

Crystallization and preliminary X-ray diffraction
analysis of thermophilic imidase from pig liverCheng-Yang Huang,^a Sheng-Kuo
Chiang,^b Yuh-Shyong Yang^{a*} and
Yuh-Ju Sun^{b*}^aDepartment of Biological Science and
Technology, College of Science, National Chiao
Tung University, Hsinchu, Taiwan, and^bInstitute of Bioinformatics and Structural
Biology and Department of Life Science,
National Tsing Hua University, Hsinchu,
Taiwan

Correspondence e-mail:

ysyang@cc.nctu.edu.tw,

yjsun@life.nthu.edu.tw

Imidase is an enzyme, also known as dihydropyrimidinase (EC 3.5.2.2), hydantoinase, dihydropyrimidine hydrase or dihydropyrimidine amidohydrolase, that catalyzes the reversible hydrolysis of 5,6-dihydrouracil to 3-ureidopropionate and many other imides. Substrate specificity, metal content and amino-acid sequence all differ significantly between bacterial and mammalian imide-hydrolyzing enzymes. In this study, a thermophilic imidase was isolated from pig liver and crystallized. Two kinds of imidase crystals were grown by the hanging-drop vapour-diffusion method using polyethylene glycol MME 5000 and 2-propanol as precipitants. One belongs to the triclinic P_1 space group, with unit-cell parameters $a = 96.35$, $b = 96.87$, $c = 154.87$ Å, $\alpha = 82.10$, $\beta = 72.54$, $\gamma = 77.19^\circ$, and the other belongs to the orthorhombic $C22_1$ space group, with unit-cell parameters $a = 113.92$, $b = 157.22$, $c = 156.21$ Å.

Received 26 November 2002

Accepted 12 March 2003

1. Introduction

Imide-hydrolyzing enzymes (EC 3.5.2.2) were first described in the 1940s as hydrolyzing hydantoin derivatives in plants and animals (Bernheim & Bernheim, 1946; Eadie *et al.*, 1949). The natural substrate of an imide-hydrolyzing enzyme isolated from calf liver was shown to be dihydropyrimidine and it was therefore named dihydropyrimidinase (Wallach & Grisolia, 1957). A detailed investigation into enzyme substrates showed that the hydrolytic cleavage of imides catalyzed by the rat liver enzyme ranges from linear imides to heterocyclic imides and includes hydantoins, dihydropyrimidines and phthalimide. Therefore, the imide-hydrolyzing enzyme was classified as an imidase (Yang *et al.*, 1993). *In vitro* data indicate that imidase prefers xenobiotics to physiological substrates (Yang *et al.*, 1993). The substrate spectrum of imidase was further shown to include the cyclic carbonates (Yang *et al.*, 1998). A recent finding shows that the imidase from pig liver is thermophilic. The optimum temperature for catalysis of this mammalian enzyme is more than 20 K higher than that of its native source and its substrate specificity is temperature dependent (Su & Yang, 2000).

All known mammalian imidases are tetramers that contain four tightly bound Zn atoms, with one Zn atom per subunit (Brooks *et al.*, 1983; Kautz & Schnackerz, 1989; Kikugawa *et al.*, 1994; Huang & Yang, 2002). However, variations are found in the structure, metal content, substrate specificity and other requirements of similar enzymes from different organisms. Imidase from *Bacillus stearothermophilus* SD-1 is a dimer and contains

manganese, with one Mn atom per dimer (Lee *et al.*, 1995). Imidase from *Blastobacter* sp. A17p-4 is a trimer (Ogawa *et al.*, 1997). Imidase from *Pseudomonas putida* 77 requires ATP for its amidohydrolysis (Ogawa *et al.*, 1995). Imidase from *Arthrobacter aureescens* DSM 3745 is a zinc enzyme (May *et al.*, 1998a) and contains 10 moles of zinc per mole of enzyme (May *et al.*, 1998b).

Recently, the crystal structures of bacterial enzymes from *Thermus* sp. (Abendroth, Niefind & Schomburg, 2002; Abendroth, Niefind, May *et al.*, 2002) and *B. stearothermophilus* (Cheon *et al.*, 2002) were resolved. They were all shown to contain bridged dimetal ions in the enzyme subunit. It has also been reported that some bacterial imide-hydrolyzing enzymes are able to hydrolyze sulfur-containing cyclic imides, simple cyclic imides and simple cyclic ureides, but that substrates do not include substituted cyclic ureides and bulky substituted cyclic imides (Ogawa *et al.*, 1997). It would be interesting to discover how nature has evolved such varied enzyme systems for imide hydrolysis in different organisms. Since the crystal structure of the bacterial enzyme is available, further structural analysis of mammalian imidase will allow the difference between these imidases to be better understood. Here, we report the first preliminary analysis of a mammalian imidase crystal.

2. Materials and methods

Pig liver imidase was purified as described previously (Su & Yang, 2000) with some modifications to obtain extra high purity for

crystallization. The thermophilic pig liver imidase was concentrated to 30 mg ml⁻¹ in 20 mM Tris-HCl. Crystallization trials were set up by the hanging-drop vapour-diffusion method (McPherson, 1982). 2 µl protein samples were equilibrated against a 500 µl reservoir solution in Linbro plates. Initial crystallization conditions were obtained using Hampton Research Crystal Screen kits (Hampton Research, California, USA) and then further optimized to obtain diffraction-quality crystals.

X-ray diffraction data from form *A* crystals (Fig. 1*a*) were measured at 0.9537 Å using a Quantum-4R CCD detector on the BL12B2 Taiwan beamline at SPring-8, Japan. The X-ray diffraction data from form *B* crystals (Fig. 1*b*) were collected on a Rigaku R-Axis IV++ image-plate detector using Cu Kα radiation from a Rigaku RU-300 rotating-anode X-ray generator operated at 50 kV and 100 mA. Thermophilic pig liver imidase form *A* crystals were grown from 10% (w/v) polyethylene glycol MME 5000; extra cryoprotectant was needed for data collection at low temperatures (100 K). The optimized cryoprotectant buffer prevented ice-ring formation in the flash-frozen crystal. Firstly, the crystals were transferred directly into a cryoprotectant buffer containing 15% (v/v) glycerol and incubated for 30 min before data collection. Higher concentrations of glycerol (>15%) melted the crystal. The form *B* crystal was

directly frozen in liquid nitrogen immediately before data collection without using any extra cryoprotectant reagent. Crystals were mounted in a nylon loop and flash-frozen in a nitrogen stream (Oxford Cryosystems). The programs *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997) were used for the X-ray diffraction data processing and analysis.

3. Results

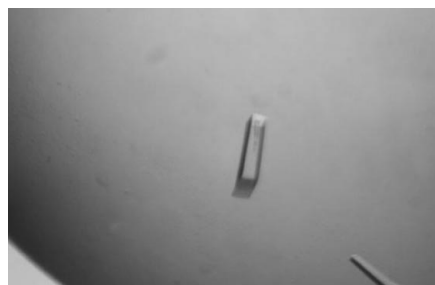
Two forms of crystal were grown under quite different crystallization conditions using

polyethylene glycol MME 5000 and 2-propanol as precipitants. Needle-shaped form *A* crystals (Fig. 1*a*) grew in 3–5 d to maximum dimensions of 0.05 × 0.05 × 0.8 mm using 10% (w/v) polyethylene glycol MME 5000, 100 mM sodium chloride, 100 mM bicine buffer pH 9.0 and 2% (w/v) 1,6-hexanediol. Form *B* crystals (Fig. 1*b*) grew over a longer period of time, 3–4 weeks, to maximum dimensions of 0.05 × 0.08 × 0.1 mm using 30% (v/v) 2-propanol, 0.2 M sodium citrate and 100 mM sodium cacodylate pH 6.5. Form *A* crystals diffracted to 2.2 Å and belonged to the

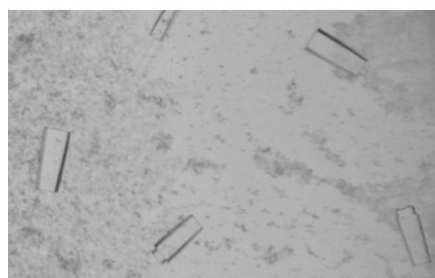
Table 1
Summary of X-ray diffraction data statistics.

Values in parentheses are for the outermost resolution shell.

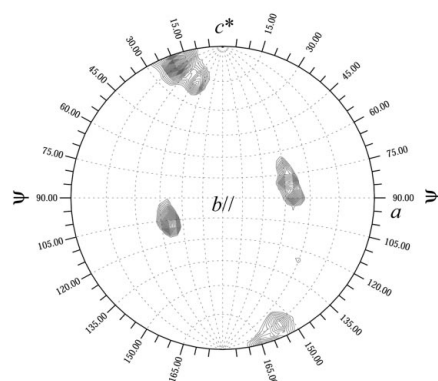
	Form <i>A</i>	Form <i>B</i>
Wavelength (Å)	0.9537	1.54
Crystal system	Triclinic	Orthorhombic
Space group	<i>P</i> ₁	<i>C</i> 222 ₁
Unit-cell parameters		
<i>a</i> , <i>b</i> , <i>c</i> (Å)	96.35, 96.87, 154.87	113.91, 157.30, 156.26
α, β, γ (°)	82.10, 72.54, 77.19	90, 90, 90
Resolution (Å)	2.2	3.0
No. of reflections collected	555227	92506
No. of unique reflections	253875	53857
Redundancy of reflections	1.8	1.72
Data completeness (%)	96.2 (92.7)	99.2 (99.9)
<i>I</i> /σ(<i>I</i>)	8.04 (1.84)	6.3 (3.5)
<i>R</i> _{merge} (%)	9.3 (46)	11.9 (20.9)



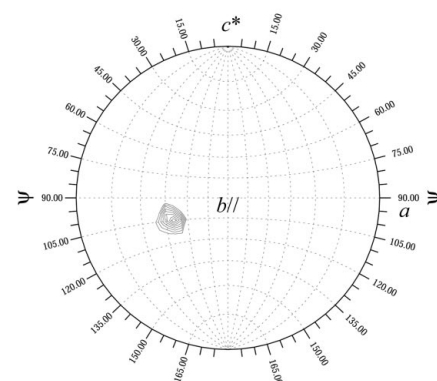
(*a*)



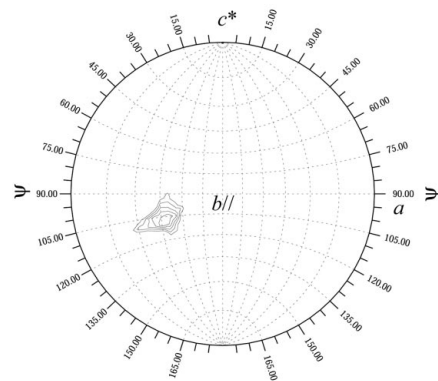
(*b*)



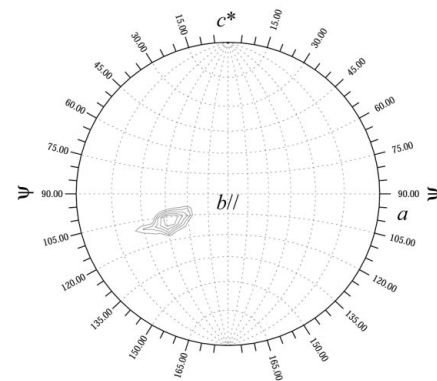
(*a*)



(*b*)



(*c*)



(*d*)

Figure 1
Photographs showing crystals of thermophilic pig liver imidase. (*a*) Form *A* crystals, triclinic *P*₁ space group. (*b*) Form *B* crystals, orthorhombic *C*222₁ space group.

Figure 2
Self-rotation functions of a form *A* crystal, space group *P*₁, with (*a*) κ = 180, (*b*) κ = 120, (*c*) κ = 90 and (*d*) κ = 60°.

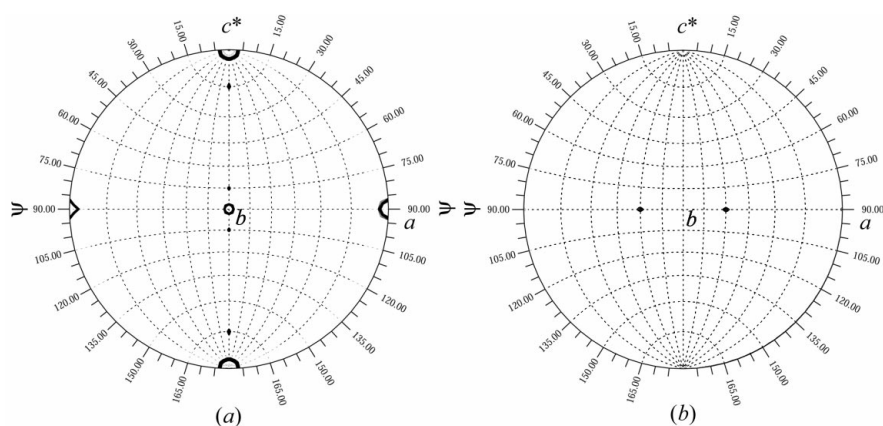


Figure 3
Self-rotation functions of a form *B* crystal space group $C222_1$, with (a) $\kappa = 180^\circ$ and (b) $\kappa = 120^\circ$.

triclinic space group, with unit-cell parameters $a = 96.35$, $b = 96.87$, $c = 154.87$ Å, $\alpha = 82.10$, $\beta = 72.54$, $\gamma = 77.19^\circ$ (Table 1). Form *B* crystals diffracted to 3.0 Å and belonged to the orthorhombic space group $C222_1$, with unit-cell parameters $a = 113.92$, $b = 157.22$, $c = 156.21$ Å (Table 1).

Most of the imidases are described in the literature as forming tetramers and a few as forming dimers or trimers, based on gel chromatography and dynamic light-scattering studies. The molecular weight for a single polypeptide chain of pig liver imidase was estimated to be around 51 kDa. However, the molecular weight of the quaternary structures of pig liver imidase was about 300 kDa as determined by gel filtration. Thus, pig liver imidase may indeed function as a tetramer (Su & Yang, 2000). In the *P1* triclinic crystal, we predicted there to be 12 molecules (three tetramers) per asymmetric unit in the crystal packing from the V_M (Matthews, 1968) constant calculation. The V_M (Matthews, 1968) was

calculated to be $2.26 \text{ \AA}^3 \text{ Da}^{-1}$ and the solvent content was estimated to be 46%. The self-rotation function was calculated using the *GLRF* program (Tong & Rossmann, 1990). The non-crystallographic symmetric relationships of molecules are shown at κ angles of 180, 120, 90 and 60° in Fig. 2. Pseudo twofold, threefold, fourfold and sixfold axes were found. For the $C222_1$ orthorhombic crystal, the V_M (Matthews, 1968) was calculated to be $2.29 \text{ \AA}^3 \text{ Da}^{-1}$, corresponding to a solvent content of 47%, assuming three molecules per asymmetric unit in the crystal. Pseudo twofolds and threefold axes were found from self-rotation function calculations. The self-rotation function peaks are shown at κ angles of 180 and 120° in Fig. 3. Structure determination by the molecular-replacement method using the imidases of *Thermus* sp. (Abendroth, Niefind & Schomburg, 2002) and *B. stearrowophilus* (Cheon *et al.*, 2002) as search models is also in progress.

References

- Abendroth, J., Niefind, K., May, O., Siemann, M., Syltatk, C. & Schomburg, D. (2002). *Biochemistry*, **41**, 8589–8597.
- Abendroth, J., Niefind, K. & Schomburg, D. (2002). *J. Mol. Biol.* **320**, 143–156.
- Bernheim, F. & Bernheim, M. L. C. (1946). *J. Biol. Chem.* **163**, 683–685.
- Brooks, K. P., Jones, E. A., Kim, B. D. & Sander, E. B. (1983). *Arch. Biochem. Biophys.* **226**, 469–483.
- Cheon, Y.-H., Kim, H.-S., Han, K.-H., Abendroth, J., Niefind, K., Schomburg, D., Wang, J. & Kim, Y. (2002). *Biochemistry*, **41**, 9410–9417.
- Eadie, G. S., Bernheim, F. & Bernheim, M. L. C. (1949). *J. Biol. Chem.* **181**, 449–458.
- Huang, C.-Y. & Yang, Y.-S. (2002). *Biochem. Biophys. Res. Commun.* **297**, 1027–1032.
- Kautz, J. & Schnackerz, K. D. (1989). *Eur. J. Biochem.* **181**, 431–435.
- Kikugawa, M., Kaneko, M., Fujimoto-Sakata, S., Maeda, M., Kawasaki, K., Takagi, T. & Tamaki, N. (1994). *Eur. J. Biochem.* **219**, 393–399.
- Lee, S.-G., Lee, D.-C., Hong, S.-P., Sung, M.-H. & Kim, H.-S. (1995). *Appl. Microbiol. Biotechnol.* **43**, 270–276.
- McPherson, A. (1982). *Preparation and Analysis of Protein Crystals*. New York: John Wiley.
- Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
- May, O., Siemann, M., Siemann, M. G. & Syltatk, C. (1998a). *J. Mol. Catal. B*, **4**, 211–218.
- May, O., Siemann, M., Siemann, M. G. & Syltatk, C. (1998b). *J. Mol. Catal. B*, **5**, 367–370.
- Ogawa, J., Kim, J. M., Nirdonoy, W., Amano, Y., Yamada, H. & Shimizu, S. (1995). *Eur. J. Biochem.* **229**, 284–290.
- Ogawa, J., Soong, C. L., Honda, M. & Shimizu, S. (1997). *Eur. J. Biochem.* **243**, 322–327.
- Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.
- Su, T.-M. & Yang, Y.-S. (2000). *Protein Exp. Purif.* **19**, 289–297.
- Tong, L. & Rossmann, M. G. (1990). *Acta Cryst.* **A46**, 783–792.
- Wallach, D. P. & Grisolia, S. (1957). *J. Biol. Chem.* **226**, 277–288.
- Yang, Y.-S., Ramaswamy, S. & Jakoby, W. B. (1993). *J. Biol. Chem.* **268**, 10870–10875.
- Yang, Y.-S., Ramaswamy, S. & Jakoby, W. B. (1998). *J. Biol. Chem.* **273**, 7814–7817.