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Purification, characterization and preliminary X-ray crystallographic studies on *Neiserria gonorrhoeae* Gly1ORF1

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Gly1ORF1 is a protein produced by the two pathogenic *Neisseria* species, *N. gonorrhoeae* and *N. meningitidis*, but not by commensal *Neisseria*, suggesting that it may be involved in pathogenesis. The protein has a signal sequence that is cleaved, is associated with outer membrane fractions of *N. gonorrhoeae* (GC) and is found in spent media and in outer-membrane fractions when expressed in *Escherichia coli*. GC strains with null mutations of the *gly1* locus have increased toxicity to human fallopian tubes in organ culture, suggesting that Gly1ORF1 may alter the amount or properties of toxic moieties produced by GC [Arvidson *et al.* (1999), *Infect. Immun.* **67**, 643–652]. In an effort to understand the function of Gly1ORF1 and its role in pathogenesis, structural biology studies have been initiated. Here, the purification, characterization by dynamic light scattering, crystallization and preliminary X-ray crystallographic studies of recombinant Gly1ORF1 are reported. Dynamic light scattering indicated the protein to be a dimer in solution. The crystals belonged to space group $P6_3$, with unit-cell parameters a = 95.2, b = 95.2, c = 83.7 Å and two molecules per asymmetric unit. The crystals diffracted to 2.4 Å using a conventional X-ray source.

1. Introduction

The *Neisseria gonorrhoeae gly1* locus was initially identified in a plasmid library screened for genes that conferred a hemolytic phenotype upon expression in *Escherichia coli*. It encodes two open reading frames, Gly1ORF1 and Gly1ORF2 (GenBank accession No. AF003941). ORF2, based on amino-acid sequence similarity, has been annotated as *hemD*, which encodes uroporphyrinogen III cosynthase (http://www.stdgen.lanl.gov). ORF1 has no homologues in the GenBank database (Arvidson *et al.*, 1999). Gly1ORF1 is expressed as a 15.7 kDa protein with a signal sequence that is cleaved. The 13.5 kDa mature product is found in the outer-membrane fractions of GC and in filtered spent media from cultures of *E. coli* expressing Gly1ORF1. *N. gonorrhoeae* strains (MS11 P^+ and P^-, which bear and lack pili, respectively) that have null mutations of their *gly1* locus show no change in their adherence to, invasion of or survival within human cells in culture. However, the null mutants show increased toxicity to human fallopian tubes in organ culture. This suggests that *gly1* may alter the amount or properties of toxic moieties produced by GC (Arvidson *et al.*, 1999).

2. Materials and methods

2.1. Protein expression and purification

A derivative of the Gly1ORF1 protein with a C-terminal His_6 tag (Gly1ORF1-H6) was constructed in the plasmid vector pET24a (Novagen, Madison, WI, USA). The recombinant construct replaces the C-terminal Arg residue of Gly1ORF1 with the amino-acid sequence AAALEHHHHHH (Arvidson *et al.*, 1999). The Gly1ORF1-H6 protein was expressed in *E. coli* strain BL21(DE3) (Studier *et al.*, 1990) and purified as described in Arvidson *et al.* (1999) with the following modifications. Briefly, bacterial cultures were induced with 400 µ*M* IPTG and grown overnight at 310 K with vigorous aeration. The signal sequence of Gly1ORF1-H6 was cleaved and the mature protein was excreted into the *E. coli* culture medium. The spent medium was clarified by three rounds of centrifugation followed by filtration through a 0.45 µm nitrocellulose filter. Proteins were precipitated from the clarified supernatant by slowly adding solid $(NH_4)_2SO_4$ with stirring to 74% saturation, collected by centrifugation, resuspended and dialyzed against buffer I (50 m*M* $NaPO_4$ pH 6.0, 300 m*M* NaCl). Dialyzed protein was fractionated by chromatography on an Ni–

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NTA column (Qiagen, Chatsworth, CA, USA). The column was washed with buffer I, equilibrated with buffer I containing 5 m*M* imidazole and was then eluted with buffer I containing a linear gradient of 5–500 m*M* imidazole. Fractions were analyzed by SDS–PAGE and those containing Gly1ORF1-H6 were pooled. At this point, Gly1ORF1-H6 was estimated to be about 85% pure. To further purify the protein, the sample was dialyzed against buffer I and reapplied to the Ni–NTA column. After washing with buffer I, the column was eluted using a pH gradient in buffer I (pH 6.0–3.5). Fractions were again analyzed by SDS–PAGE, Gly1ORF1-H6-containing fractions were pooled and the resulting protein was concentrated using Centricon 3 filtration units (Millipore Corporation, Bedford, MA, USA). To produce a selenomethionine derivative of Gly1ORF1-H6, pGly1ORF1-H6 was transformed into *E. coli* strain B834(DE3), a methionine auxotroph derived from BL21(DE3). Bacteria were grown in M9 minimal medium containing 0.4% glucose, a mixture of all amino acids except methionine at 0.2 g l^{-1} and 35 mg l^{-1} selenomethionine. When cell density reached $\sim 5\times 10^8$ colony-forming units per millilitre, Gly1ORF1-H6 expression was induced by the addition of 500 µ*M* IPTG. Following growth overnight at 310 K, the protein was purified as described above.

2.2. Dynamic light scattering

Gly1ORF1-H6 in buffer I pH 4.0 was filtered through 0.1 µm Anotop filters (Whatman, Kent, UK) to remove particulates. Dynamic light scattering was performed using a DynaPro Dynamic Light Scattering Instrument (Protein Solutions, Lakewood, NJ, USA) with runs of 25 scans of 15 s each at 295 K. Data were analyzed using *DYNAMICS* v. 3.30.

2.3. Crystallization

Crystals were grown by the hanging-drop vapor-diffusion method. Hanging drops were prepared by mixing 2 µl of protein solution in buffer I pH 4.0 with 2 µl of reservoir solution on silanized glass cover slips. The cover slips were placed over the reservoirs of VDX plates (Hampton Research) and were sealed with vacuum grease. The drops were then equilibrated with 700 µl reservoir solution at 294 K. Crystallization conditions were screened using Crystal Screen Cryo (Hampton Research, Laguna Niguel, CA, USA). Condition 3 (0.26 *M* Na_2HPO_4 pH 5.7, 35% glycerol) yielded numerous hexagonal crystals in an otherwise clear drop. Grid screening of salt and glycerol concentrations was used to find optimum conditions, which were 0.2 *M* Na_2HPO_4 pH 5.7, 25% glycerol. To prepare crystals for cryocrystallography, an additional 300 µl of glycerol was added to each reservoir and the drops were allowed to re-equilibrate for 4 d. The final solution conditions were confirmed to be cryoprotective by the absence of ice rings in diffraction data.

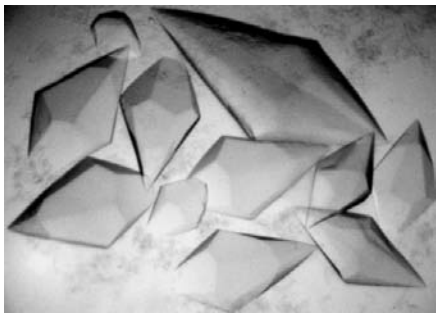

Figure 1
Hexagonal bipyramidal crystals of Gly1ORF1-H6. The largest crystals had dimensions of 1.3 × 0.6 mm.

2.4. X-ray data collection and processing

Crystals were mounted in nylon loops and frozen in a cryogenic (~100 K) nitrogen stream. Data were collected using an R-AXIS IV image-plate system and a Rigaku RU400-H rotating-anode generator with Yale mirrors operating at 50 kV and 100 mA with Cu $K\alpha$ (λ = 1.5418 Å) radiation. Data were processed using the *Processing GUI* (Nielsen *et al.*, 1998) as a front end for *MOSFLM* (Leslie, 1992) and *SCALA* (Kabsch, 1988).

3. Results and discussion

The Gly1ORF1-H6 protein was purified to electrophoretic homogeneity using a three-step procedure: ammonium sulfate precipitation of the protein from the spent medium was followed by elution of the His-tagged protein from an Ni–NTA column using an imidazole gradient and a pH gradient in two successive chromatography steps. The final preparation appears as a single band after SDS–PAGE. Approximately 4 mg of purified Gly1ORF1-H6 protein was recovered from each litre of spent growth medium.

Dynamic light-scattering measurements of a Gly1ORF1-H6 sample had a baseline error of 1.001 and 17% relative polydispersity. A monomodal fit of the data predicted a molecular weight of 24 kDa, indicating that Gly1ORF1-H6 (MW 14.6 kDa) was a monodisperse dimeric complex.

Table 1
Data-collection statistics.

Values in parentheses are for the highest resolution shell (2.53–2.4 Å).

Resolution range (Å)	23.8–2.4
No. of reflections	56458
No. of unique reflections	16791
R_{sym}†	0.039 (0.37)
Completeness (%)	98.9 (98.9)
Multiplicity	3.4 (2.9)
I/SD‡	14.2 (2.1)

† $R_{sym} = \sum(|I - \langle I\rangle|)/\sum(I)$. ‡ I = intensity; SD = standard deviation.

Hanging-drop vapor diffusion was used as described above to grow hexagonal bipyramidal crystals of Gly1ORF1-H6. The crystals appeared overnight and grew to as large as 1.3 × 0.6 mm in about three weeks (Fig. 1). The crystals were of data quality and diffracted to 2.4 Å using a conventional X-ray source. Data-collection statistics from a Gly1ORF1-H6 crystal are summarized in Table 1. The unit-cell parameters of the crystal were a = 95.2, b = 95.2, c = 83.7 Å, α = 90, β = 90, γ = 120°. The Laue symmetry and systematic absences are consistent with the $P6_3$ space group. The Matthews volume was 3.8 $Å^3\,Da^{-1}$ (67% solvent) with two molecules in the asymmetric unit (Matthews, 1968). More recently, a selenomethionine-containing derivative of Gly1ORF1-H6 has been produced, purified and crystallized. We anticipate that MAD phasing will allow us to determine the structure. The analysis of this structure may help to reveal the function of the Gly1ORF1 protein and its role in pathogenesis.

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