mosquito® crystal
application note

rapid screening and optimisation of crystallisation conditions of the bacterial periplasmic binding protein, HbpA role as a glutathione import protein

glutathione: its role in metabolism

Glutathione is one of the major antioxidants/redox molecules in most aerobic cells, playing an important role in cell metabolism, differentiation, proliferation, and apoptosis. The reduced form of glutathione (GSH) is required for the detoxification of drugs and reactive compounds of both endogenous and exogenous origin, the delivery of its constituent amino acids to other tissues and the secretion of hepatic bile.

glutathione import and export proteins

In eukaryotic cells, GSH is made constitutively within the cell and is exported across the plasma membrane by transporter proteins. The main GSH export proteins identified belong to the ATP binding cassette (ABC) protein family and a number of studies have been carried out to characterise and define the molecular identity of these proteins [1]. As GSH is made constitutively in eukaryotic cells, there was not thought to be a role for GSH import proteins. Recent interest, however, in its transport across intracellular membranes, such as mitochondria, suggests that there is indeed a role for these proteins in eukaryotic cells [2]. Although GSH import proteins have been identified in some species of *Saccharomyces* [3,4], research on the presence of import proteins in humans has been limited.

Bacteria which are auxotrophic for cysteine and GSH need to acquire these molecules by import mechanisms and studies of bacterial import proteins reveal that they belong to the ABC protein family, similar to that described for GSH export proteins [5]. In studies with mutants of *Haemophilus*, which lack the ability to import GSH, it was observed that these bacteria are more susceptible to oxidative and disulfide stress. It was proposed that the inability of these mutants to sustain stresses may impact on their pathogenicity and ability to colonise certain host niches.

identification of GbpA

Recently, Vergauwen and colleagues identified a bacterial periplasmic lipoprotein in H. *influenzae* Rd, a global human pathogen that specifically binds both reduced glutathione (GSH) and oxidised glutathione (GSSG) with physiologically relevant affinity [6].

This discovery came as a complete surprise as this protein was previously thought to be a heme-binding protein A (HbpA) serving as a binding platform for heme. Vergauwen and colleagues employed a combination of structural, biochemical and cellular studies in establishing the physiological role of this protein, as a periplasmic binding protein that primes glutathione import via its cognate ABC-like dipeptide transporter DppBCDF.

Accordingly, HbpA has now been renamed as glutathione-binding protein A (GbpA) to reflect its biological function accurately and to facilitate the correct annotation of GbpA-like proteins in bacterial genomes [6].

Arguably, the defining experimental undertaking that helped to dispel any doubts about the proposed function of GbpA resulted from crystallographic studies of GbpA in complex with glutathione. To achieve this, Vergauwen *et al.* were able to successfully co-crystallise GbpA from *Haemophilus parasuis*, a protein highly homologous to that of H. *influenzae* Rd [6].

This application note describes the use of mosquito® crystal to screen and optimise crystallisation conditions for GbpA using limited amounts of protein, thus highlighting its ability to address complex biological questions in structural biology quickly and efficiently.

methods

Purified recombinant GbpA from H. *parasuis*, (10 mg/mL in 10 mM Tris-HCl pH 8.0, 100 mM NaCl) was used to carry out an extensive crystallisation screen in the presence and absence of 1 mM GSSG using a mosquito crystal (SPT Labtech).

key benefits

- **low protein consumption**
- **only 25 µL reservoir used per drop**
- **high throughput co-crystallisation**
- **fast, <2 minutes per plate**
- **accuracy of drop placement at 50 nL**
- **no cross-contamination**

The crystallisation screen was based on 100 nL crystallisation droplets (50 nL protein sample and 50 nL crystallisation condition) equilibrated in sitting-drop geometry over 25 µL reservoirs in Triple Drop Plates (Molecular Dimensions Ltd) containing a given crystallisation condition. This approach allowed the efficient screening of nearly 2000 unique crystallisation conditions starting from a very limited amount of protein sample (~1 mg purified GbpA).

sptlabtech

results

This screen led to the identification of two lead conditions (0.1 M $MgCl_2$, 0.1 M sodium citrate pH 5.0, 15% wt/vol PEG4000 and 4% Tascimate pH 4.0, 20% wt/vol PEG3350) starting with protein samples containing 1 mM GSSG. Upon optimisation of the screen conditions, diffraction quality crystals of GbpA could be grown overnight as rectangular rods typically measuring 0.05 x 0.05 x 0.2 mm from both lead conditions using the sitting drop geometry. For data collection under cryogenic conditions (100 K), single crystals were flash cooled in liquid nitrogen using a nylon loop after a very brief incubation (typically <30 seconds) in a cryoprotection solution containing 4% Tascimate pH 4.0, 20% wt/vol PEG3350, 20% vol/vol glycerol or 0.1 M MgCl2, 0.1 M sodium citrate pH 5.0, 15% wt/vol PEG4000, 20% vol/vol glycerol. The structure of GbpA was determined by maximum-likelihood molecular replacement as implemented in the program suite PHASER and crystallographic refinement and structure validation was described by Vergauwen *et al.* [6].

discussion

The application of state-of-the-art crystallisation robotic platforms has emerged as a powerful technical advancement in structural biology in the post-genomic era, allowing targeting of challenging biological questions. Here, the mosquito crystal was successfully employed to facilitate crystallographic studies towards the structural basis of the proposed novel function for GbpA-like proteins. mosquito crystal proved to be a tremendously efficient tool in setting up close to 2000 crystallisation trials using a limited amount of sample. The speed (less than two minutes per plate) and reproducibility in pipetting 50 nL droplets, without the possibility for sample cross-contamination, offered a clear experimental advantage.

Structural studies of GbpA from *H. parasuis* in complex with GSSG has revealed the structural basis of the proposed novel function for GbpAlike proteins. GbpA adopts the pear-shaped, two-domain α/ß-fold characteristic for bacterial dipeptide and oligopeptide binding proteins (Fig 1). GSSG is bound at full occupancy at a large solvent-filled interface between the N-terminal and C-terminal halves of *H. parasuis* GbpA (Fig 1A). The two glutathione legs (designated as GS-I and G-II) are bound asymmetrically in two well-defined compartments and each make numerous specific interactions with both the N-and C-terminal domains of Hp_HbpA and ordered solvent molecules (Fig 1B).

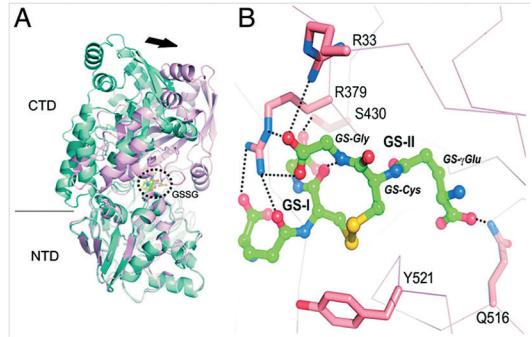

Fig 1. Structure of GbpA from H. parasuis in complex with GSSG.

(A) Ribbon diagram showing an overlay of unliganded Dpp (PDB entry 1DPE) with GbpA from H. parasuis. The structures were superposed with respect to their N-terminal domains

(B) Binding of GSSG to the GbpA interdomain interface. The two glutathione legs are labeled as GS-I and GS-II. For clarity some interactions have been omitted.

The figure was created with PyMOL (The PyMOL Molecular Graphics System, Schrödinger, LLC).

The amino acids involved in numerous specific interactions between GSSG and *H. parasuis* GbpA can be traced to well-conserved sequence fingerprints across the entire GbpA family of proteins.

The first snapshot of a physiologically relevant complex of a GbpAfamily protein with its ligand cargo, has provided the missing link for understanding at the atomic level how GbpA-family proteins could serve as glutathione-binding platforms for priming their cognate dipeptide permeases. In its GSSG-bound form, *H. parasuis* GbpA adopts a collapsed conformation about the hinge region connecting the N-and C-terminal domains to sandwich a single GSSG molecule. This is consistent with the general mode of action of bacterial periplasmic binding proteins, whereby substrate binding between the N-and C-terminal domains shifts the equilibrium towards a closed state, in a process often referred to as a Venus flytrap mechanism. In their closed, ligandbound forms periplasmic binding proteins associate with their cognate membrane-embedded pore to deliver the cargo for translocation. Taken together, the structural features of GSSG binding by GbpA-family proteins highlight the versatility of the dpp fold whereby a handful of key mutations on either side of the binding interface has led to a gain of function.

references

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specifications

SPT Labtech's mosquito® crystal is a bench-top liquid handler which has been integrated with a wide array of other laboratory instrumentation, ranging from simple stacking robots through to complete plate preparation solutions, to achieve walkaway operation in many application areas.

Dispense range:	25 nL – 1200 nL
Plate capacity:	2 or 5
Plate format:	All SBS footprint plates and crystallisation plates
Min accessible volume:	10 nL
Dimensions:	390 mm x 470 mm x 690 mm (15.5" x 18.5" x 27") (w x d x h)
Weight:	27 kg (59 lbs)
Services:	110 V/220 V single phase 50.60 Hz
Noise:	64dBA peak noise during operation

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