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Crystallization of recombinant *Bacteroides fragilis* glutamine synthetase (GlnN) isolated using a novel and rapid purification protocol

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ABSTRACT

Glutamine synthetase enzymes (GSs) are large oligomeric enzymes that play a critical role in nitrogen metabolism in all forms of life. To date, no crystal structures exist for the family of large (~1 MDa) type III GS enzymes, which only share 9% sequence identity with the well characterized GSI and GSII enzymes. Here we present a novel protocol for the isolation of untagged *Bacteroides fragilis* GlnN expressed in an auxotrophic *Escherichia coli* strain. The rapid and scalable two-step protocol utilized differential precipitation by divalent cations followed by affinity chromatography to produce suitable quantities of homogenous material for structural characterization. Subsequent optimizations to the sample stability and solubility led to the discovery of conditions for the production of the first diffraction quality crystals of a type III GS enzyme.

Introduction

Glutamine synthetases (GSs)[1] are large oligomeric enzymes that play a central role in nitrogen assimilation, catalysing the condensation of ammonium and glutamate to form glutamine, a precursor for the synthesis of many critical bio-molecules. The ancient [1] and ubiquitous [2] GS superfamily is, therefore, evolutionarily diverse and can be divided into three main groupings, namely GSI, GSII and GSIII, on the basis of amino-acid divergence [3].

GSI and GSII enzymes have been purified from numerous bacterial and eukaryotic sources, respectively, over the past five decades of biochemical and structural investigations into their functioning and regulation [4]. The first crystal structure of a GSI enzyme [5] was solved using material prepared from an adenylylation deficient *Salmonella typhimurium* strain using a combination of simple differential precipitation techniques and nucleotide-analogue affinity chromatography [6]. Since then, advances in recombinant DNA technology and the advent of affinity purification technologies have greatly facilitated the isolation of GS material suitable for crystallization. The first crystal structure of a GSII enzyme was of the *Zea mays* GS1a protein [7], which was heterologously expressed in *Escherichia coli* and purified using a combination of anion exchange and affinity chromatography steps [8]. More recently, several structures of GSII enzymes purified with affinity tags [9,10] have been reported.

In comparison, GSIII enzymes have only been isolated and characterized from a small number of sources [11–13] despite the widespread occurrence of homologues in a number of evolutionarily divergent organisms [14–16], including, the anaerobic pathogenic protozoan *Trichomonas vaginalis* [17]. To date, the only structural information describing the large GSIII enzymes (~1 MDa), which share only ~9% sequence identity with the GSI enzymes, is from a low-resolution cryo-EM reconstruction of the GlnN enzyme from the opportunistic human pathogen *Bacteroides fragilis* [3]. A detailed understanding of the functioning and evolution of these divergent enzymes in light of the known structure–function relationships of the other GS families is, therefore, still lacking. Although the current purification protocols [3,11] are sufficient for the preparation of small quantities of material for microscopy and biochemical studies, the poor yields, resulting from protein solubility and stability issues, represent a barrier to the attainment of suitable material for crystallization and the achievement of these goals.

Here we present a novel scheme for the rapid isolation of suitable quantities of pure GlnN protein and the subsequent sample optimizations that have lead to the first diffraction quality crystals of a GSIII enzyme.

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[1] *Abbreviations used:* GSs, glutamine synthetases; CFE, cell-free extract; AGB, Affi-Gel Blue; MetSox, methionine sulphoximine; GGT, γ-glutamyl transferase; ANX, anion-exchange chromatography; SEC, size-exclusion chromatography; HIC, hydrophobic interaction chromatography; EM, electron microscopy.

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Materials and methods

All reagents and chemicals were sourced from Sigma–Aldrich unless otherwise stated.

Expression and cell lysis

The glutamine synthetase from *B. fragilis* BF-1, GlnN, was expressed constitutively from a pEcoR1-derived low copy number plasmid, pJS139, in an auxotrophic *E. coli* strain, YMC11 (glnA$^-$, ntrB$^-$, ntrC$^-$, ApS) [18] as described previously [3] but without the low-nitrogen induction and CTAB steps. Following 16 h growth in Luria broth [19] containing ampicillin (100 μg/ml) at 37 °C with aeration, cells were collected by centrifugation for 15 min at 7500 rpm at 4 °C and resuspended in 1/50th of the original culture volume of extraction buffer (50 mM imidazole, 50 mM KCl, pH 7.1). Cells were then disrupted by sonication for 4 min on ice using a Misonix sonicator fitted with a microtip operating at a power output of 9 W and cell-free extract (CFE) was then prepared by centrifugation at 15,000 rpm for 30 min at 4 °C. For long-term storage, CFE was diluted 1:2 with glycerol, aliquoted into Sterilin® plastic tubes, flash-frozen in liquid nitrogen, and then placed at −80 °C.

Protein purification

Frozen CFE was thawed and diluted 1:2 with extraction buffer (50 mM imidazole, 50 mM KCl, pH 7.1). $ZnCl_2$ was then added to a final concentration of 2 mM and the preparation was left to precipitate for 1 h at room temperature. Precipitated material was collected by centrifugation at 15,000 rpm at 4 °C for 30 min and resuspended, using a Dounce homogenizer, in ice-cold wash buffer (15 mM imidazole, 2 mM $MgCl_2$, pH 7.1). Following recollection of the washed material, a solubilization step was performed in resuspension buffer (50 mM imidazole, 500 mM KCl, 10% glycerol, pH 7.1). Insoluble material remaining after 1 h at 4 °C was removed by a 30-min centrifugation step at 14,000 rpm in a desktop centrifuge at 4 °C. Before loading onto the pre-equilibrated 5 ml Affi-Gel Blue (AGB) HiTrap affinity column (GE Healthcare), the supernatant was diluted 1:10 with ice-cold AGB equilibration buffer (50 mM imidazole, pH 7.1, 50 mM KCl, 20 mM $MgCl_2$, 10% glycerol, and 0.02% sodium azide) and filtered through a 0.45-μm filter. The column was then washed with 5 volumes of equilibration buffer before elution of the bound protein with 2 volumes of equilibration buffer containing 10 mM ATP. A period of 5 min was allowed to elapse between the application of the first and second volumes of elution buffer. All chromatography steps were carried out at a flow rate of 1 ml/min at ambient temperature.

After chromatographic purification, methionine sulphoximine (MetSox) was added to the eluted fraction at a final concentration of 8 mM and the sample was stored for 16 h at 4 °C. This final preparation was concentrated to 6 mg/ml using Microsep and Nanosep 10 kDa MWCO centrifugal concentrators (Pall Corporation). The final preparation was divided into 10 μl aliquots and flash-frozen in NUNC™ CryoTubes™ (Thermo Fisher) for storage under liquid nitrogen.

Biochemical assays and protein identification

Protein concentrations were estimated using the Bradford assay [20] with Bio-Rad reagents and bovine serum albumin standards. Results of assays prepared in 96 well NUNC™ plates were read using a Titertek Multiscan plus MKII plate reader fitted with a 595-nm filter and data processing was carried out with Genesis Lite.

GS activity was assayed by the γ-glutamyl transferase (GGT) assay as described by Bender et al. [21] and subsequently modified by Southern et al. [11]. Total assay volumes including reaction mixtures and samples were also reduced from 1.5 ml to 250 μl to allow measurement in the Titertek Multiscan plate reader. Specific activity was expressed as μmoles of glutamyl hydroxamate formed per min, per mg protein, and 1 μmol of glutamyl hydroxamate gave 2.916 absorbance units at a wavelength of 540 nm.

Discontinuous SDS–PAGE was carried out according to Laemmli [22] using a Mini-PROTEAN 3 cell from Bio-Rad. Acrylamide (19:1 monomer:cross-linker) was used to prepare 5% stacking gels and 7.5 or 10% separating gels. Pre-stained molecular weight markers (Fermentas Life Sciences) were included in all electrophoretic separations. Gels were stained either with 0.25% Coomassie Brilliant Blue R250 in 45% methanol and 10% acetic acid or by a non-ammoniacal silver-staining protocol [23] using reagents from the GE Healthcare PlusOne™ Silver-Staining Kit. Protein purity was estimated by densitometry using the program IMAGEJ [24]. Peak areas, corresponding to bands in the lane under investigation, were calculated after baseline subtraction, and the purity of the GlnN band was determined from the ratio of its peak area to the total for all the bands in the lane.

Crystallization and preliminary diffraction characterization

GlnN, stored under liquid nitrogen, was thawed, diluted to 2 mg/ml with AGB equilibration buffer, and clarified by centrifugation at 14,000 rpm in desktop centrifuge prior to the setting up of modified microbatch crystallization conditions [25]. Equal volumes (1 μl each) of protein and precipitant (reagent 20 from Hampton Research Crystal Screen diluted 1:2 with distilled H_2O) were dispensed under 5 ml Al's oil (Hampton Research) in 72 well microbatch plates (Greiner) and stored at ambient temperature (21–23 °C). All assessments of crystal growth and manipulations were performed on a Leica MZ6 stereo microscope fitted with a CLS 150X cold light source. Photographs of crystals were captured with a Zeiss Axiocam attached to a Nikon Diaphot inverted microscope fitted with a 20× short working distance objective lens.

The identity of the proteins comprising the crystals was confirmed using silver-stained SDS–PAGE. Crystals were washed serially in four droplets (2 μl) of crystallization solution (Hampton condition 20 diluted 1:2 with H_2O) placed under oil in the microbatch plate and finally dissolved in a drop of diluted SDS–PAGE loading buffer (1:2 H_2O) before electrophoretic analysis.

Diffraction analyses were carried out on BM14 at the ESRF (Grenoble). Crystals were looped using LithoLoops™ fitted in Hampton HT Crystal Caps and cryo-protected with Paratone-N oil (Hampton Research) prior to being flash-cooled in liquid nitrogen. Data (1° oscillations) were collected at 100 K using 0.95373 Å wavelength X-rays (200 mA beam current) with an exposure of 120 s and a crystal-to-detector distance of 324.8 mm.

Electron microscopy of GlnN crystals

Several microlitres of crystallization solution were removed and applied to EM grids coated with a glow-discharged thin carbon support film, before being stained with 2% uranyl acetate solution using the droplet method [26]. Electron micrographs were recorded by a Proscan 2048 × 2048 slow-scan CCD camera, with 14 μm pixels, using a Leo 912 TEM operating at 120 kV with zero-loss energy filtering. Fourier transforms were calculated using a FFT plug-in [27] in Paint Shop Pro® and patch correlation averages were calculated in 2DX after Fourier filtering [28].