

Catleya Rojviriyi,^a Thunyaluck Pratumrat,^a Mark A. Saper^b and Jirundon Yuvaniyama^{a*}

^aDepartment of Biochemistry and Center for Excellence in Protein Structure and Function, Faculty of Science, Mahidol University, Rama 6 Road, Phayathai, Bangkok 10400, Thailand, and ^bDepartment of Biological Chemistry, University of Michigan, Ann Arbor, Michigan 48109-1055, USA

Correspondence e-mail: scjyv@mahidol.ac.th

Received 21 July 2011

Accepted 1 October 2011

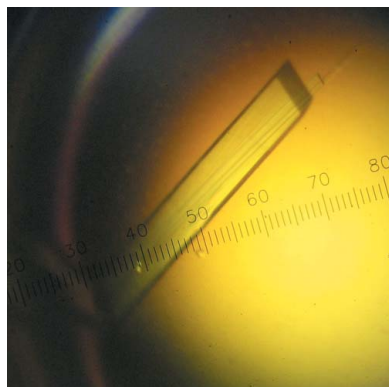
Improved X-ray diffraction from *Bacillus megaterium* penicillin G acylase crystals through long cryosoaking dehydration

Penicillin G acylase from *Bacillus megaterium* (*BmPGA*) is currently used in the pharmaceutical industry as an alternative to PGA from *Escherichia coli* (*EcPGA*) for the hydrolysis of penicillin G to produce 6-aminopenicillanic acid (6-APA), a penam nucleus for semisynthetic penicillins. Despite the significant differences in amino-acid sequence between PGAs from Gram-positive and Gram-negative bacteria, a representative PGA structure of Gram-positive origin has never been reported. In this study, crystallization and diffraction studies of *BmPGA* are described. Poor diffraction patterns with blurred spots at higher resolution were typical for *BmPGA* crystals cryocooled after a brief immersion in cryoprotectant solution. Overnight soaking in the same cryo-solution substantially improved both the mosaicity and resolution limit through the establishment of a new crystal-packing equilibrium. A crystal of *BmPGA* diffracted X-rays to 2.20 Å resolution and belonged to the monoclinic space group $P2_1$ with one molecule of *BmPGA* in the asymmetric unit.

1. Introduction

Penicillin G acylase (PGA; penicillin amidohydrolase; EC 3.5.1.11), also referred to as penicillin acylase class II, is an important enzyme in the industrial production of semisynthetic penicillins, a widely used class of β -lactam antibiotics (Kresse *et al.*, 2007). PGA hydrolyzes the amide bond between the phenylacetyl side chain and the β -lactam nucleus of natural penicillin G to produce 6-aminopenicillanic acid (6-APA), which is a key intermediate in the manufacture of a wide range of semisynthetic penicillins. Its high selectivity in this single-step conversion reaction makes the enzyme a cost-effective and environmentally friendly alternative to conventional processes, which involve multiple chemical reactions (Elander, 2003; Chandel *et al.*, 2008). Currently, the majority of deacylations in β -lactam production processes depend on PGA. Present developments indicate that the enzyme can also be successfully exploited in a synthetic direction (Bruggink *et al.*, 1998; Alkema *et al.*, 2003; Gabor *et al.*, 2005; Giordano *et al.*, 2006). Subsequent production processes of valuable antibiotics such as amoxicillin and ampicillin are based on the condensation of an appropriate D(-)-amino-acid derivative with 6-APA (Youshko & Svedas, 2000; Gonçalves *et al.*, 2002; Gabor & Janssen, 2004; Giordano *et al.*, 2006).

PGA is a heterodimeric protein that belongs to the N-terminal nucleophile (Ntn) hydrolase family, a class of enzymes containing a distinctive $\alpha\beta\beta\alpha$ structural motif with the N-terminal amino-acid residue of the β -chain serving as the nucleophile for catalysis (Duggleby *et al.*, 1995). It is produced as either an intracellular or an extracellular enzyme by a variety of microorganisms. PGAs from Gram-negative bacteria such as *Escherichia coli* (Cole, 1969), *Kluyvera cryocrescens* (formerly *K. citrophila*; Barbero *et al.*, 1986), *Providencia rettgeri* (Klei *et al.*, 1995) and *Alcaligenes faecalis* (Verhaert *et al.*, 1997) accumulate in the periplasmic space, whereas PGAs from Gram-positive bacteria such as *Arthrobacter viscosus* (Ohashi *et al.*, 1988) and *Bacillus megaterium* (Chiang & Bennett, 1967) are generally secreted outside of the cells. Interestingly, primary-structure analysis indicates that the PGAs of Gram-positive and Gram-negative bacteria are distinct, although they belong to the



© 2011 International Union of Crystallography
 All rights reserved

same protein family. The observed differences suggest that PGA from Gram-positive bacteria might represent a different evolutionary branch of the β -lactam acylases, such as the cephalosporin acylases (Martín *et al.*, 1995). To date, only crystal structures of PGAs from Gram-negative bacteria such as *E. coli* and *P. reuteri* have been determined (Duggleby *et al.*, 1995; Done *et al.*, 1998; Alkema *et al.*, 2000; McVey *et al.*, 2001).

Currently, PGA from *B. megaterium* ATCC14945 (*BmPGA*) is preferred for industrial processes (Rajendhran & Gunasekaran, 2004). Its biotechnological applications have emerged as an alternative to the well known PGA from *E. coli* ATCC11105 (*EcPGA*) because its extracellular secretion simplifies the downstream purification process. *BmPGA* has only 30% amino-acid sequence identity to *EcPGA* as well as to other PGAs from Gram-negative bacteria, but has at least 97% identity to other PGAs from Gram-positive counterparts. Recent research efforts have been focused on protein engineering to improve the enzyme stability and to extend its substrate specificity for synthetic applications. The lack of an experimental structure of *BmPGA* has restricted such rational design to information obtained from homology models (Yang *et al.*, 2000; Rajendhran & Gunasekaran, 2004; Wang *et al.*, 2007; Chandel *et al.*, 2008), the reliability of which might be limited by the low sequence similarity between *BmPGA* and the template structures. Therefore, in this study an effort has been made to crystallize *BmPGA* in order to elucidate its three-dimensional structure, which would facilitate protein engineering. Unfortunately, poor diffraction patterns were initially obtained from these *BmPGA* crystals. Instead of finding new crystal forms that might exhibit better X-ray diffraction, a long cryo-soak was tried, which substantially improved the diffraction quality of the crystals. Here, we present the crystallization, post-crystallization soaking and preliminary crystallographic studies of *BmPGA*.

2. Methods

2.1. Protein production and purification

BmPGA was produced as a secreted protein from the plasmid pBA402 containing the PGA-producing gene (*pac*) of *B. megaterium* UN1 in the *pac*⁻ mutant strain *B. megaterium* UN-cat (Panbangred *et al.*, 2000) with the following modifications. The bacterial cells were

grown overnight at 301 K with shaking at 250 rev min⁻¹ in Luria-Bertani medium (LB) containing 10 $\mu\text{g ml}^{-1}$ kanamycin. A 2 ml preculture was transferred into a culture flask of 200 ml modified LB (1% peptone, 0.5% yeast extract, 0.1% sodium chloride) containing kanamycin for batch preparation of the enzyme. Following continued shaking for an additional 48 h, the extracellular medium was separated from the cell culture by centrifugation for 20 min at 5000g. After adjustment of the pH to 5.50, the supernatant was filtered through a 0.45 μm PVDF membrane (Durapore, Millipore) to remove any fine particles as well as residual cells. The filtered *BmPGA* was purified to homogeneity using an SP-Sepharose HP (GE Healthcare) column pre-equilibrated with 50 mM sodium phosphate pH 5.50. The bound *BmPGA* was eluted with a linear gradient of 0–1 M sodium chloride in 50 mM sodium phosphate pH 5.50 at room temperature. The purity of the *BmPGA* was determined by SDS-PAGE (Laemmli, 1970) and the enzyme activity was assayed as described previously (Balasingham *et al.*, 1972). Fractions containing *BmPGA* were pooled based on *BmPGA* purity and then dialyzed against 0.4 M sodium chloride in 50 mM sodium phosphate pH 5.50 at 277 K. The purified protein was concentrated to 8–10 mg ml⁻¹ by ultrafiltration using 10 kDa cutoff centrifugal filter devices (Amicon, Millipore) and stored at 277 K for use in crystallization trials.

2.2. Crystallization of *BmPGA*

Prior to crystallization-drop setup, the concentrated protein was passed through a 0.22 μm centrifugal filter (Ultrafree-MC, Millipore) to remove dust, microparticles and aggregated protein. A wide range of conditions for *BmPGA* crystallization were initially screened in 60-well minitray plates (Nunc) using the modified microbatch method (Chayen *et al.*, 1992; D'Arcy *et al.*, 1996). Crystallization drops were set up by mixing an equal volume (1 μl) of protein solution with each crystallization solution under mineral oil containing vitamin E (Babi Mild Natural 'N Mild baby oil, Thailand; Chitnumsub *et al.*, 2004). Crystallization conditions producing crystals were further optimized systematically in ComboPlates (Greiner Bio-One) using the hanging-drop vapour-diffusion method (McPherson, 1999; Benvenuti & Mangani, 2007). For data collection, *BmPGA* crystals were obtained from drops consisting of 1 μl 8.6 mg ml⁻¹ protein solution and 1 μl 27% (m/v) PEG 4000, 0.2 M CaCl₂, 0.1 M imidazole pH 6.50 that were equilibrated against a 500 μl reservoir containing the same crystallization solution. All crystallization trials for *BmPGA* were set up at 295 K.

2.3. X-ray data collection and processing

A crystal was scooped up in a nylon loop and soaked in a cryo-protectant solution overnight prior to flash-cooling in a 98 K nitrogen stream generated by an X-Stream 2000 low-temperature system (Rigaku/MS). X-ray diffraction data were collected at the Center for Excellence in Protein Structure and Function (CPSF), Faculty of Science, Mahidol University, Thailand. The X-ray radiation was generated by a Rigaku RU-H3R rotating-anode X-ray generator (Cu K α ; $\lambda = 1.5418 \text{ \AA}$) running at 50 kV and 100 mA equipped with Osmic Confocal Max-Flux multi-layer optics and a 0.3 mm collimator. Diffraction images were recorded on an R-AXIS IV⁺⁺ image-plate system (Rigaku/MS). All diffraction data were indexed, integrated and scaled using the *CrystalClear/d*TREK* program suite (Pflugrath, 1999).

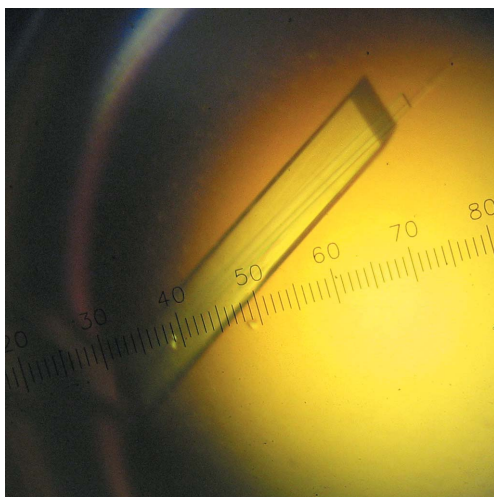


Figure 1
A monoclinic crystal of *B. megaterium* PGA of length 750 μm . These *BmPGA* crystals typically reached approximate dimensions of 130 \times 500 \times 70 μm in 7 d. At this magnification, a 60-unit length on the scale corresponds to an actual length of 1 mm.

3. Results and discussion

BmPGA was purified from extracellular medium to 95% homogeneity using a single-step purification protocol. The initial crystallization trials produced clusters of layered plate-shaped crystals within 2 d. Combinations of either sodium chloride or calcium chloride, PEG 4000 and buffers in the pH range 6.0–7.5 represented the best crystallizing solutions in the microbatch screening. Single plate-shaped crystals of *BmPGA* were successfully grown after grid refinement of selected conditions using the hanging-drop vapour-diffusion technique. Using the optimized conditions, crystals appeared within 3 d and grew to maximum dimensions in 7 d (Fig. 1).

A number of cryoprotectant solutions were tested by flash-cooling the crystals under a cold nitrogen stream at 98 K prior to data collection, with unsuccessful results. Although most of the tested cryoprotectants allowed sample vitrification with no ice rings, the crystals showed either no or very poor diffraction. A brief immersion (5–30 s) of the crystals in cryoprotectant solutions that were similar to the reservoir solution but with a 7–40% increase in the corresponding crystallizing agent concentration and with or without 12–15% (v/v) glycerol yielded diffraction patterns with streaked or blurred reflections at higher resolution despite sharp spots at low resolution (Fig. 2). The high-resolution reflections appeared to be slightly less smeared with a shorter cryoprotectant soak, yet the diffraction was still unsuitable for data collection and structure determination. One of the better diffracting crystals could be indexed in the monoclinic space group $P2_1$, with unit-cell parameters $a = 86.22$, $b = 78.15$, $c = 117.02$ Å, $\beta = 100.20^\circ$. The molecular volume (Matthews coefficient; Matthews, 1968) was estimated to be 2.3 Å³ Da⁻¹ with a calculated solvent content of 46.3% assuming the presence of two molecules per asymmetric unit. Attempts to minimize the soaking time did not improve the diffraction. The smearing patterns led us to think that there might be global conformational changes, perhaps with minor

rearrangement of the crystal packing, upon contact with the cryo-solution, as the high-resolution data were more dramatically affected. We then decided to leave some crystals to soak for longer in the cryosolutions (150–200 µl in a sealed well), allowing the establishment of a new conformation/packing equilibrium. Crystals soaked for longer in the cryoprotectant solution showed brighter birefringence under a polarized microscope and exhibited improved diffraction, with sharp spots to beyond 2.5 Å resolution after overnight soaking. One of the *BmPGA* crystals diffracted X-rays to 2.20 Å resolution in-house with a 185 mm crystal-to-detector distance (Fig. 3). A total of 180 diffraction images were collected with an oscillation angle of 1° and an exposure time of 720 s per image. The crystal belonged to the primitive monoclinic space group $P2_1$, with unit-cell parameters $a = 58.10$, $b = 77.84$, $c = 84.04$ Å, $\beta = 101.02^\circ$. A molecular volume of 2.2 Å³ Da⁻¹ was obtained with a calculated solvent content of 44.0% assuming one *BmPGA* molecule per asymmetric unit. The change in unit-cell parameters supports our hypothesis of molecular rearrangement. In this case, the *BmPGA* molecules apparently reoriented such that the noncrystallographic symmetry that might have been slightly off the b axis in the original lattice was moved parallel to the b axis and essentially became the crystallographic symmetry in the new packing. Thus, this allowed the new lattice to be indexed with half the original unit-cell volume. Statistics of data collection for the new cell are summarized in Table 1.

Initial phases for this *BmPGA* structure were solved with the molecular-replacement method using the *AMoRe* program (Navaza, 1994) in the *CCP4* program package (Winn *et al.*, 2011; Dodson *et al.*, 1997). A known structure of native *EcPGA* (PDB entry 1pnm; Duggleby *et al.*, 1995), the amino-acid sequence of which shares 71% homology (30% identity) with *BmPGA*, was used as a search model. Using 15–3 Å resolution data, *AMoRe* successfully located a molecule in the asymmetric unit with a correlation coefficient and R factor

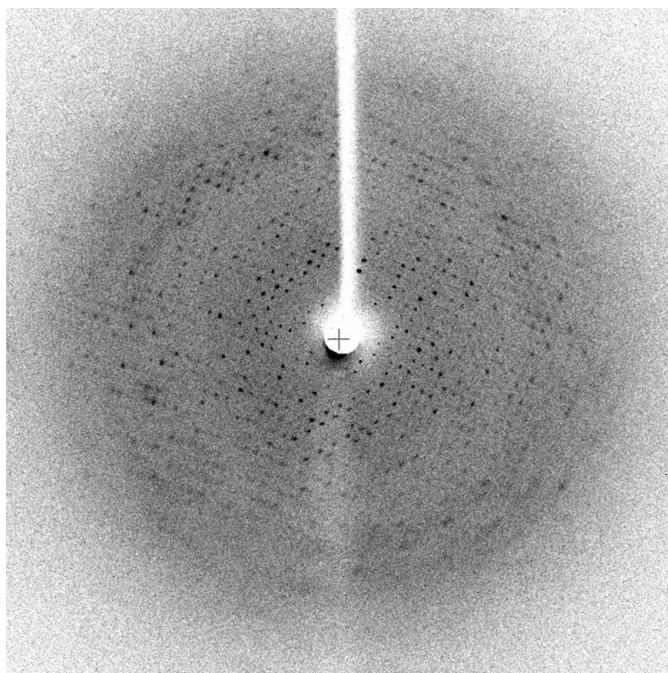


Figure 2 One of the best 1° oscillation photographs showing an X-ray diffraction pattern of a *BmPGA* crystal obtained from a short soak in cryoprotectant prior to flash-cooling under cold N₂ gas. The resolution at the edges of the detector is 2.89 Å. The diffraction spots at higher resolution are typically fuzzier than those at lower resolution.

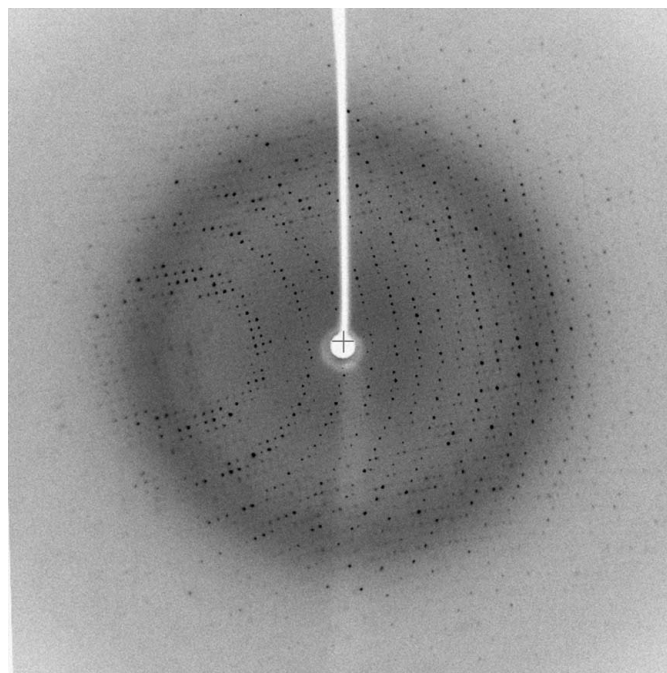


Figure 3 A 1° oscillation image showing an X-ray diffraction pattern of a *BmPGA* crystal soaked overnight in cryoprotectant solution consisting of 29% (m/v) PEG 4000, 0.2 M calcium chloride, 0.1 M imidazole pH 6.50 and 15% (v/v) glycerol. The resolution at the edges of the detector is 1.98 Å. Data were processed to 2.20 Å resolution.

Table 1Data-collection statistics for a *BmPGA* crystal.

Values in parentheses are for the highest resolution shell.

Resolution limits (Å)	56.6–2.20 (2.28–2.20)
No. of observed reflections	133201
No. of unique reflections	68640
Completeness (%)	93.5 (82.3)
Multiplicity	1.93 (1.67)
$R_{\text{merge}}^{\dagger}$ (%)	4.0 (20.6)
$\langle I/\sigma(I) \rangle$	9.8 (2.1)

$\dagger R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $I_i(hkl)$ is the intensity of the i th measurement of an equivalent reflection with indices hkl .

of 30.12% and 55.34%, respectively (the corresponding values for the second-best solution were 24.2% and 57.4%, respectively). Rigid-body refinement and minimization of the resulting solution carried out in the resolution range 50–2.8 Å using the *CNS* program (Brünger *et al.*, 1998) resulted in a drop in the R factor to 51.24%, with an R_{free} of 55.22%. The initial σ_A -weighted $2mF_o - DF_c$ electron-density map showed well defined backbone density, allowing us to build a three-dimensional structure of *BmPGA*. Crystallographic model rebuilding and refinement are in progress.

This crystal form exemplifies how extended soaking time in a cryoprotectant solution can improve X-ray diffraction quality in a case where a short cryosoak does not work. Reduction in the solvent content indicated that the overnight-soaked crystals were slightly dehydrated by the cryosolution. A similar treatment was previously reported for three unrelated proteins where crystals were dried for 15 min to several hours in small volumes of cryosolutions exposed to open air (Abergel, 2004). Such an improvement of diffraction upon crystal desiccation is thought to initiate rearrangement of protein conformation throughout the crystals similar to that seen in the dehydration of an HIV-1 reverse transcriptase crystal (Esnouf *et al.*, 1998; Abergel, 2004). Other successful crystal dehydration attempts involved exposure to air, addition of dehydrating agents or equilibration with dehydrating solutions using a hanging-drop setup over a period of minutes to months (Haebel *et al.*, 2001; Heras *et al.*, 2003; Kuo *et al.*, 2003; Heras & Martin, 2005; Sam *et al.*, 2006). Some of these techniques require multiple steps of crystal transfer or successive increments of dehydrating agent concentration to allow gradual changes in the crystal environment. Some were also followed by additional cryoprotecting steps prior to crystal vitrification. In our case, the increase in crystallizing agent concentration in the cryoprotectant solution was originally aimed to prevent crystal dissolution as no additional *BmPGA* was present in the soaking solution. This has been successfully used in our general crystal harvesting/preservation protocol for many proteins crystallized with salt and PEG combinations. Some crystal forms do not require such a gradual change of crystal environment and can endure transfer with excess liquid from the crystallization drops (2–10 μ l) to the soaking solution (100–200 μ l) with no sacrifice of crystal mosaicity. However, the increase in the concentration of crystallizing agent unintentionally dehydrated the *BmPGA* crystals, inducing a conformational change, and cryoprotected the crystal in the single soaking step. In addition, our method of soaking in a sealed container offers better control of the final conditions as well as reproducibility compared with an open-air treatment, which may result in detrimental dehydration. The presence of glycerol in the soaking of *BmPGA* crystals helped to eliminate the occasional appearance of faint shadows from ice rings. In crystal dehydration, the time required to reach equilibrium of the new packing environment may vary for different crystal forms. The soaking of *BmPGA* crystals was originally left overnight for convenience, although it was subsequently found with other similarly

treated crystals that improved birefringence could be observed after a couple of hours of soaking. Hence, crystal birefringence under polarized light may also serve as a crude detection of improvement in crystal integrity. From these results, we propose that a long soaking in cryoprotectant be tried as an alternative treatment for vitrification of crystals in routine data collection, especially in cases where a short cryoprotectant soak gives similar diffraction characteristics as were observed in our case.

We thank Vitthaya Meevootisom for his kind provision of the bacterial strain used in this study. Financial support was received from Mahidol University and Thailand Research Fund (TRF). CR is a Royal Golden Jubilee PhD Fellow of the TRF.

References

- Abergel, C. (2004). *Acta Cryst.* **D60**, 1413–1416.
- Alkema, W. B., de Vries, E., Floris, R. & Janssen, D. B. (2003). *Eur. J. Biochem.* **270**, 3675–3683.
- Alkema, W. B., Hensgens, C. M., Kroezinga, E. H., de Vries, E., Floris, R., van der Laan, J. M., Dijkstra, B. W. & Janssen, D. B. (2000). *Protein Eng.* **13**, 857–863.
- Balasingham, K., Warburton, D., Dunnill, P. & Lilly, M. D. (1972). *Biochim. Biophys. Acta*, **276**, 250–256.
- Barbero, J. L., Buesa, J. M., González de Buitrago, G., Méndez, E., Péz-Aranda, A. & García, J. L. (1986). *Gene*, **49**, 69–80.
- Benvenuti, M. & Mangani, S. (2007). *Nature Protoc.* **2**, 1633–1651.
- Bruggink, A., Roos, E. C. & de Vroom, E. (1998). *Org. Process Res. Dev.* **2**, 128–133.
- Brünger, A. T., Adams, P. D., Clore, G. M., DeLano, W. L., Gros, P., Grosse-Kunstleve, R. W., Jiang, J.-S., Kuszewski, J., Nilges, M., Pannu, N. S., Read, R. J., Rice, L. M., Simonson, T. & Warren, G. L. (1998). *Acta Cryst.* **D54**, 905–921.
- Chandel, A. K., Rao, L. V., Narasu, M. L. & Singh, O. V. (2008). *Enzyme Microb. Technol.* **42**, 199–207.
- Chayen, N. E., Shaw Stewart, P. D. & Blow, D. M. (1992). *J. Cryst. Growth*, **122**, 176–180.
- Chiang, C. & Bennett, R. E. (1967). *J. Bacteriol.* **93**, 302–308.
- Chitnumsub, P., Yavaniyama, J., Vanichtanankul, J., Kamchonwongpaisan, S., Walkinshaw, M. D. & Yuthavong, Y. (2004). *Acta Cryst.* **D60**, 780–783.
- Cole, M. (1969). *Biochem. J.* **115**, 733–739.
- D'Arcy, A., Elmore, C., Stihle, M. & Johnston, J. E. (1996). *J. Cryst. Growth*, **168**, 175–180.
- Dodson, E. J., Winn, M. & Ralph, A. (1997). *Methods Enzymol.* **277**, 620–633.
- Done, S. H., Brannigan, J. A., Moody, P. C. & Hubbard, R. E. (1998). *J. Mol. Biol.* **284**, 463–475.
- Duggleby, H. J., Tolley, S. P., Hill, C. P., Dodson, E. J., Dodson, G. & Moody, P. C. E. (1995). *Nature (London)*, **373**, 264–268.
- Elander, R. P. (2003). *Appl. Microbiol. Biotechnol.* **61**, 385–392.
- Esnouf, R. M., Ren, J., Garman, E. F., Somers, D. O'N., Ross, C. K., Jones, E. Y., Stammers, D. K. & Stuart, D. I. (1998). *Acta Cryst.* **D54**, 938–953.
- Gabor, E. M., de Vries, E. J. & Janssen, D. B. (2005). *Enzyme Microb. Technol.* **36**, 182–190.
- Gabor, E. M. & Janssen, D. B. (2004). *Protein Eng. Des. Sel.* **17**, 571–579.
- Giordano, R. C., Ribeiro, M. P. & Giordano, R. L. (2006). *Biotechnol. Adv.* **24**, 27–41.
- Gonçalves, L. R. B., Fernández-Lafuente, R., Guisán, J. M. & Giordano, R. L. C. (2002). *Enzyme Microb. Technol.* **31**, 464–471.
- Haebel, P. W., Wichman, S., Goldstone, D. & Metcalf, P. (2001). *J. Struct. Biol.* **136**, 162–166.
- Heras, B., Edeling, M. A., Byriel, K. A., Jones, A., Raina, S. & Martin, J. L. (2003). *Structure*, **11**, 139–145.
- Heras, B. & Martin, J. L. (2005). *Acta Cryst.* **D61**, 1173–1180.
- Klei, H. E., Daumy, G. O. & Kelly, J. A. (1995). *Protein Sci.* **4**, 433–441.
- Kresse, H., Belsey, M. J. & Rovini, H. (2007). *Nature Rev. Drug Discov.* **6**, 19–20.
- Kuo, A., Bowler, M. W., Zimmer, J., Antcliff, J. F. & Doyle, D. A. (2003). *J. Struct. Biol.* **141**, 97–102.
- Laemmli, U. K. (1970). *Nature (London)*, **227**, 680–685.
- Martín, L., Prieto, M. A., Cortés, E. & García, J. L. (1995). *FEMS Microbiol. Lett.* **125**, 287–292.
- Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.

- McPherson, A. (1999). *Crystallization of Biological Macromolecules*, 1st ed. New York: Cold Spring Harbor Laboratory Press.
- McVey, C. E., Walsh, M. A., Dodson, G. G., Wilson, K. S. & Brannigan, J. A. (2001). *J. Mol. Biol.* **313**, 139–150.
- Navaza, J. (1994). *Acta Cryst.* **A50**, 157–163.
- Ohashi, H., Katsuta, Y., Hashizume, T., Abe, S. N., Kajiura, H., Hattori, H., Kamei, T. & Yano, M. (1988). *Appl. Environ. Microbiol.* **54**, 2603–2607.
- Panbangred, W., Weeradechapon, K., Udomvaraphant, S., Fujiyama, K. & Meevootisom, V. (2000). *J. Appl. Microbiol.* **89**, 152–157.
- Pflugrath, J. W. (1999). *Acta Cryst.* **D55**, 1718–1725.
- Rajendhran, J. & Gunasekaran, P. (2004). *J. Biosci. Bioeng.* **97**, 1–13.
- Sam, M. D., Abbani, M. A., Cascio, D., Johnson, R. C. & Clubb, R. T. (2006). *Acta Cryst.* **F62**, 825–828.
- Verhaert, R. M., Riemens, A. M., van der Laan, J. M., van Duin, J. & Quax, W. J. (1997). *Appl. Environ. Microbiol.* **63**, 3412–3418.
- Wang, Z., Wang, L., Xu, J.-H., Bao, D. & Qi, H. (2007). *Enzyme Microb. Technol.* **41**, 121–126.
- Winn, M. D. *et al.* (2011). *Acta Cryst.* **D67**, 235–242.
- Yang, S., Huang, H., Li, S. Y., Ye, Y. Z., Wan, L., Zhang, F. W. & Yuan, Z. Y. (2000). *Sheng Wu Hua Xue Yu Sheng Wu Wu Li Xue Bao (Shanghai)*, **32**, 581–585.
- Youshko, M. I. & Svedas, V. K. (2000). *Biochemistry*, **65**, 1367–1375.