mucosa identified it as a manganese-activated enzyme (Bergmann & Fruton, 1937). The prolidases subsequently isolated from other organisms were assumed to be similar (Yaron & Naider, 1993). The homodimeric prolidase from *P. furiosus*, on the other hand, contains one Co^{2+} ion per subunit as isolated and requires either a second Co^{2+} ion or a Mn^{2+} ion for catalytic activity (Ghosh *et al.*, 1998). In requiring two metal ions per subunit, *P. furiosus* prolidase resembles two related *Escherichia coli* enzymes, methionine aminopeptidase (MAP) and proline aminopeptidase (AMPP). The crystal structures of MAP (Roderick & Matthews, 1993) and AMPP (Wilce *et al.*, 1998) have closely similar active sites with a dinuclear metal cluster bridged by a hydroxide ion. The residues that coordinate the cluster in the two enzymes are chemically equivalent. In MAP, the metal ions at the dinuclear active site are Co^{2+} (Roderick & Matthews, 1993), while AMPP is most active in the presence of Mn^{2+} but is activated almost as efficiently by Co^{2+} (Yaron & Mlynar, 1968).

There remain doubts concerning the nature and number of metal ions that these enzymes use *in vivo*. The *in vivo* concentrations of Mn^{2+} and Co^{2+} are much lower than that of Zn^{2+}, so that a preference for Zn^{2+} might be advantageous. At first sight this cannot be the case, since *P. furiosus* prolidase (Ghosh *et al.*, 1998), *Xanthomonas maltophilia* prolidase (Suga *et al.*, 1995), *Aureobacterium esteraromaticum* prolidase (Fujii *et al.*, 1996) and AMPP (Rusu & Yaron, 1992; Yaron & Berger, 1970; Zhang *et al.*, 1998) are reported to be active in the presence of Mn^{2+} or Co^{2+}, but to be inactive in the presence of Zn^{2+}. However, the cited observations of enzyme activity were all made after treating the enzyme with an excess of the metal ion. Thus, there is a logical possibility that the enzymes are Zn^{2+} proteins, but are inhibited when Zn^{2+} is present in excess. There is experimental evidence that *Saccharomyces cerevisiae* MAP is such an enzyme (Walker &

Table 1
Data-collection statistics for PF prolidase.

Values in parentheses refer to the highest resolution shells (3.3–3.2 Å for form I and 2.02–1.95 Å for form II).

	Form I	Form II
X-ray wavelength (Å)	1.5418	1.240
Temperature (K)	113	100
Space group	$P2_1$	$P2_1$
Unit-cell parameters		
a (Å)	130.4	56.5
b (Å)	97.5	97.3
c (Å)	129.9	70.0
β (°)	118.3	97.1
Resolution (Å)	3.2	1.95
Mosaicity (°)	0.61	0.32
Observations	98160	189407
Unique reflections	43017	51810
Redundancy	2.3 (2.2)	3.7 (3.4)
Completeness (%)	92.5 (94.9)	94.9 (94.1)
$I/\sigma(I)$	6.6 (1.95)	26.8 (3.0)
$R_{\rm merge}$	0.091 (0.367)	0.035 (0.334)

Bradshaw, 1998). On the other hand, in the case of *E. coli* MAP it has been confirmed that both low and high concentrations of Zn^{2+} produce only trace activity, that the enzyme is partially active in the presence of Mn^{2+} and fully active in the presence of Fe^{2+} and Co^{2+} (D'Souza & Holz, 1999) and that the physiologically active metal is most likely to be Fe^{2+} (D'Souza *et al.*, 2000). The functional significance of the dinuclear metal sites that have been characterized crystallographically and spectroscopically in most of these enzymes is also in doubt. Recent studies have shown that only one metal atom per active site is required for full activity in *E. coli* MAP (Fe^{2+}; D'Souza *et al.*, 2000), human cytosolic AMPP (Mn^{2+}; Cottrell, Hooper *et al.*, 2000) and porcine membrane-bound AMPP (Mn^{2+}; Cottrell, Hyde *et al.*, 2000). These observations are consistent with previously unexplained EPR data which indicated that only one Mn^{2+} atom at the active site of *E. coli* AMPP is tightly bound, whereas the other Mn^{2+} atom is in labile equilibrium with the solvent (Zhang *et al.*, 1998).

Despite their similarities in primary structure, AMPP and *P. furiosus* prolidase have different quaternary structures. The active form of *E. coli* AMPP is tetrameric (Wilce *et al.*, 1998), whereas that of *P. furiosus* prolidase is dimeric (Ghosh *et al.*, 1998). It has been suggested that the difference between the substrate specificities of the two types of enzyme is related to the difference between their quaternary structures (Wilce *et al.*, 1998).

We seek to solve the structure of *P. furiosus* prolidase in order to find the basis of its specificity for dipeptides and to find structural reasons for its enhanced stability at high temperature. *P. furiosus* prolidase is fully active for extended periods at 373 K. In this paper, we report the crystallization and preliminary X-ray analysis.

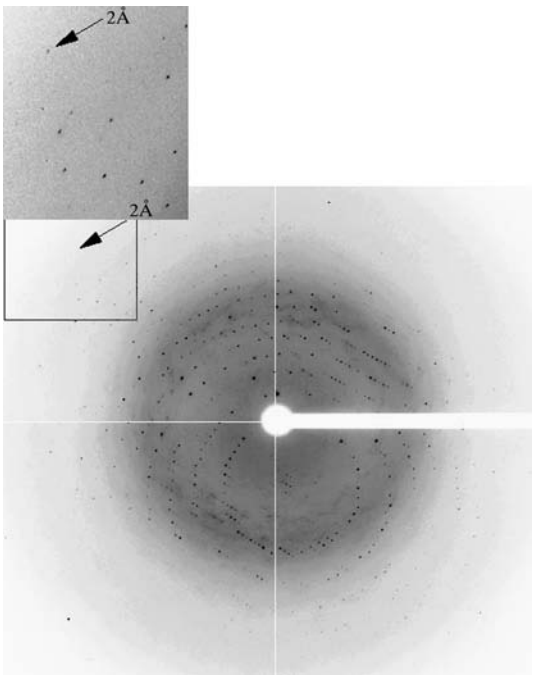

Figure 1
Oscillation image recorded from a form II crystal of PF prolidase on beamline 9-2 at SSRL. The data were recorded on an ADSC Q4 detector with a crystal-to-detector distance of 110 mm. The inset shows data near the limit of diffraction.

2. Materials and methods

2.1. Crystallization

The gene encoding *P. furiosus* prolidase was expressed in *E. coli* and the recombinant enzyme was purified as described previously (Ghosh *et al.*, 1998). Preliminary crystallization conditions were found using factorial screens (Jancarik & Kim, 1991) by hanging-drop vapor diffusion. Each of the solutions from Hampton Crystal Screens 1 and 2 (Hampton Research, CA, USA) (2 µl) was mixed with a solution of *P. furiosus* prolidase (2 µl, 10 mg ml^{-1}, 250 m*M* MOPS pH 7) at room temperature (293 K). Crystals were observed after 1 d under eight different conditions (Hampton Screen 1 conditions 9, 15, 17, 18, 22, 28, 40 and 46). The common factor in these eight conditions is that they each contain a high molecular-weight polyethylene glycol, PEG 8K or PEG 4K, as the precipitant.

Initial refinement from the Hampton Screen 1 condition 18 (0.1 *M* sodium cacodylate pH 6.5, 0.2 *M* magnesium acetate) yielded the best crystals. Further attempts to improve the crystal quality included changing the crystallization technique (sitting drop, dialysis, microbatch under oil), growth temperature, buffer type, drop size, protein concentration, the concentration of magnesium acetate and the addition of various Co^{2+} salts. The crystals which diffracted to the highest resolution were obtained from hanging drops with a well solution consisting of 0.1 *M* Tris–HCl pH 8.5, 0.2 *M* magnesium acetate, 13–15% PEG 8K. The drop contained 2.5 µl of well solution mixed with 2.5 µl of 10 mg ml^{-1} *P. furiosus* prolidase at room temperature. After more than six months, crystals with a new morphology appeared in trays using the same crystallization conditions and the same protein sample that had resulted in the growth of the original crystal form. The form II crystals grew without the prior formation of form I and can now be grown routinely and rapidly by streak seeding.

2.2. Diffraction data and crystallographic calculations

Diffraction data were recorded on an R-AXIS IIc image-plate detector with Cu $K\alpha$ X-rays from a Rigaku RU-200 rotating-anode generator focused using mirror optics (Z. Otwinowski & G. Johnson, Yale University, as marketed by Rigaku, Texas, USA). High-resolution data were recorded on beamline 9-2 of the Stanford Synchrotron facility using an ADSC Q4 CCD detector. All data were recorded using cryoprotected crystals that were kept at low temperature in a cold gas stream. Crystals were cryoprotected by replacing the mother liquor with solutions of mother liquor containing increasing concentrations of 2-methyl-2,4-pentanediol (MPD). Crystals were transferred rapidly from the drop directly into the cold gas stream. Diffraction data were integrated and scaled with *DENZO* and *SCALEPACK* from the *HKL* program suite (Otwinowski & Minor, 1997).

Self-rotation functions were calculated with the *POLARRFN* program from the *CCP*4 package (Collaborative Computational Project, Number 4, 1994).

3. Results and discussion

Crystals of form I are prisms with maximum dimensions 0.4 × 0.1 × 0.05 mm. The symmetry and systematic absences in the diffraction pattern show that the crystals are

monoclinic, space group $P2_1$, with unit-cell parameters a = 130.4, b = 97.4, c = 129.9 Å, β = 118.3°. Laboratory data at 113 K from a single form I crystal were integrated and scaled to a resolution of 3 Å; however, the statistics in the highest resolution shells suggest that a more reasonable estimate of the resolution is 3.2 Å (Table 1). Crystals of form II are generally less elongated than those of form I, with maximum dimensions 0.25 × 0.18 × 0.075 mm. Crystals of form II diffract to 2.2 Å on a rotating-anode source and to 1.95 Å at a synchrotron (Table 1; Fig. 1). They are monoclinic, space group $P2_1$, with unit-cell parameters a = 56.5, b = 97.3, c = 70.0 Å, β = 97.1°. The unit cells of the two crystal forms appear to be related in a way that may reflect the underlying molecular packing. They are both monoclinic and the unit-cell dimensions in the direction of the twofold screw axes are nearly identical. An overlay of the two unit cells shows there is an approximate relationship of the following form: $a_{II} = 2a_I + 2c_I$, $b_{II} = b_I$ and $c_{II} = -2a_I + 2c_I$. The low overall values of $I/\sigma(I)$ for form I crystals compared with those for form II crystals (Table 1) indicate weaker diffraction from more poorly ordered crystals, consistent with higher mosaicity, the larger unit-cell volume and the use of a laboratory source instead of synchrotron radiation. The data for form II include no obvious systematically weak reflections with simple parity relationships.

If it is assumed that there is a dimer in the asymmetric unit of the form II crystals and that the density of the protein is 1.36 g cm^{-3} then the Matthews coefficient is 2.42 and the solvent content is 49%. If there is a monomer in the asymmetric unit then the Matthews coefficient is 4.9 and the solvent content is 74%. These calculations strongly suggest that there a dimer in the asymmetric unit of the form II crystals. This would be consistent with gel-filtration analysis, which shows that *P. furiosus* prolidase forms homodimers in solution (Ghosh *et al.*, 1998). If the solvent content were the same for both crystal forms, then the form I crystals would have eight molecules or four dimers in the asymmetric unit.

A self-rotation function for the form II crystals (Fig. 2) shows the presence of a twofold rotation axis almost perpendicular to the crystallographic twofold screw axis. This function was calculated in the resolution range 15–4 Å with a 30 Å radius of integration. The strongest peak lies at ω = 90.0, φ = 13.3° on the section with κ = 180°. This peak and its symmetry equivalents are one-third as high as the origin peak and 50% larger than the next highest peak. Attempts to solve the structure using molecular replacement with various models derived from the AMPP structure have been unsuccessful. Structure solution by MAD phasing methods is currently in progress.

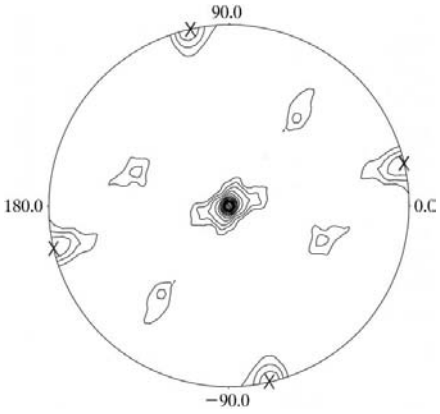

Figure 2
Plot of the κ = 180° section of the self-rotation function for form II crystals. The orthogonalization code was chosen in such a way as to place the twofold axis parallel to the pole; that is, with x parallel to c, y parallel to $b^* \times c$ and z parallel to b^*. The origin peak, at the center, is along the b axis. The lowest contour is drawn at two root-mean-square deviations (r.m.s.) of the mean density, with subsequent contours at 0.5 r.m.s. Crosses indicate the positions of the major peak and its symmetry-related equivalents.

The work in Sydney was supported by grant A29906004 to HCF and JMG from the Australian Research Council. MJM is the recipient of an Australian Research Council postdoctoral Fellowship. Access to the facilities of Stanford Synchrotron Radiation Laboratory was made possible by a travel grant from the Access to Major National Facilities Programme administered by the Australian Nuclear Science and Technology Organization. The research in the laboratory of MWWA was supported by grants from the National Science Foundation (BCS-9632657 and MCB-9809060).

References

Bergmann, M. & Fruton, J. S. (1937). *J. Biol. Chem.* **117**, 189–202.
Collaborative Computational Project, Number 4 (1994). *Acta Cryst.* D**50**, 760–763.
Cottrell, G. S., Hooper, N. M. & Turner, A. J. (2000). *Biochemistry*, **39**(49), 15121–15128.
Cottrell, G. S., Hyde, R. J., Lim, J., Parsons, M. R., Hooper, N. M. & Turner, A. J. (2000). *Biochemistry*, **39**(49), 15129–15135.
D'Souza, V. M., Bennett, B., Copik, A. J. & Holz, R. C. (2000). *Biochemistry*, **39**(13), 3817–3826.
D'Souza, V. M. & Holz, R. C. (1999). *Biochemistry*, **38**(34), 11079–11085.
Endo, F. & Matsuda, I. (1991). *Mol. Biol. Med.* **8**(1), 117–127.
Fujii, M., Nagaoka, Y., Imamura, S. & Shimizu, T. (1996). *Biosci. Biotechnol. Biochem.* **60**(7), 1118–1122.
Ghosh, M., Grunden, A. M., Dunn, D. M., Weiss, R. & Adams, M. W. W. (1998). *J. Bacteriol.* **180**(18), 4781–4789.
Jancarik, J. & Kim, S.-H. (1991). *J. Appl. Cryst.* **24**, 409–411.
Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.
Roderick, S. L. & Matthews, B. W. (1993). *Biochemistry*, **32**, 3907–3912.
Rusu, I. & Yaron, A. (1992). *FEBS Lett.* **210**, 93–100.
Suga, K., Kabasima, T., Ito, K., Tsuru, D., Okamura, H., Kataoka, J. & Yoshimoto, T. (1995). *Biosci. Biotechnol. Biochem.* **59**(11), 2087–2090.
Walker, K. W. & Bradshaw, R. A. (1998). *Protein Sci.* **7**(12), 2684–2687.
Wilce, M. C. J., Bond, C. S., Dixon, N. E., Freeman, H. C., Guss, J. M., Lilley, P. E. & Wilce, J. A. (1998). *Proc. Natl Acad. Sci. USA*, **95**, 3472–3477.
Yaron, A. & Berger, A. (1970). *Methods Enzymol.* **19**, 521–534.
Yaron, A. & Mlynar, D. (1968). *Biochem. Biophys. Res. Commun.* **32**(4), 658–663.
Yaron, A. & Naider, F. (1993). *Crit. Rev. Biochem. Mol. Biol.* **28**(1), 31–81.
Zhang, L., Crossley, M. J., Dixon, N. E., Ellis, P. J., Fisher, M. L., King, G. F., Lilley, P. E., MacLachlan, D., Pace, R. J. & Freeman, H. C. (1998). *J. Biol. Inorg. Chem.* **3**, 470–483.