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Recombinant expression, purification, and crystallization of the glutaminyl-tRNA synthetase from *Toxoplasma gondii*

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ABSTRACT

Aminoacyl tRNA synthetases play a critical role in protein synthesis by providing precursor transfer-RNA molecules correctly charged with their cognate amino-acids. The essential nature of these enzymes make them attractive targets for designing new drugs against important pathogenic protozoans like Toxoplasma. Because no structural data currently exists for a protozoan glutaminyl-tRNA synthetase (QRS), an understanding of its potential as a drug target and its function in the assembly of the Toxoplasma multi-aminoacyl tRNA (MARS) complex is therefore lacking. Here we describe the optimization of expression and purification conditions that permitted the recovery and crystallization of both domains of the Toxoplasma QRS enzyme from a heterologous *Escherichia coli* expression system. Expression of full-length QRS was only achieved after the addition of an N-terminal histidine affinity tag and the isolated protein was active on both cellular and *in vitro* produced Toxoplasma tRNA. Taking advantage of the proteolytic susceptibility of QRS to cleavage into component domains, N-terminal glutathione S-transferase (GST) motif-containing domain fragments were isolated and crystallization conditions discovered. Isolation of the C-terminal catalytic domain was accomplished after subcloning the domain and optimizing expression conditions. Purified catalytic domain survived cryogenic storage and yielded large diffraction-quality crystals over-night after optimization of screening conditions. This work will form the basis of future structural studies into structural–functional relationships of both domains including potential targeted drug-design studies and investigations into the assembly of the Toxoplasma MARS complex.

Introduction

Aminoacyl tRNA-synthetases (aaRSs[1]) play an essential role in all life by charging tRNA molecules with their cognate amino acids. This two-step esterification reaction, requiring ATP, proceeds through an aminoacyl-adenylate intermediate and ends with the amino acid transferred to the 2′ or 3′ hydroxyl of the tRNA. The importance of the charging step dictates a high level of fidelity of aminoacylation, a feat accomplished with auxiliary editing domains which ensure correct translation of the genetic code during protein synthesis. By nature, most tRNA synthetases are therefore large modular proteins comprising catalytic, tRNA binding, and in some cases editing domains [1].

The essential role of aaRSs has led to these enzymes being targeted in a number of structure-based drug design initiatives [2]. Inspired by the success of the commercially available bacterial isoleucyl-tRNA synthetase inhibitor, mupirocin, attention is now turning to the enzymes from eukaryotic protozoan pathogens in an attempt to discover new drugs against the important human pathogens [3]. Despite the progress made with a number of protozoan aaRSs, no structural information exists for the glutaminyl-tRNA synthetase (QRS) and only recently has a yeast enzyme been solved [4].

In addition to the widespread catalytic and editing domains, several aaRSs, particularly those from higher eukaryotes, possess additional non-catalytic motifs [5]. The function of these N and C-terminal appended domains, like their primary sequences, is varied and, to date, roles including supplementary tRNA binding [6],

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[1] *Abbreviations used:* QRS, glutaminyl-tRNA synthetase; MARS, multi-aminoacyl tRNA; aaRSs, aminoacyl tRNA-synthetases; YRS, tyrosyl-tRNA synthetase; MRS, methionyl-tRNA synthetase; PBS, phosphate-buffered saline; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; HFF, human foreskin fibroblast.

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subcellular localization [7], and cytokine genesis [8] have been assigned. In eukaryotes, auxiliary domains also appears responsible for driving a subset of aaRSs to assemble into multi-aminoacyl tRNA synthetase (MARS) complexes [9,10,6], which are thought to increase the overall efficiency of protein synthesis by channeling charged tRNA directly to the translation machinery [11]. We have recently discovered that a subset of tRNA synthetases: methionyl- (MRS), glutaminyl-, glutamyl- (ERS), and tyrosyl-tRNA synthetase (YRS), are found in a unique MARS complex in the protozoan, *Toxoplasma gondii*, [12].

In order to address our poor understanding of the molecular assembly of the MARS complex, we have undertaken to overexpress and biophysically characterize both domains of the Toxoplasma QRS enzyme. Atomic resolution structural details of the catalytic domain of QRS will also be invaluable in assessing the potential of the enzymes as a target for selective inhibition by novel drugs.

Materials and methods

All chemicals were sourced from Sigma–Aldrich unless otherwise specified.

Bioinformatics

In silico disorder predictions of the QRS sequence (ToxoDB: TGME49_217460) were calculated with Genesilico MetaDisorder2 [13] and schematics of domain arrangements were derived from NCBI conserved domain searches [14] with an *E*-value threshold of 1. Predictions were performed and rendered by the tRNAscan-SE web server [15].

Construct design, cloning, and expression

Full-length QRS was prepared by heterologous (*Escherichia coli*) expression of the codon-optimized *QRS* gene (ToxoDB: *TGME49_217460*) cloned into a pET-28 b (+) vector (Novagen) as described previously [12]. For the larger-scale protein purification in this experiment, cultures were scaled up to 0.8 l, induction was initiated at an optical density at 600 nm (OD_{600}) of 0.65 using 1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) for 4 h at 37 °C with shaking before cells were collected by centrifugation at 5000 rcf for 10 min at 4 °C, washed twice in phosphate-buffered saline (PBS), and stored at −20 °C.

In order to produce the C-terminal catalytic domain construct (QRS-C) of the *QRS* gene, a region corresponding to residues 255–805 of the annotated full-length *gene was subcloned* into the *NdeI* and *BamHI sites of* pET-28 b (+) vector (Novagen) to give an N-terminal 6xHis-tagged construct. Conditions for optimal expression of QRS-C in *E. coli* BL21 (DE3) cells, grown in Luria broth (3 ml containing kanamycin and shaking at 200 rpm), were optimized by varying culture and induction conditions including starting inoculum volumes, culture temperatures, IPTG concentrations, and incubation times. Protein expression yields were measured by Western blot (see below) following separation of cell-free extracts by SDS–PAGE. Final larger-scale expression took place in 0.5 l cultures grown to an OD_{600} of 1 before IPTG (0.5 mM final concentration) was added to induce protein expression. Induced cultures were left shaking at room temperature (~21 °C) overnight before cells were collected and stored as described above.

Protein purification

For lysis, cell pellets were thawed and resuspended in either 40 ml (full-length QRS purification) or 25 ml (QRS-C) of 25 mM Tris–HCl pH 8.0, 5 mM EDTA, and 50 mM NaCl containing protease inhibitors (Complete Protease Inhibitor Tablets, Roche). Lysozyme was then added to 0.1 mg/ml and samples left shaking at 37 °C for 30 min before carrying out three freeze–thaw cycles. A second 30 min incubation followed the addition of $MgCl_2$ to 20 mM and DNAse I from Bovine pancreas (Sigma–Aldrich) to 0.1 mg/ml.

Both full-length and QRS-C proteins were recovered from clarified (by passage through 0.22 μm filter) cell-free extracts by immobilized metal affinity chromatography on a 10-ml nickel-nitrilotriacetic acid agarose column (Qiagen) pre-equilibrated with lysis buffer and operated at 1 ml/min. Unbound proteins were removed by washing with 5 column volumes (CVs) of the same buffer containing 20 mM imidazole and elution of tagged-proteins was achieved with three CVs of buffer containing 175 mM imidazole. Fractions were pooled and concentrated by ultrafiltration (Amicon Ultra 15 device – Millipore; 10 kDa MWCO 4 °C) to 10 mg/ml for QRS full-length and 25 mg/ml for QRS-C before final purification by SEC (as described below). Following concentration of peak fractions to 24 mg/ml for full-length QRS and 40 mg/ml for the QRS-C construct, using a 10 kDA MWCO Microsep ultrafiltration filter (Pall Corporation), and passage through 0.22 μm Nanosep filter, samples were flash-frozen in liquid nitrogen for long-term storage at –80 °C.

Biophysical characterization

Size-exclusion chromatography was performed on a Superdex 200 10/300 GL column (GE Healthcare) using the AKTA Purifier system (GE Healthcare). Separations were carried out in 25 mM Tris pH 7.5, 150 mM KCl, and 10 mM $MgCl_2$ at 4 °C with a flow rate of 0.5 ml/min and fractions were collected every minute. The column was calibrated using high-molecular weight standards (Sigma–Aldrich). Multi-angle laser light scattering measurements were carried out on an identical S200 column (operated under the same conditions calibrated with the same standards as above) attached to a dedicated chromatography system with an in-line Dawn EOS detector (Wyatt).

Protein analysis and identification

Eluates from chromatographic separations were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) using pre-cast gradient gels (NuPAGE® Novex® 4–12% Bis–Tris polyacrylamide gel, Life Technologies) with MES-SDS running buffer using the XCell SureLock® Mini-Cell as per the manufacturer's instructions. Proteins were then visualized with the Colloidal Blue Staining Kit from Life Technologies or the ProteoSilver Plus Silver Stain Kit. Identification of the proteins in the excised bands by MS–MS was carried out as previously described [16]. Alternatively, proteins were electro-blotted onto polyvinylidene difluoride membranes (Immobilon-P; Millipore) with the XCell II™ Blot Module (Life Technologies) following separation by SDS–PAGE and the detected by Western blot with a 1:1000 dilution of anti-6xHis antiserum (BPS Bioscience) and 1:1000 anti-mouse secondary antibody (Promega) before development with BCIP/NBT (Amresco).

Native tRNA recovery and transcription of recombinant $tRNA^{Gln}$

Total small RNA, including tRNA, was recovered from lysates of egressed *T. gondii* tachyzoites using the method of von Ehrenstein [17] with slight modification. Briefly, parasites were cultured by infection of confluent monolayers of human foreskin fibroblast (HFF) grown in Dulbecco's modified Eagle's medium (Life Technologies) supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 10 mM HEPES buffer (pH 7.2), 4 mM glutamine,