Expression, purification and crystallisation of *Helicobacter pylori* L-asparaginase

Prathusha Dhavala,[a] Julya Krasotkina,[b] Christine Dubreuil[a] and Anastassios C. Papageorgiou[a*]

[a]*Turku Centre for Biotechnology, University of Turku and Åbo Akademi, Turku 20521, Finland, and* [b]*Institute for Biomedical Sciences, Russian Academy of Medical Sciences, Moscow, Russia. E-mail: tassos.papageorgiou@btk.fi*

Synopsis L-asparaginase from *Helicobacter pylori* was overexpressed in *E. coli*, purified and crystallized. The crystals belong to the *I*222 space group with unit-cell parameters *a*=63.6, *b*=94.9, *c*=100.2 Å and one molecule in the asymmetric unit. A complete data set to 1.6 Å resolution was collected using synchrotron radiation.

Abstract L-asparaginases from *Escherichia coli* and *Erwinia chrysanthemi* are effective drugs used in the treatment of acute childhood lymphoblastic leukemia for over thirty years. However, despite their therapeutic potential they can cause serious side-effects owing to their intrinsic glutaminase activity that leads to L-glutamine depletion in the blood. Consequently, new asparaginases with low glutaminase activity, fewer side-effects and high activity towards L-asparagine are highly desirable as better alternatives in cancer therapy. L-asparaginase from *Helicobacter pylori* was overexpressed in *E. coli* and purified for structural studies. The enzyme was crystallized at pH 7.0 in the presence of PEG 4000 16-19% (*w/v*) and 0.1 *M* magnesium formate. Data to 1.6 Å resolution were collected at 100 K in a synchrotron radiation source from a single crystal. The crystals belong to the *I*222 space group with unit-cell dimensions *a*=63.6, *b*=94.9, *c*=100.2 Å, and one molecule of L-asparaginase in the asymmetric unit. Elucidation of the crystal structure will provide insights into the active site of the enzyme and a better understanding of the structure-activity relationship amongst L-asparaginases.

Keywords: Leukaemia treatment; enzyme therapy; asparaginase; glutaminase; *Helicobacter pylori*

1. Introduction

Bacterial L-asparaginases (EC 3.5.1.1) catalyze the conversion of L-asparagine to L-aspartic acid and ammonia. Two types of L-asparaginases, namely I and II, have been identified. Type II (periplasmic) L-asparaginases have been intensively studied for years owing to their wide use in the treatment of acute childhood lymphoblastic leukaemia since the early '70s (Duval *et al*., 2002; Avramis & Tiwari, 2006). The antineoplastic effect of type II L-asparaginases stems from their ability to deplete L-asparagine circulated in blood and tissues. Asparagine is a crucial amino acid for protein, DNA, and RNA synthesis, and a cell cycle-specific requirement for the G1 phase of cell

division (Stams *et al*., 2005). In contrast to normal cells, leukaemic cells are characterized by a generally low expression of asparagine synthetase (Richards & Kilberg, 2006). As a result, they are unable to synthesize their own L-asparagine and rely on extracellular supplies of this amino-acid for survival and growth (Verma *et al*. 2007).

L-asparaginases from *Escherichia coli* (EcAII) and *Erwinia chrysanthemi* (ErA) are currently used in clinical practice. Recent statistics have shown that L-asparaginases in combination with other drugs, such as corticosteroids, have improved significantly the cure rates of leukaemia patients (Holcenberg, 2005; Apostolidou *et al*., 2007). Despite their beneficial effect, EcAII and ErA can also cause serious side-effects, including liver disorders, acute pancreatitis, hyperglycemia, immunosuppression and other dysfunctions. Their toxicity is partially attributed to their intrinsic low glutaminase activity that leads to a significant reduction of L-glutamine concentration in the blood (Carlsson *et al*., 1995). Indeed, the glutaminase activity of EcAII and ErA amounts to 2 and 10 % of their asparaginase activity, respectively. Hence, L-asparaginases with high activity against L-asparagine and low specificity towards L-glutamine are thought to be more efficient and induce fewer side effects (Distasio *et al*., 1982).

Crystal structures of bacterial L-asparaginases from *Escherichia coli* and *Erwinia chrysanthemi* (Swain *et al*., 1993; Aghaiypour *et al*., 2001; Sanches *et al*., 2002; Lubkowski *et al*., 2002; Kozak *et al*., 2002), and those of L-glutaminase-asparaginases from *Pseudomonas 7a*, *Acinetobacter glutaminasificans* and *Wolinella succinogenes* (Lubkowski *et al*., 1994ab; Lubkowski *et al*., 1996) have been elucidated. The majority of L-asparaginases crystallizes as homotetramers with 222 symmetry and exhibit high similarity in their tertiary and quaternary structures. The enzyme monomer consists of ~330 residues arranged in two domains, a larger one at the N-terminal and a smaller one at the C-terminal of the enzyme. The active site is located between the N- and C-terminal domains of two adjacent monomers. Residues responsible for ligand binding form the rigid part of the active site. The flexible part of the active site controls the access to the binding pocket and carries a highly conserved for all L-asparaginases Thr residue as the catalytic nucleophile (Aghaiypour *et al*., 2001). In contrast to type II, type I (cytoplasmic) L-asparaginases display allosteric behavior and reduced affinity for L-asparagine despite the overall structural similarity between the two types of the enzymes (Yun *et al*., 2007). Recently identified plant L-asparaginases comprise a structurally and evolutionarily distinct family of L-asparaginases that differ in their substrate specificity and affinity profiles from the bacterial enzymes (Borek *et al*., 2004).

In an effort to identify and characterize new L-asparaginases with better therapeutic properties, we have initiated structural and biochemical studies on *H. pylori* type II L-asparaginase (HpA) and report here on its cloning, overexpression and crystallization. The expressed enzyme contains 333 amino acids with a calculated MW of 35686 Da based on the amino acid sequence, and exhibits ~50% sequence identity with the periplasmic *W. succinogenes* L-asparaginase. The availability of pure enzyme will allow a full biochemical characterisation, including studies on its oligomerization stage, and assessment of its potential in therapeutic settings. Preliminary kinetic data have shown a significantly low L-glutaminase:L-asparaginase ratio (data not shown).

2. Materials and methods

2.1. Expression and purification

A DNA fragment encoding the mature form of *Helicobacter pylori J99* L-asparaginase (GeneID: 890242) was amplified from genomic DNA with Pfu polymerase (Fermentas) using 3'-CAGCCATGGCTCAAAATTTACCCACCATTGC-5' and 3'-CTGCGGCCGCTTAATACTCTTCAAACATTTC-5′ as forward and reverse primers, respectively. NcoI and NotI restrictions sites (underlined) were included in the 3'-ends of the oligonucleotides, correspondingly. The resulting PCR product was digested with *NcoI* and *NotI* restriction enzymes and cloned into a pET22b vector (Novagen) immediately after the *E. coli* signal peptide sequence. The fidelity of the resulting construct was verified by DNA sequencing. The resultant plasmid was named *pET22b/HpA* and it was transformed into BL21(DE3) *E. coli* cells. Cell cultures were grown at 310 K in LB medium containing 100 μg ml^{-1} ampicillin until the optical density at 600 nm reached 0.8. HpA expression was induced with 0.5 m*M* isopropyl-1-thio-β-D-galactopyranoside (IPTG) for a further 16 h at 310 K. Cells from a 2 L culture were harvested by centrifugation at 4 000g (277 K) for 15 min, suspended in 70 ml 20 m*M* potassium phosphate buffer, pH 6.0 and sonicated. Cell debris was removed by centrifugation at 15 000 g (277 K) for 30 min and the soluble fraction of cell-free extract was loaded onto an SP-Sepharose column (1cm x 5cm) equilibrated with 20 m*M* potassium phosphate buffer, pH 6.0. The resin was washed with 5 volumes of column buffer to remove non-bound proteins. Bound HpA was eluted with a 0-0.6 *M* KCl linear gradient in column buffer. L-asparaginase samples were desalted with PD-10 pre-packed columns (Amersham Pharmacia Biotech). The purity of the resultant enzyme solution was confirmed by 12% SDS-PAGE after staining with Coomassie Brilliant Blue R-250. A single major band corresponding to a MW of approximately 35 kDa was found (Fig. 1). The final yield is approximately 6 mg from 1 L of bacterial culture.

2.2. Crystallization and data collection

The purified protein was concentrated by ultra-filtration using Amicon YM-10 filters and stored in 10 m*M* HEPES-NaOH pH 7.0 after buffer exchange. The final protein concentration was 3.7 mg ml^{-1} as measured by the Bradford method (Bradford, 1976) with bovine serum albumin as a standard. Initial crystallization trials were performed with the INDEX crystal screen (Hampton Research) using the sitting drop vapour diffusion method at 289 K in a 96-well Corning plate. The drops contained 1.0 μl protein solution and an equal volume of precipitant solution. Crystals were found after 2 d in condition 92 containing PEG 4000 15% (*w/v*) and 0.1 *M* magnesium formate. Optimization of the crystallization conditions was performed with the hanging drop vapour diffusion method. Crystals suitable for X-ray crystallographic analysis (Fig. 2) were grown at 289 K by mixing 2.5 μl of a protein stock solution with an equal volume of a reservoir solution containing PEG 4000 16-19% (*w/v*), 0.1 *M* magnesium formate, 0.1 *M* HEPES-NaOH, pH 7.0. The hanging drops were equilibrated against 800 μl reservoir solution and crystals grew in about 4 d. Data to 1.6 Å were collected on the X13 beamline in EMBL Hamburg (c/o DESY) from a single crystal soaked for a few seconds in a crystallization solution containing 20% (*v/v)* glycerol as cryoprotectant and flash-cooled to 100 K in a nitrogen-gas cold stream. A total of 400 images were collected with 0.5º rotation per image at a wavelength of 0.8081

Å using a MAR CCD detector, a crystal-to-detector distance of 140 mm, and an average exposure time of 10 seconds per image. Data were processed with the *HKL* package (Otwinowski & Minor, 1997) and final statistics are summarized in Table 1.

3. Results and Discussion

Crystals of HpA belong to the *I*222 space group with unit-cell dimensions *a*=63.6, *b*=94.9, *c*=100.2 Å. Assuming one molecule of the enzyme in the asymmetric unit and a MW of 35686 Da, the Matthews coefficient V_M (Matthews, 1968) is 2.27 $Å^3$ Da^{-1} corresponding to a solvent content of ~46 %. The presence of one molecule in the asymmetric unit is in contrast to most other bacterial type II L-asparaginases which crystallize as non-crystallographic homotetramers. Similarly to HpA, L-asparaginase-gluataminase from *Acinetobacter glutaminasificans* also crystallizes as a monomer in the asymmetric unit. In this case, the active homotetramer could be generated by crystal symmetry operations (Lubkowski *et al*., 1994b). Structure determination of HpA will be pursued by molecular replacement using the co-ordinates of the monomer from the homotetrameric *W. succinogenes* L-asparaginase (pdb accession code: 1WSA) (Lubkowski *et al*., 1996). In combination with biochemical and mutagenesis data, the crystal structure of HpA will provide new clues for protein engineering efforts to improve the therapeutic potential of L-asparaginases.

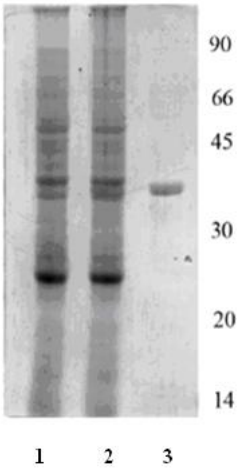

Figure 1 Purity of *H. pylori* L-asparaginase in 12% SDS-PAGE. Crude lysate of *E.coli* BL21(DE3) cells harboring *pET22b/HpA* plasmid (1) before and (2) after 16-h induction with 0.5 m*M* IPTG; (3) purified HpA. Molecular weight standards are shown on the right (in kDa).

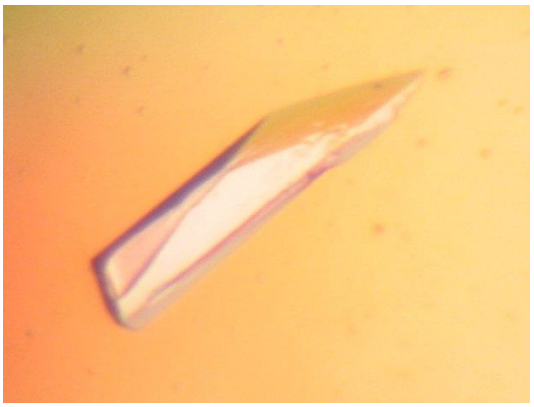

Figure 2 Crystal of *H. pylori* L-asparaginase. Typical dimensions are 0.35 x 0.10 x 0.10 mm.

Table 1 Crystal parameters and data-processing statistics.

Space group	*I*222
Unit-cell parameters	
a (Å)	63.6
b (Å)	94.9
c (Å)	100.2
Resolution range (Å)	99-1.6 (1.63-1.60)
Wavelength (Å)	0.8081
Temperature (K)	100
Total reflections	1153231 (57700)
Unique reflections	40100 (1973)
Completeness (%)	98.9 (97.8)
Mosaic spread (°)	0.6
R_{merge} (%)[a]	9.0 (36.7)
$\langle I/\sigma(I)\rangle$	31.4 (5.3)

Numbers in parentheses correspond to the highest resolution shell.

[a] $R_{merge} = \Sigma_{\mathbf{h}} \Sigma_l |I_{\mathbf{h}l} - \langle I_{\mathbf{h}}\rangle| / \Sigma_{\mathbf{h}} \Sigma_l \langle I_{\mathbf{h}}\rangle$, where I_l is the lth observation of reflection **h** and $\langle I_{\mathbf{h}}\rangle$ is the weighted average intensity for all observations l of reflection **h**..

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