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Crystallization and preliminary X-ray analysis of aspartate racemase from *Pyrococcus horikoshii* OT3

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Aspartate racemase from *Pyrococcus horikoshii* OT3 (*P.* AspR) has been crystallized in three crystal forms by the sitting-drop vapour-diffusion method. The crystals belong to the space groups $P2_1$, $P2_12_12_1$ and $P3_121$ (or $P3_221$). The crystals of space group $P2_1$ diffract X-rays beyond 1.7 Å resolution under 90 K liquid-nitrogen cryo-conditions with synchrotron radiation and were selected for structure determination. Two heavy-atom derivatives of this crystal form were obtained by the soaking method, which afforded the initial electron-density map.

1. Introduction

As it is directly related to the origin of the homochirality in proteins, amino-acid chirality has attracted much attention in recent years (Keszthelyi, 1995). Contrary to the opinion that all naturally occurring amino acids *in vivo* were L-enantiomers, D-amino acids have been reported to be present as free or combined in peptides not only in bacteria but also in the tissues of higher plants and animals (D'Aniello, Di Fiore *et al.*, 1998). Much biological work has already shown that D-amino acids play crucial roles in many life phenomena. It is well known that D-amino acids are basic substrates for the construction of the bacterial peptidoglycan of bacterial cell walls (Walsh, 1989). They are associated with some specific and exquisite immune phenomena (Michael & Einat, 1997) and they also participate in the activities of the nervous and endocrine systems (D'Aniello, Lee *et al.*, 1998).

Amino-acid racemases/epimerases are responsible for the racemization/epimerization of amino acids in bacteria. These enzymes can be grouped into two families, pyridoxal 5′-phosphate (PLP) dependent and PLP independent, based on whether or not PLP is required for the catalysis as a cofactor. Aspartate racemase, glutamate racemase and diaminopimelate epimerase are representatives of the PLP-independent enzyme family (Yamauchi *et al.*, 1992).

Recently, crystal structures of *Haemophilus influenzae* diaminopimelate epimerase and *Aquifex pyrophilus* glutamate racemase (*A.* GluR) were solved at 2.7 and 2.3 Å, respectively (Cirilli *et al.*, 1998; Hwang *et al.*, 1999). In spite of the fact that they showed very different primary and three-dimensional structures, crystallographic information indicated that both enzymes fold into two α/β domains, supporting a conjugated acid–base mechanism, which was subjected to PLP-independent racemases/epimerases. It is worth mentioning that the two cysteine residues and their respective surrounding amino-acid residues are highly conserved among all presently cloned and sequenced aspartate and glutamate racemases (Yamauchi *et al.*, 1992), which suggests that the racemization mechanism may be analogous, even if *P.* AspR shares only about 26% identity of sequence with *A.* GluR. Interestingly, in contrast to the two above-mentioned enzymes which exist as biological monomers, aspartate racemase was proved to be present as a biologically homologous dimer (Okada *et al.*, 1991) and it has also been suggested that the dimeric structure might be accountable for the racemization mechanism (Yamauchi *et al.*, 1992).

Crystallographic study will provide evidence to help in understanding the racemization mechanism of aspartate racemase. The three-dimensional structure of the aspartate racemase may be helpful to clarify their consistency and discrimination amongst PLP-independent racemases/epimerases. Here, we report the crystallization and the preliminary crystallographic study of aspartate racemase from the archaeon *Pyrococcus horikoshii*. It should also be mentioned that there are few reports on racemases from archaea, although they are widely found in bacteria (Yohda *et al.*, 1996, Matsumoto *et al.*, 1999).

2. Experimental

2.1. Cloning, expression and purification

The expression vector pET23c (Novagen) was used for *P.* AspR expression. The open reading frame encoding *P.* AspR was amplified

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by PCR from a shotgun clone containing the full-length gene of PH0670 with the oligonucleotide primers 5′-CATATGAAAACGATAGGTATACTTGG-3′ and 5′-GGATCCTTACTTTTCTAATGCAACC-3′; it was then subcloned into the pT7Blue T vector (Novagen). After sequence confirmation, the gene was excised and introduced into pET23c to construct a plasmid, pPH0670E. *Escherichia coli* BL21(DE3) transformed with pPH0670E was cultured at 310 K in Luria–Bertani medium containing 100 µg ml^{-1} ampicillin. The *E. coli* cells were collected by centrifugation and disrupted by sonication. The crude extract, which exhibited racemase activity against aspartate at 343 K (manuscript in preparation), was heated at 343 K for 30 min to denature host proteins. After removing most host proteins by centrifugation, the extract was applied to a butyl-Toyopearl column (Tosoh, Japan) equilibrated with buffer *A* (50 m*M* Tris–HCl pH 8.0, 1 m*M* 2-mercaptoethanol) containing 1.6 *M* ammonium sulfate and the column was eluted with a linear ammonium sulfate gradient from 1.6 to 0 *M*. Fractions containing *P.* AspR were merged, concentrated and equilibrated with buffer *A* by ultrafiltration (Centriprep YM10, Millipore, USA), then applied to a UnoQ6 column (Bio-Rad, USA) equilibrated with buffer *A*. Finally, the column was eluted with buffer *A* with a linear NaCl gradient from 0 to 500 m*M* and the fractions containing *P.* AspR were merged and concentrated to 30 mg ml^{-1} in buffer *A* for crystallization.

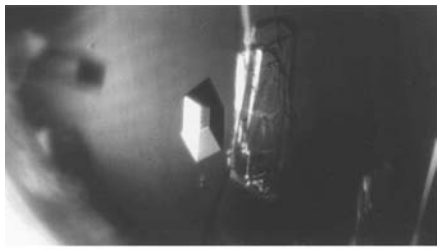
2.2. Crystallization

Crystallization was performed by the sitting-drop vapour-diffusion method at 293 K. In all experiments the crystallization drops, containing 3 µl protein solution (30 mg ml^{-1}) and 3 µl precipitant solution, were equilibrated against 0.8 ml reservoir solution (same as precipitant solution).

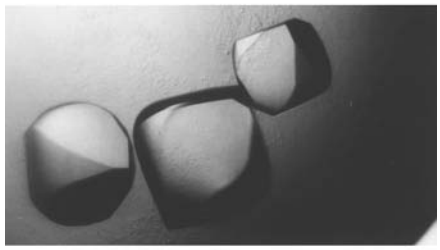
Initially crystallization conditions were surveyed by the Hampton screening series prior to optimization. Three types of crystals were obtained (Fig. 1). Type I crystals grew when precipitant solution (3.5 *M* ammonium sulfate, 10 m*M* magnesium acetate, 50 m*M* MES pH 5.6) was used, but this condition was not easily reproduced. Some dot-shaped type II crystals occurred when a different precipitant solution (3.0–4.0 *M* NaCl, 0.10 *M* sodium citrate pH 4.0) was used. On decreasing the NaCl concentration from 4.0 to 3.0 *M*, the crystal shape became more regular but the crystallization time increased from two weeks to a six months. Type III crystals could be obtained on substituting PEG 6000 for the inorganic salt precipitants. A precipitant solution consisting of 16.5% PEG 6000, 0.10 *M* sodium citrate pH 4.0 proved to be efficient for obtaining perfect single crystals with high diffraction ability. Interestingly, in this case 30 m*M* calcium acetate introduced to the precipitant dramatically accelerated the crystallization, which caused precipitation of calcium citrate but did not affect the crystal quality. Type III crystals were used for structure determination.

2.3. Derivative search

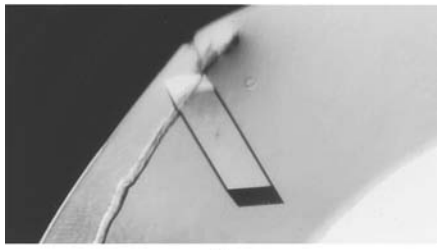
More than 20 kinds of heavy-atom reagent, directly introduced to the reservoir solution at concentrations of 0.25, 0.50, 1.0 and 2.0 m*M*, were screened in the heavy-atom derivative search. Finally, two derivatives were successfully obtained when crystals were separately soaked in 1.0 m*M* $HgCl(C_6H_4COOH)$ and 1.0 m*M* $KAu(CN)_2$ solutions for 3 d.



(*a*)



(*b*)



(*c*)

Figure 1
Crystals of aspartate racemase from *P. horikoshii* OT3: (*a*) type I, (*b*) type II, (*c*) type III. Approximate dimensions are 0.1 × 0.1 × 0.2, 0.4 × 0.4 × 0.4 and 0.15 × 0.15 × 0.6 mm for type I, II and III crystals, respectively.

2.4. X-ray diffraction study

Diffraction data from all the native and derivative crystals were collected under liquid-nitrogen cryoconditions of 90 K. Glycerol at a concentration of 15, 12 and 20% was introduced to the corresponding reservoir solutions as cryoprotectant for type I, II and III crystals, respectively. Crystals were flash-cooled by rapidly moving them into the cold nitrogen stream. Simply rinsing crystals in reservoir solution containing cryoprotectant for about 10 s could avoid damage on freezing. X-ray diffraction data were collected with an R-AXIS IV imaging-plate area detector using Cu $K\alpha$ radiation from a Rigaku ultraX18 rotating-anode generator working at 40 kV and 100 mA, and also with an ADSC CCD and a MAR CCD using synchrotron radiation on beamline BL6A at the Photon Factory (KEK, Tsukuba, Japan) and on beamline BL44B2 at SPring-8 (Harima, Japan), respectively. Diffraction data were processed using *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997) and *DPS* and *SCALA* (Rossmann & van Beek, 1999).

3. Results and discussion

The processed diffraction data of type I crystals showed the space group to be $P2_12_12_1$, with unit-cell parameters a = 68.1, b = 135.0, c = 151.5 Å. It was also found that type II and III crystals belong to the space groups $P3_121$ (or $P3_221$), with unit-cell parameters $a = b$ = 80.6, c = 70.3 Å, and $P2_1$, with unit-cell parameters of a = 65.5, b = 58.7, c = 67.0 Å, β = 109.6°, respectively. As *P.* AspR has a molecular mass of 25 kDa, the V_M values were calculated to be 2.36 Å^3 Da^{-1} for six molecules per asymmetric unit, 2.61 Å^3 Da^{-1} for one molecule per asymmetric unit and 2.48 Å^3 Da^{-1} for two molecules per asymmetric unit, for type I, II and III crystals, respectively (Matthews, 1968). Type I, II and III crystals diffract X-rays to at least 3.0, 2.5 and 1.7 Å resolution, respectively, and type III crystals were used for further structure determination. Table 1 shows the data-collection and processing statistics for three types of native crystals. The self-rotation function for type III crystals from *POLARRFN* (Collaborative Computational Project, Number 4, 1994) showed the existence of twofold non-crystallographic symmetry (NCS) between two molecules in the same asymmetric unit. The initial phases were successfully obtained by the multiple isomorphous replacement with anomalous scattering (MIRAS) using

Table 1
Data-collection and processing statistics for three types of *P.* AspR crystals.

Crystal	Type I	Type II	Type III
X-ray source	Rigaku ultraX18	BL6A (PF)	BL44B2 (SPring-8)
Space group	$P2_12_12_1$	$P3_121/P3_221$	$P2_1$
Unit-cell parameters† (Å, °)	a = 68.1 (1), b = 135.0 (1), c = 151.5 (2)	$a = b$ = 80.6 (1), c = 70.3 (1)	a = 65.5 (1), b = 58.7 (1), c = 67.0 (1), β = 109.6 (1)
No. of molecules in asymmetric unit	6	1	2
V_M (Å^3 Da^{-1})	2.36	2.61	2.48
X-ray source		BL6A (PF)	BL44B2 (Spring-8)
Wavelength (Å)		1.0000	1.0000
Resolution limit‡ (Å)		20–2.5 (2.59–2.5)	20–1.7 (1.76–1.7)
No. of measured reflections‡		96719 (9628)	420764 (29410)
No. of unique reflections‡		9466 (924)	52887 (5201)
Completeness‡ (%)		99.9 (100.0)	97.5 (95.5)
R_{sym}‡§ (%)		3.5 (5.6)	4.4 (8.5)

† Values in parentheses are the estimated deviations of the unit-cell parameters. ‡ Values in parentheses refer to the highest resolution shells. § $R_{sym} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I_i(hkl)\rangle|/\sum_{hkl}\sum_i I_i(hkl)$. i is the multiplicity of symmetry-related reflections of (hkl) and $\langle I_i(hkl)\rangle$ is the mean intensity of the i reflections.

two derivatives and *MLPHARE* (Collaborative Computational Project, Number 4, 1994). The MIRAS phases were significantly improved after density modification by *DM* (Collaborative Computational Project, Number 4, 1994). Construction of the model is now in progress.

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References

Cirilli, M., Zheng, R., Scapin, G. & Blanchard, J. S. (1998). *Biochemistry*, **37**, 16452–16458.
Collaborative Computational Project, Number 4 (1994). *Acta Cryst.* D**50**, 760–763.
D'Aniello A., Di Fiore, M. M. & Fisher, G. (1998). *Trends Comput. Biochem. Physiol.* **4**, 1–21.
D'Aniello A., Lee, J. M., Petrucelli, L. & Di Fiore, M. M. (1998). *Neurosci. Lett.* **250**, 131–134.
Hwang, K. Y., Cho, C.-S., Kim, S. S., Sung, H.-C., Yu, Y. G. & Cho, Y. (1999). *Nature Struct. Biol.* **6**, 422–426.
Keszthelyi, L. (1995). *Quart. Rev. Biophys.* **28**, 473–507.
Matsumoto, M., Homma, H., Long, Z., Imai, K., Iida, T., Maruyama, T., Aikawa, Y., Endo, I. & Yohda, M. (1999). *J. Bacteriol.* **181**, 6560–6563.
Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
Michael, S. & Einat, Z. (1997). *FASEB J.* **11**, 449–456.
Okada, H., Yohda, M., Giga-Hama, Y., Ueno, Y., Ohdo, S. & Kumagai, H. (1991). *Biochim. Biophys. Acta*, **1078**, 377–382.
Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.
Rossmann, M. G. & van Beek, C. G. (1999). *Acta Cryst.* D**55**, 1631–1640.
Walsh, C. T. (1989). *J. Biol. Chem.* **264**, 2393–2396.
Yamauchi, T., Choi, S.-Y., Okada, H., Yohda, M., Kumagai, H., Esaki, N. & Soda, K. (1992). *J. Biol. Chem.* **267**, 18361–18364.
Yohda, M., Endo, I., Abe, Y., Ohta, T., Iida, T., Maruyama, T. & Kagawa, Y. (1996). *J. Biol. Chem.* **271**, 22017–22021.