Cloning, expression, purification, crystallization and preliminary X-ray diffraction analysis of glyceraldehyde-3-phosphate dehydrogenase from *Streptococcus agalactiae* NEM316

Revathi Nagarajan and Karthe Ponnuraj*

Centre of Advanced Study in Crystallography and Biophysics, University of Madras, Guindy Campus, Chennai 600 025, India

Correspondence e-mail: pkarthe@hotmail.com

Received 7 April 2014

Accepted 17 May 2014

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is an essential enzyme involved in glycolysis. Despite lacking the secretory signal sequence, this cytosolic enzyme has been found localized at the surface of several bacteria and fungi. As a surface protein, GAPDH exhibits various adhesive functions, thereby facilitating colonization and invasion of host tissues. *Streptococcus agalactiae*, also known as group B streptococcus (GBS), binds onto the host using its surface adhesins and causes sepsis and pneumonia in neonates. GAPDH is one of the surface adhesins of GBS binding to human plasminogen and is a virulent factor associated with host colonization. Although the surface-associated GAPDH has been shown to bind to a variety of host extracellular matrix (ECM) molecules in various bacteria, the molecular mechanism underlying their interaction is not fully understood. To investigate this, structural studies on GAPDH of *S. agalactiae* were initiated. The *gapC* gene of *S. agalactiae* NEM316 encoding GAPDH protein was cloned into pET-28a vector, overexpressed in *Escherichia coli* BL21(DE3) cells and purified to homogeneity. The purified protein was crystallized using the hanging-drop vapour-diffusion method. The GAPDH crystals obtained in two different crystallization conditions diffracted to 2.8 and 2.6 Å resolution, belonging to two different space groups $P2_1$ and $P2_12_12_1$, respectively. The structure was solved by molecular replacement and structure refinement is now in progress.

1. Introduction

The Lancefield group B streptococcus (GBS) is a human commensal Gram-positive coccus that causes pneumonia, sepsis and meningitis in neonates (Arisoy *et al.*, 2003; Puopolo *et al.*, 2005). The transmission of the bacterium to neonates takes place by colonization of the gastrointestinal and urogenital tracts of mothers, and up to 40% of healthy women are colonized with GBS. 1–2% of colonized neonates develop acute infections (Rosenau *et al.*, 2007). *Streptococcus agalactiae* infections are usually treated with penicillin. Nevertheless, with the presence of many penicillin-allergic individuals, macrolides and clindamycin remain important drug alternatives in these cases, and an increase in resistance to these has been described in several countries (Sherman *et al.*, 2012; Lambiase *et al.*, 2012; Kimura *et al.*, 2013; Wang *et al.*, 2013; Fröhlicher *et al.*, 2014).

Adherence followed by colonization of human tissues is the primary step in any bacterial infection. In general, bacterial adhesion is mediated by several cell-surface structures, mostly proteins and lipoproteins termed adhesins that bind to components of the extracellular matrix (ECM). The ECM includes glycoproteins such as collagen, fibrinogen, fibronectin and laminin that form the underlying basement membrane of the epithelial and endothelial cells (Hay, 1991). Several interactions between *S. agalactiae* surface proteins and ECM components have been reported and it has been suggested that these interactions are critical for bacterial tissue adhesion and invasion (Maisey *et al.*, 2008; Tazi *et al.*, 2012).

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH; EC 1.2.1.12) is a glycolytic enzyme that catalyses the conversion of glyceraldehyde 3-phosphate (G3P) to 1,3-biphosphoglycerate (1,3-BPG). Besides its glycolytic activity in the cytosol, electron-microscopic and fluorescence-activated cell-sorter (FACS) studies of

GAPDHs of various organisms revealed its localization on the cell surface. The surface-localized GAPDH acts as a binding partner with proteins of the human extracellular matrix, playing a key role in bacterial infection (Pancholi & Chhatwal, 2003; Lama *et al.*, 2009; Dumke *et al.*, 2011). The surface-bound enzymatically active GAPDH of GBS has been described as a secretory protein despite lacking a signal peptide, and the transport mechanism of the cytosolic protein to the cellular surface still remains unidentified (Seifert *et al.*, 2003).

The GBS GAPDH is highly homologous to the plasmin receptor protein of group A streptococcus (GAS; Itzek *et al.*, 2010). It interacts with the human plasminogen system to increase the proteolytic activity on the bacterial surface. The enzyme has been partially characterized and found to bind to actin and fibrinogen (Seifert *et al.*, 2003). It has been designated as a virulence-associated immunomodulatory protein (VIP) as higher concentrations of IL-10, an immunosuppressor, were seen in the serum of mice treated with recombinant GBS GAPDH (Madureira *et al.*, 2007). This implies successful host colonization of *S. agalactiae*, thereby highlighting the direct role of GAPDH in this process. Recently, it has been shown that maternal vaccination with recombinant GAPDH (rGAPDH) protects mice neonates from GBS infection (Madureira *et al.*, 2011). These data and the presence of GAPDH in all GBS strains strongly suggest that GAPDH is an ideal candidate for global vaccine development.

GBS GAPDH consists of 336 residues comprising two domains: a cofactor (NAD⁺) binding domain (residues 1–150) and a catalytic domain (residues 151–336). Although GAPDHs of many organisms have been extensively studied by X-ray diffraction methods, the binding mechanism of GAPDH to various ECM molecules still remains unidentified. In this direction, we initiated structural studies on the GAPDH protein from *S. agalactiae*. Here, we report its cloning, expression, purification, crystallization and preliminary X-ray analysis. Understanding the structure of GAPDH and the underlying molecular mechanism of binding to host plasminogen and fibrinogen will be useful in the development of drugs for treating and preventing streptococcal infections.

2. Materials and methods

2.1. Cloning and expression of rGAPDH

The *gapC* gene (gbs1811; <http://genotype.pasteur.fr/SagaList>) was PCR-amplified from *S. agalactiae* NEM316 chromosomal DNA using the forward and reverse primers GAP-*NcoI* (CGGccatggTAGT-TAAAGTTGGTATTAACGGTTTC) and GAP-*XhoI* (CGG-ctcgagTGCAATTTTTCGAAAGTATTCAAG), respectively (the restriction sites used for cloning are shown in lower case). The 1008 base pair *gapC* gene was digested with *NcoI* and *XhoI* and cloned into pET-28a vector which was linearized with the same enzymes to produce rGAPDH with a carboxylic histidyl tag. *Escherichia coli* DH5 α cells were transformed with the plasmid pET-28a(+)-*gapC* and grown on Luria broth agar plates containing 50 μ g ml⁻¹ kanamycin. The positive clones were verified by DNA sequencing. The resulting plasmid was transformed into the expression host *E. coli* BL21(DE3) and protein expression was carried out in Luria–Bertani (LB) medium. 10 ml overnight culture was prepared and transferred to 500 ml LB medium supplemented with 50 μ g ml⁻¹ kanamycin and allowed to grow at 310 K with shaking (180 rev min⁻¹) until an optimum optical density (OD₆₀₀ of 0.5–0.6) was reached. At this point, expression of the recombinant protein was induced with 1 mM isopropyl β -D-1-thiogalactopyranoside (IPTG) at 310 K. After 5 h of incubation with shaking, bacterial cells were harvested by centrifuga-

tion using a REMI CPR-30 cooling centrifuge (rotor R-236) at 4000 rev min⁻¹ for 20 min at 277 K.

2.2. Purification

The cell pellet was resuspended in 25 ml cold lysis buffer (20 mM Tris–HCl pH 8.0, 300 mM NaCl, 5% glycerol) with 1 mM phenylmethanesulfonylfluoride (PMSF) and ultrasonicated for 2 min at 25% amplitude (20 s on, 10 s off) on ice. This lysate solution was spun down at 10 000 rev min⁻¹ for 45 min at 277 K using a REMI C-24BL cooling centrifuge (rotor R-241). The supernatant and pellet fractions were checked on a 12.5% SDS–PAGE gel and most of the over-expressed protein was found in the soluble fraction. The supernatant was passed through a 0.22 μ m membrane filter to remove any cell debris from the preparation for protein purification. The first step involved in the protein purification was immobilized metal-affinity chromatography (IMAC) using an Ni–NTA column (5 ml HisTrap column, GE Healthcare) which was equilibrated in the lysis buffer to capture the target 6 $\times$ His-tagged fusion protein from the supernatant. The bound protein was eluted stepwise with buffer A (20 mM Tris–HCl pH 8.0, 300 mM NaCl and 5% glycerol) containing increasing concentrations of imidazole (100, 200, 300, 500, 700 mM and 1 M). The elution fractions were checked on 12.5% SDS–PAGE gel and the rGAPDH protein was found in the 200 and 300 mM imidazole fractions. These fractions were pooled, concentrated to approximately 1.5 ml and subjected to size-exclusion chromatography using Superdex 75 prep-grade matrix in a 10/300 C column (GE Healthcare Biosciences) equilibrated with buffer A on an ÄKTA system (GE Healthcare Biosciences). 1.5 ml fractions were collected at a flow rate of 0.5 ml min⁻¹. The peak fractions containing the desired protein were checked on 12.5% SDS–PAGE, pooled and concentrated to 300 μ l by centrifugal ultrafiltration (Pierce; 10 kDa cutoff). The concentration of the protein was measured using a UV spectrophotometer (A_{280}) using a calculated absorption coefficient of 0.640 (ProtParam; Gasteiger *et al.*, 2005).

2.3. Crystallization

Crystallization experiments were carried out using the hanging-drop vapour-diffusion method with Hampton Research screens (Crystal Screen, Crystal Screen 2, PEG/Ion, PEG/Ion 2 and SaltRx) and home-made screens such as ammonium sulfate *versus* pH and polyethylene glycol (PEG) *versus* pH. Initial crystallization trials were carried out by mixing 1 μ l protein solution and 1 μ l reservoir solution and equilibrating the drop against 1 ml reservoir solution at 287 K. Although crystals with different morphologies were obtained in the various Hampton Research screens, none were of diffraction quality. The home-made screens such as PEG 3350 and 4000 *versus* pH also gave small crystals and the initial diffraction of these crystals was weak, with typical diffraction limits of $\sim$ 4–6 Å resolution. Conditions consisting of 40% (w/v) PEG 3350 and PEG 4000, 0.1 M Tris–HCl pH 8, 8.5 and 9 were optimized by a repeated microseeding technique which yielded good diffraction quality crystals. For microseeding, seed stocks were prepared in volume ratios of 1:200 and 1:300 from a crystallization drop containing tiny crystals and reservoir solution, respectively. A human hair dipped into the seed stock solution was streaked onto a drop comprised of 1 μ l protein solution and 1 μ l reservoir solution, and long, sharp needle-like crystals and thick rod-shaped crystals were obtained within one week.

Table 1
Data-collection statistics.

Values in parentheses are for the highest resolution shell.

Wavelength (Å)	1.5418	0.97
Space group	$P2_1$	$P2_12_12_1$
Unit-cell parameters (Å, °)	$a = 67.79, b = 102.13, c = 88.8, \beta = 96.98$	$a = 88.58, b = 124.42, c = 125.31$
Resolution range (Å)	49.71–2.8 (2.95–2.80)	36.17–2.60 (2.74–2.60)
$R_{\text{merge}}^{\dagger}$ (%)	9.2 (42.2)	15.7 (43.0)
$R_{\text{meas}}^{\ddagger}$ (%)	12.8 (54.8)	17 (47.5)
$R_{\text{p.i.m.}}^{\S}$ (%)	6.6 (28.2)	4.9 (15.2)
Total No. of reflections	100663	499846
No. of unique reflections	27540	43293
Mean $I/\sigma(I)$	11.4 (2.9)	13 (5.7)
Completeness (%)	93.1 (86.2)	99.9 (99.9)
Multiplicity	3.7 (3.4)	11.5 (9.5)
Detector type	MAR345 image plate	CCD
Detector distance (mm)	200	200
Temperature (K)	100	100
Exposure time per frame (s)	160	15
Oscillation range (°)	1	1
Total rotation range (°)	100–280	1–360

$\dagger R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$. $\ddagger R_{\text{meas}}$ is defined as $\sum_{hkl} \{N(hkl) / [N(hkl) - 1]^{1/2} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)\}$. $\S R_{\text{p.i.m.}}$ is defined as $\sum_{hkl} \{1 / [N(hkl) - 1]^{1/2} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)\}$.

2.4. Data collection and processing

For X-ray data collection, rGAPDH crystals grown in 40%(w/v) PEG 3350, 0.1 M Tris–HCl pH 9.0 were flash-cooled in a nitrogen-gas stream at 100 K. Diffraction data were collected at our in-house data-collection facility using a MAR345 image-plate detector and a Bruker MICROSTAR copper rotating-anode generator operating at 60 mA and 45 kV. A total of 180 frames were collected with an oscillation step of 1° and an exposure of 160 s per frame. The crystal-to-detector distance was maintained at 200 mm. The diffraction images were indexed, integrated, merged and scaled using *iMosflm* (Battye *et al.*, 2011) and *SCALA* (Winn *et al.*, 2011).

The crystals grown in PEG 4000 [40%(w/v) PEG 4000, 0.1 M Tris–HCl pH 9.0] diffracted better than the crystals grown in PEG 3350 and a complete data set was collected on beamline XRD1 at Elettra, Trieste, Italy using a CCD detector. All frames were collected at

100 K using a 1° oscillation angle and an exposure of 15 s per frame. The crystal-to-detector distance was set to 200 mm. The diffraction data were processed using *iMosflm* (Battye *et al.*, 2011) and *SCALA* (Winn *et al.*, 2011).

In both cases, prior to data collection crystals were soaked for 30 s in a reservoir solution supplemented with 20% glycerol and flash-cooled in a nitrogen-gas stream. The data-collection and processing statistics are given in Table 1.

3. Results and discussion

The *gapC* gene of *S. agalactiae* encoding GAPDH protein was cloned into pET-28a vector, expressed in *E. coli* and purified using affinity and size-exclusion chromatography. The purified protein was concentrated to 12 mg ml^{−1} prior to crystallization trials. The 37 kDa protein migrates apparently around 45 kDa on SDS–PAGE (Fig. 1). Crystals of different morphology such as plate-like, needle-like and hexagon-shaped were observed in various conditions with Hampton Research Crystal Screen and Crystal Screen 2 and with PEG 3350 and PEG 4000 as precipitants. However, single crystals that were suitable for X-ray diffraction measurement grew in drops consisting of 40%(w/v) PEG 3350, 0.1 M Tris–HCl pH 9.0 and also in drops consisting of 40%(w/v) PEG 4000, 0.1 M Tris–HCl pH 9.0 in one week with several rounds of microseeding (Fig. 2). The crystals obtained in the PEG 3350 condition belonged to two different crystal systems, namely monoclinic and orthorhombic. The crystals corresponding to the former system were thinner and longer (0.7–1 mm) whereas the latter were broader and shorter, and the monoclinic form of the crystal diffracted better than the orthorhombic form even though the crystals grew in identical conditions in different drops. For a monoclinic crystal a complete data set was collected to a resolution of 2.8 Å. rGAPDH crystals were also obtained with PEG 4000 as a precipitant and these crystals belonged to the orthorhombic form. Although the orthorhombic crystals obtained using both PEG 3350 and PEG 4000 were similar in morphology, the latter diffracted to a resolution of 2.6 Å.

Analysis of the symmetry and the systematic absences in the recorded diffraction patterns indicated that the crystals belonged to the monoclinic $P2_1$ and orthorhombic $P2_12_12_1$ space groups. Deter-

Figure 1
12.5% SDS–PAGE of purified GAPDH. Lane 1, molecular-weight marker (labelled in kDa); lane 2, final purified rGAPDH at a concentration of 12 mg ml^{−1}.

Figure 2
Crystals of rGAPDH. (a) Needle-like crystals of approximately 0.03 × 0.05 × 1.0 mm in size obtained in 40%(w/v) PEG 3350, 0.1 M Tris–HCl pH 9.0. (b) Crystals obtained in 40%(w/v) PEG 4000, 0.1 M Tris–HCl pH 9.0. The rod-like crystals shown are about 0.1 × 0.1 × 0.8 mm in size.

mination of the Matthews coefficient suggested the presence of 47.28 and 40.4% solvent content in the unit cell ($V_M = 2.33$ and $2.06 \text{ \AA}^3 \text{ Da}^{-1}$) with four molecules in the asymmetric unit for the two crystal systems, respectively (Matthews, 1968).

The crystal structure of homologous enzyme from various Gram-positive species such as *Staphylococcus aureus* (PDB entry 3k73; Mukherjee *et al.*, 2010) and *Bacillus stearothermophilus* (PDB entry 1gd1; Skarzynski *et al.*, 1987), Gram-negative species such as *Thermatoga maritima* (PDB entry 1hdg; Korndorfer *et al.*, 1995), *Borrelia burgdorferi* (PDB entry 3hja; Seattle Structural Genomics Center for Infectious Disease, unpublished work) and *E. coli* (PDB entry 1s7c; Berkeley Structural Genomics Center, unpublished work) and fungal species as *Saccharomyces cerevisiae* (PDB entry 4iq8; Liu *et al.*, 2012) and *Kluyveromyces marxianus* (PDB entry 2i5p; Ferreira-da-Silva *et al.*, 2006) are available. They exhibit sequence identity ranging from 50 to 69% with the *S. agalactiae* enzyme.

The structure of rGAPDH of *S. agalactiae* was solved by molecular-replacement with *Phaser* (McCoy *et al.*, 2007) using the crystal structure of GAPDH from *S. aureus* (Mukherjee *et al.*, 2010) as the search model. Final model building and refinement are in progress.

KP thanks the Department of Biotechnology (DBT), Government of India for financial support. KP also thanks the Abdus Salam International Centre for Theoretical Physics (ICTP), Trieste, Italy for financial support to visit the Elettra synchrotron facility for the data collection. RN thanks the University Grants Commission (UGC), Government of India for the fellowship.

References

- Arisoy, A. S., Altinşik, B., Tünger, O., Kurutepe, S. & Ispahi, C. (2003). *Infection*, **31**, 244–246.
- Battye, T. G. G., Kontogiannis, L., Johnson, O., Powell, H. R. & Leslie, A. G. W. (2011). *Acta Cryst.* **D67**, 271–281.
- Dumke, R., Hausner, M. & Jacobs, E. (2011). *Microbiology*, **157**, 2328–2338.
- Ferreira-da-Silva, F., Pereira, P. J., Gales, L., Roessle, M., Svergun, D. I., Moradas-Ferreira, P. & Damas, A. M. (2006). *J. Biol. Chem.* **281**, 33433–33440.
- Fröhlicher, S., Reichen-Fahrni, G., Müller, M., Surbek, D., Droz, S., Spellerberg, B. & Sendi, P. (2014). *Swiss Med. Wkly*, **144**, w13935.
- Gasteiger, E., Hoogland, C., Gattiker, A., Duvaud, S., Wilkins, M. R., Appel, R. D. & Bairoch, A. (2005). *The Proteomics Protocols Handbook*, edited by J. M. Walker, pp. 571–607. Totowa: Humana Press.
- Hay, E. D. (1991). *Cell Biology of Extracellular Matrix*. New York: Plenum Press.
- Itzek, A., Gillen, C. M., Fulde, M., Friedrichs, C., Rodloff, A. C., Chhatwal, G. S. & Nitsche-Schmitz, D. P. (2010). *PLoS One*, **5**, e13826.
- Kimura, K., Nagano, N., Nagano, Y., Suzuki, S., Wachino, J., Shibayama, K. & Arakawa, Y. (2013). *J. Antimicrob. Chemother.* **68**, 539–542.
- Korndorfer, I., Steipe, B., Huber, R., Tomsch, A. & Jaenicke, R. (1995). *J. Mol. Biol.* **246**, 511–521.
- Lama, A., Kucknoor, A., Mundodi, V. & Alderete, J. F. (2009). *Infect. Immun.* **77**, 2703–2711.
- Lambiasi, A., Agangi, A., Del Pezzo, M., Quaglia, F., Testa, A., Rossano, F., Martinelli, P. & Catania, M. R. (2012). *Infect. Dis. Obstet. Gynecol.* **2012**, 913603.
- Liu, Q., Wang, H., Liu, H., Teng, M. & Li, X. (2012). *Acta Cryst.* **F68**, 978–980.
- Madureira, P., Andrade, E. B., Gama, B., Oliveira, L., Moreira, S., Ribeiro, A., Correia-Neves, M., Trieu-Cuot, P., Vilanova, M. & Ferreira, P. (2011). *PLoS Pathog.* **7**, e1002363.
- Madureira, P., Baptista, M., Vieira, M., Magalhães, V., Camelo, A., Oliveira, L., Ribeiro, A., Tavares, D., Trieu-Cuot, P., Vilanova, M. & Ferreira, P. (2007). *J. Immunol.* **178**, 1379–1387.
- Maisey, H. C., Doran, K. S. & Nizet, V. (2008). *Expert Rev. Mol. Med.* **10**, e27.
- Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
- McCoy, A. J., Grosse-Kunstleve, R. W., Adams, P. D., Winn, M. D., Storoni, L. C. & Read, R. J. (2007). *J. Appl. Cryst.* **40**, 658–674.
- Mukherjee, S., Dutta, D., Saha, B. & Das, A. K. (2010). *J. Mol. Biol.* **401**, 949–968.
- Pancholi, V. & Chhatwal, G. S. (2003). *Int. J. Med. Microbiol.* **293**, 391–401.
- Puopolo, K. M., Madoff, L. C. & Eichenwald, E. C. (2005). *Pediatrics*, **115**, 1240–1246.
- Rosenau, A., Martins, K., Amor, S., Gannier, F., Lanotte, P., van der Meer, N., Mereghetti, L. & Quentin, R. (2007). *Infect. Immun.* **75**, 1310–1317.
- Seifert, K. N., McArthur, W. P., Bleiweis, A. S. & Brady, L. J. (2003). *Can. J. Microbiol.* **49**, 350–356.
- Sherman, K., Whitehead, S., Blondel-Hill, E., Wagner, K. & Cheeptham, N. (2012). *Can. J. Infect. Dis. Med. Microbiol.* **23**, 196–198.
- Skarzynski, T., Moody, P. C. & Wonacott, A. J. (1987). *J. Mol. Biol.* **193**, 171–187.
- Tazi, A., Bellais, S., Tardieux, I., Dramsi, S., Trieu-Cuot, P. & Poyart, C. (2012). *Curr. Opin. Microbiol.* **15**, 44–49.
- Wang, H., Zhao, C., He, W., Zhang, F., Zhang, L., Cao, B., Sun, Z., Xu, Y., Yang, Q., Mei, Y., Hu, B., Chu, Y., Liao, K., Yu, Y., Hu, Z. & Ni, Y. (2013). *Antimicrob. Agents Chemother.* **57**, 1538–1541.
- Winn, M. D. *et al.* (2011). *Acta Cryst.* **D67**, 235–242.