Acta Crystallographica Section D
Biological Crystallography
ISSN 0907-4449

Expression, purification, crystallization and preliminary crystallographic analysis of *Leishmania mexicana* phosphoglycerate mutase

Buabarn Poonperm,[a,b] Daniel G. Guerra,[c] Iain W. McNae,[a] Linda A. Fothergill-Gilmore[a] and Malcolm D. Walkinshaw[a]*

[a]Institute of Cell and Molecular Biology, University of Edinburgh, Edinburgh EH9 3JR, Scotland, [b]Beamline Division, Siam Photon Laboratory (SPL), Nakhon Ratchasima 30000, Thailand, and [c]Research Unit for Tropical Diseases, Christian de Duve Institute of Cellular Pathology and Department of Biochemistry, Université Catholique de Louvain, Brussels, Belgium

Correspondence e-mail: malcolm.walkinshaw@ed.ac.uk

Received 13 March 2003
Accepted 9 May 2003

Bacterially expressed 2,3-bisphosphoglycerate-independent phosphoglycerate mutase (iPGAM) from *Leishmania mexicana* with a six-His tag fused at its C-terminus was expressed from plasmid pET28a after IPTG induction in *Escherichia coli* cells and gave a yield of 20 mg of highly purified iPGAM per litre of cell culture. Crystals of the protein complexed with 3-phosphoglycerate were obtained by the hanging-drop method of vapour diffusion with PEG 4000 as the precipitating agent in the presence of cobalt chloride and diffracted synchrotron radiation to beyond 1.90 Å. The crystals belong to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters a = 62.46, b = 72.27, c = 129.68 Å. A model of *Bacillus stearothermophilus* iPGAM (33% identity) was used to provide an initial molecular-replacement solution. X-ray data to 2.05 Å for the structure of *L. mexicana* iPGAM complexed with 2-phosphoglycerate have also been collected.

1. Introduction

Phosphoglycerate mutase (PGAM) is an enzyme of the glycolytic pathway that catalyses the interconversion of 2- and 3-phosphoglycerates. There are two types of PGAM: one is dependent on the cofactor 2,3-bisphosphoglycerate (dPGAM) and the other is cofactor-independent (iPGAM). All vertebrates contain only dPGAM, whereas PGAM from the parasitic protozoan *Leishmania mexicana* belongs to the iPGAM class. The chain length of iPGAM is much larger than that of dPGAM and there is no sequence similarity between iPGAM and dPGAM (Chevalier *et al.*, 2000).

There is significantly more information available for the dPGAM family compared with the iPGAM family. dPGAMs from a variety of organisms have been well studied and the enzyme is present in all vertebrates, most invertebrates and some fungi and bacteria. It is found in monomeric, dimeric and tetrameric combinations of identical subunits of about M_r = 30 000 (Fothergill-Gilmore & Watson, 1989; Graña *et al.*, 1995). The catalytic mechanism of dPGAM involves the formation and hydrolysis of a phosphohistidine intermediate (Fothergill-Gilmore & Michels, 1993). A potent inhibitor of dPGAM is vanadate, which has no effect on iPGAM (Chevalier *et al.*, 2000; Fraser *et al.*, 1999). X-ray crystal structures of the yeast *Saccharomyces cerevisae* dPGAM at 2.3 and 2.12 Å resolution (Rigden *et al.*, 1998, 1999) and complexed with 3-phosphoglycerate (3PGA) at 1.7 Å (Crowhurst *et al.*, 1999) have been reported.

In contrast, information on iPGAM is rather sparse. The enzyme is a member of the alkaline phosphatase (AlkP) superfamily, which is composed of a wide range of enzymes that catalyse the intramolecular transfer of phospho or sulfo groups (Fraser *et al.*, 1999). All characterized iPGAMs are monomers of M_r = 55 000–75 000 (Fothergill-Gilmore & Michels, 1993) with sequences that are not apparently homologous with dPGAM. An X-ray structure of *Bacillus stearothermophilus* iPGAM complexed with 3PGA and two Mn^{2+} cations has been solved at 1.9 Å resolution (Chander *et al.*, 1999; Jedrzejas *et al.*, 2000*a*) and the structure of a complex with 2-phosphoglycerate (2PGA) and two Mn^{2+} cations has been solved at 1.7 and 1.4 Å resolution (Jedrzejas *et al.*, 2000*b*; Rigden *et al.*, 2003). The catalytic mechanism of this enzyme appears to involve a phosphoserine intermediate (Jedrzejas *et al.*, 2000*a*; Rigden *et al.*, 2002). iPGAMs from bacteria require Mn^{2+} for activity and are also very sensitive to pH (Chander *et al.*, 1998).

It has been noted that when iPGAM from the trypanosomatid parasite *Trypanosoma brucei* is inactivated by EDTA, it can only be fully reactivated by Co^{2+}. In contrast, Mn^{2+} only restored activity to about 10% (Collet *et al.*, 2001; Guerra *et al.*, 2003). Moreover, Collet *et al.* (2001) conclude from kinetic results that the mutase reaction is unlikely to proceed *via* a phosphoserine, but that a metaphosphate intermediate is more likely. *T. brucei* iPGAM shares 74% sequence identity with *L. mexicana*

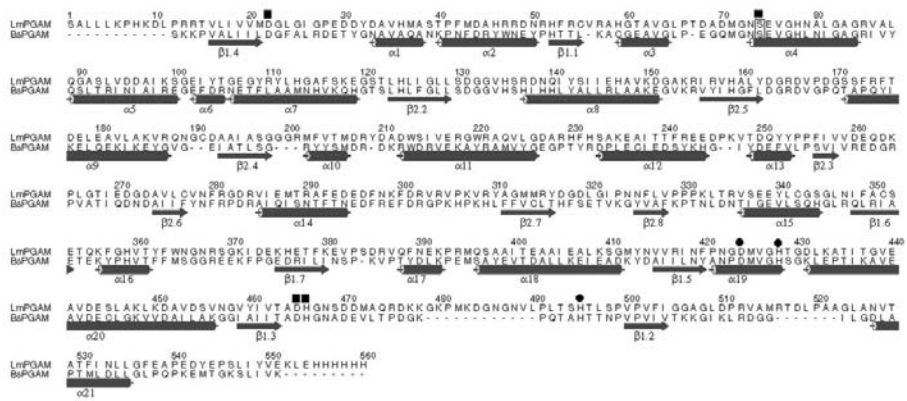

Figure 1
Sequence alignment of *L. mexicana* and *B. stearothermophilus* iPGAMs. Residue numbering is according to the *L. mexicana* iPGAM (accession No. AJ544274) and secondary structure is presented for the *B. stearothermophilus* iPGAM. The box indicates a putative phosphoserine residue and circles and squares show metal-coordinating residues for Mn1 and Mn2 of PDB entry 1ejj, respectively. The figure was prepared with *ALSCRIPT* (Barton, 1993).

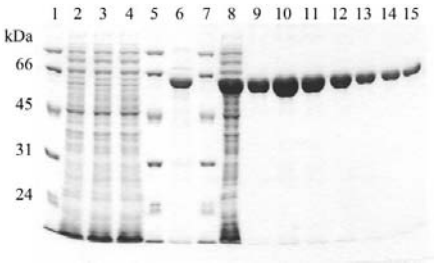

Figure 2
SDS–PAGE analysis of purified fractions from the Talon column. A 12% gel was used and marker proteins (lanes 1, 5 and 7) were from a low-molecular-weight calibration kit (Amersham Pharmacia Biotech). Lanes 2, 3 and 4, proteins not bound by TALON; lane 6, pre-eluted fraction with 10 m*M* imidazole; lane 8, crude extract; lanes 9–15, purified protein fractions eluted with 50 m*M* imidazole.

iPGAM (Lm iPGMA; Chevalier *et al.*, 2000; Guerra *et al.*, 2003).

In this report, the expression, purification, crystallization and preliminary crystallographic analysis of iPGAM from *L. mexicana* are described.

2. Methods and results

2.1. Overexpression

The recombinant plasmid pET28a-Lm iPGAM carried by *Escherichia coli* strain BL21 (pDLmPGAM)pZLm37 encodes iPGAM with a six-His tag at the C-terminus. The expressed iPGAM contains 561 amino-acid residues (Fig. 1), with a calculated M_r of 61 623 (excluding the initiator methionine and including the His tag LEHHHHHH) and a theoretical pI of 5.26 (http://www.expasy.ch). Cells harbouring the recombinant plasmid were grown at 310 K in Luria–Bertani (LB) medium containing 30 µg ml^{-1} kanamycin and expression was induced by the addition of isopropyl thio-β-D-galactoside (IPTG) when the cell culture reached an OD_{600} of approximately 0.6. Cell growth was continued at 290 K for 20 h. Cell pellets were collected by centrifugation at 4200*g* for 30 min and then stored at 253 K.

2.2. Purification of bacterially expressed Lm iPGAM

Frozen pellets from 1 l cell cultures were defrosted and completely resuspended in 20 ml lysis buffer consisting of 100 m*M* triethanolamine–HCl (TEA) buffer pH 8.0 containing 10% glycerol, 500 m*M* NaCl, 10 µ*M* $CoCl_2$ and two tablets of 'Complete, Mini, EDTA-free' (Roche) protease-inhibitor mixture. Cobalt was included at 10 µ*M* throughout the purification and crystallization because it is essential for optimal activity and stability (data not shown). 40 mg of lysozyme (Sigma) was added to the cell culture, which was incubated at room temperature for 10 min, sonicated and centrifuged at 11 000*g* for 30 min at 277 K. 200 mg protamine sulfate (Sigma) was added to the soluble fraction, which was then mixed gently on a circular rotor at 277 K for 30 min and centrifuged as before. The supernatant was collected and filtered through a 0.45 µm membrane.

A metal-affinity Talon (Clontech) column was used with 50 m*M* imidazole in 100 m*M* TEA buffer pH 8.0 containing 10% glycerol, 500 m*M* NaCl and 10 µ*M* $CoCl_2$ as eluting solution. The column was washed extensively with the same buffer lacking imidazole prior to elution. Lm iPGAM was prepared for crystallization trials by removal of glycerol and imidazole salts using a PD-10 pre-packed size-exclusion column (Amersham Pharmacia Biotech) which was eluted with 20 m*M* TEA buffer pH 7.4 containing 50 m*M* NaCl and 10 µ*M* $CoCl_2$. The sample was then concentrated with a 20 ml Vivaspin centrifugal concentrator tube (Vivasciences) with a molecular-weight cutoff of 30 000. The purified protein was shown to be homogeneous by SDS–PAGE (Fig. 2), with a recovery of 20 mg per litre of culture. The M_r of the purified Lm iPGAM was found to be 61 698 by MALDI–TOF mass spectrometry, compared with the calculated M_r from the sequence (61 623) plus the mass of one cobalt to give a total of mass of 61 682. No attempt was made to remove the His tag prior to crystallization.

2.3. Crystallization, data collection and preliminary structure analysis

Well formed orthorhombic crystals of Lm iPGAM complexed with 3PGA and cobalt were grown in 24-well Limbro plates by vapour diffusion using the hanging-drop method (Ducruix & Giegé, 1992) at 290 K. Crystallization drops contained equal volumes (1.5 µl) of reservoir and enzyme solutions (5 mg ml^{-1} Lm iPGAM, 1.5 m*M* 3PGA, 50 m*M* NaCl and 10 µ*M* $CoCl_2$ in 20 m*M* TEA buffer pH 7.4). Crystals were initially obtained from a preliminary screen using the Structure Screen I kit from Molecular Dimensions: 0.20 *M* ammonium acetate, 0.10 *M* trisodium citrate dihydrate pH 5.6 with 30%(*w*/*v*) PEG 4000 as the precipitating agent. In the optimized crystallization condition, the well solution consisted of 0.08 *M* ammonium acetate, 0.04 *M* trisodium citrate dihydrate pH 6.0 and 24%(*w*/*v*) PEG 4000. Streak-seeding with a cat's whisker was necessary to obtain well formed crystals suitable for X-ray diffraction (Fig. 3) (Ducruix & Giegé, 1992). The crystals were cryoprotected using crystallizing solution and flash-frozen. X-ray data were collected at 100 K in a nitrogen flow using a Cryostream Cooler (Oxford Cryosystems, Oxford, England), using a Cu $K\alpha$ rotating-anode source mounted on a Nonius FR591 generator operating at 40 kV and 110 mA connected to a MAR 345 area detector. 1.0° oscillation images were exposed for about 30 min each. Under these conditions, the crystal diffracted X-rays to beyond 2.3 Å. Analysis of diffraction data using the autoindexing procedure of *DENZO* (Otwinowski & Minor, 1997) indicated that the crystal belongs to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters $a = 62.75$, $b = 72.23$, $c = 129.74$ Å. A total of 28 496 unique reflections were processed with 99.3% completeness (Table 1). The relative molecular volume, V_M, is 2.4 Å^3 Da^{-1} and is consistent with one

Table 1
Data-collection details and statistics.

Values in parentheses are for the highest resolution shell.

	Lm iPGAM–3PGA–Co	Lm iPGAM–3PGA–Co	Lm iPGAM–2PGA–Co
X-ray source	Rotating anode	SRS station 14.2	SRS station 14.2
Wavelength (Å)	1.5418	0.979	0.979
Resolution (Å)	2.25	1.90	2.05
Unit-cell parameters			
a (Å)	62.75	62.46	63.58
b (Å)	72.23	72.27	74.41
c (Å)	129.74	129.68	131.59
$\alpha = \beta = \gamma$ (°)	90.00	90.00	90.00
No. of total reflections	257597	189715	120394
No. of unique reflections	28496	46330	38662
Multiplicity	9.2	4.1	3.1
$R_{\rm merge}$† (%)	10.2 (45.8)	8.9 (25.9)	10.1 (26.6)
Completeness (%)	99.3 (99.5)	98.7 (98.7)	97.0 (97.0)
$I/\sigma(I)$	21.9 (3.9)	5.7 (11.4)	4.4 (10.2)

† $R_{\rm merge} = \sum_h \sum_i |I_{hi} - \langle I_h \rangle| / \sum_h \sum_i I_{hi}$.

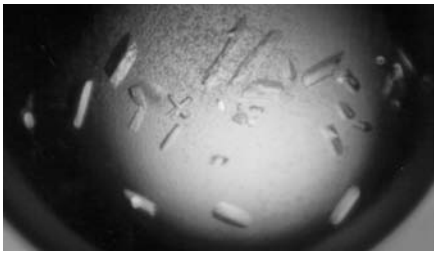

Figure 3
Crystals of Lm iPGAM complexed with 3PGA and cobalt. See text for details.

molecule of protein in the asymmetric unit and a solvent content of 48%.

A model of *B. stearothermophilus* iPGAM (PDB code 1ejj; Berman *et al.*, 2000) was used to provide an initial solution using *MOLREP* (Vagin & Teplyakov, 1997). However, the solution was of relatively poor quality because the sequence identity of the model and Lm iPGAM is low (33% identity, Fig. 1) and the connectivity of the electron-density map was also not always apparent. An improved search model was generated by threading the Lm iPGAM sequence onto the published *B. stearothermophilus* iPGAM structure using 3*DPSSM* (Kelley *et al.*, 2000). After removal of poorly defined loops in the structure, the model gave a molecular-replacement solution with an R value of 56.7% (Collaborative Computational Project, Number 4, 1994).

X-ray data were also collected at the Daresbury SRS (station 14.2, λ = 0.979 Å) with an ADSC Q4 CCD detector using a φ scan with a step size of 0.5° and 60 s exposures. The crystal-to-detector distance was 140 mm and the crystal diffracted to beyond 1.90 Å. The data were processed with *MOSFLM* (Leslie, 1992).

High-quality crystals of Lm iPGAM–2PGA–Co were also obtained in 0.04 *M* ammonium acetate, 0.02 *M* trisodium citrate dihydrate pH 6.2 with 30%(*w*/*v*) PEG 4000 as the precipitating agent. Data were collected at the Daresbury SRS (station 14.2, λ = 0.979 Å) to 2.05 Å resolution. Details of synchrotron data collection and statistics are shown in Table 1.

3. Discussion

Leishmaniasis, one of the diseases caused by trypanosomatid protozoan parasites, affects about 12 million people worldwide and efficacious drugs and vaccines are not currently available. The parasites are transmitted by the bite of an insect vector, the phlebotomine sandfly. In humans, the disease occurs in at least four major forms; cutaneous, diffuse cutaneous, mucocutaneous and visceral (World Health Organization, 2002). Current treatments include meglumine antimonate, sodium stibogluconate and amphotericine B, which can cause severe toxic side effects and can even be fatal. Our work is part of an effort to provide three-dimensional crystal structures of validated target proteins.

In trypanosomes, *Leishmania* species and other kinetoplastid organisms, part of the glycolytic pathway is compartmentalized in specialized peroxisomes, called glycosomes, which are not present in other eukaryotic organisms. Bloodstream forms of trypanosomes exclusively obtain energy through glycolysis and the inhibition of any of the glycolytic enzymes thus provides a potential therapeutic approach. Phosphoglycerate mutase is a particularly attractive target for the design of selective inhibitors because the parasite and human enzymes are not homologous (Verlinde *et al.*, 2001).

In this study, *L. mexicana* iPGAM expression has been optimized and the protein purified, characterized and crystallized. The crystal structure of an Lm iPGAM–3PGA complex is being analysed. Crystal structures will be used as templates for database mining to identify ligands/inhibitors. Combinatorial chemistry and *in vitro* assays will be used to produce tight-binding drug-like molecules. Any drugs thus produced are also likely to be effective against diseases caused by trypanosomes because of the high structural similarity of their iPGAMs.

This project is part of a collaborative project financially supported by the European Commission through its INCO-DEV programme (contract ICA4-CT-2001-10075). Buabarn Poonperm is supported by a Royal Thai Government Scholarship, Ministry of Science, Technology and Environment, Thailand. Daniel Guerra acknowledges a scholarship from the Commission pour la Coopération Universitaire au Développement (CUD), Commission Permanente du Conseil Interuniversitaire de la Communauté Française (CIUF) and a travel grant from the COST B9 Action of the European Commission for a two-month work visit to the University of Edinburgh. We are grateful to Professor P. A. M. Michels (Université Catholique de Louvain, Brussels) for providing facilities and for a long-term valuable collaboration. We thank Dr J. Martinez-Oyanedel of the Structural Biochemistry Group at Edinburgh University for helpful discussions and assistance with data collection, processing and structure refinement, the Edinburgh Protein Interaction Centre (EPIC) for support, H. Florance for mass analysis and the staff at the Synchrotron Radiation Source (SRS) at Daresbury.

References

Barton, G. J. (1993). *Protein Eng.* **6**, 37–40.
Berman, H. M., Westbrook, J., Feng, Z., Gilliland, G., Bhat, T. N., Weissig, H., Shindyalov, I. N. & Bourne, P. E. (2000). *Nucleic Acids Res.* **28**, 235–242.
Chander, M., Setlow, B. & Setlow, P. (1998). *Can. J. Microbiol.* **44**, 759–767.
Chander, M., Setlow, P., Lamani, E. & Jedrzejas, M. J. (1999). *J. Struct. Biol.* **126**, 156–165.
Chevalier, N., Rigden, D. J., Van Roy, J. V., Opperdoes, F. R. & Michels, P. A. M. (2000). *Eur. J. Biochem.* **267**, 1464–1472.
Collaborative Computational Project, Number 4 (1994). *Acta Cryst.* D**50**, 760–763.
Collet, J., Stroobant, V. & Schaftingen, E. V. (2001). *FEMS Microbiol. Lett.* **204**, 39–44.
Crowhurst, G. S., Dalby, A. R., Isupov, M. N., Campbell, J. W. & Littlechild, J. A. (1999). *Acta Cryst.* D**55**, 1822–1826.

Ducruix, A. & Giegé, R. (1992). *Crystallization of Nucleic Acids and Proteins: A Practical Approach.* Oxford University Press.
Fothergill-Gilmore, L. A. & Michels, P. A. M. (1993). *Prog. Biophys. Mol. Biol.* **59**, 105–235.
Fothergill-Gilmore, L. A. & Watson, H. C. (1989). *Adv. Enzymol. Relat. Areas. Mol. Biol.* **62**, 227–313.
Fraser, H. I., Kvaratskhelia, M. & White, F. M. (1999). *FEBS Lett.* **455**, 344–348.
Graña, X., Ossa, P. P., Broceño, C., Stöcker, M., Garriga, J., Puidomènech, P. & Climent, F. (1995). *Comput. Biochem. Physiol. B*, **112**, 287–293.
Guerra, D. G., Fothergill-Gilmore, L. A., Opperdoes, F. R. & Michels, P. A. M. (2003). Submitted.
Jedrzejas, M. J., Chander, M., Setlow, P. & Krishnasamy, G. (2000*a*). *EMBO J.* **19**, 14–19.
Jedrzejas, M. J., Chander, M., Setlow, P. & Krishnasamy, G. (2000*b*). *J. Biol. Chem.* **275**, 23146–23153.
Kelley, L. A., MacCallum, R. M. & Sternberg, M. J. E. (2000). *J. Mol. Biol.* **299**, 499–520.
Leslie, A. G. W. (1992). *Jnt CCP4/ESF–EAMCB Newsl. Protein Crystallogr.* **26**.
Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.
Rigden, D. J., Alexeev, D., Phillips, S. E. V. & Fothergill-Gilmore, L. A. (1998). *J. Mol. Biol.* **276**, 449–459.
Rigden, D. J., Lamani, E., Mello, L. V., Littlejohn, J. E. & Jedrzejas, M. J. (2003). *J. Mol. Biol.* **328**, 909–920.
Rigden, D. J., Mello, L. V., Setlow, P. & Jedrzejas, M. J. (2002). *J. Mol. Biol.* **135**, 1129–1143.
Rigden, D. J., Phillips, S. E. V. & Fothergill-Gilmore, L. A. (1999). *J. Mol. Biol.* **286**, 1507–1517.
Vagin, A. & Teplyakov, A. (1997). *J. Appl. Cryst.* **30**, 1022–1025.
Verlinde, C. L. M. J., Hannaert, V., Blonski, C., Wilson, M., Périé, J. J., Fothergill-Gilmore, L. A., Opperdoes, F. R., Gelb, M. H., Hol, W. G. J. & Michels, P. A. M. (2001). *Drug Resist. Updates*, **4**, 50–65.
World Health Organization (2002). *Leishmaniasis*, http://www.who.int/health_topics/leishmaniasis/.