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Cloning, purification and preliminary crystallographic analysis of the *Helicobacter pylori* leucyl aminopeptidase–bestatin complex

Helicobacter pylori is an important human pathogenic bacterium associated with numerous severe gastroduodenal diseases, including ulcers and gastric cancer. Cytosolic leucyl aminopeptidase (LAP) is an important housekeeping protein that is involved in peptide and protein turnover, catabolism of proteins and modulation of gene expression. LAP is upregulated in metronidazole-resistant *H. pylori*, which suggests that, in addition to having an important housekeeping role, LAP contributes to the mechanism of drug resistance. Crystals of *H. pylori* LAP have been grown by the hanging-drop vapour-diffusion method using polyethylene glycol as a precipitating agent. The crystals belonged to the primitive triclinic space group *P*1, with unit-cell parameters $a = 97.5$, $b = 100.2$, $c = 100.4$ Å, $\alpha = 75.4$, $\beta = 60.9$, $\gamma = 81.8^\circ$. An X-ray diffraction data set was collected to 2.8 Å resolution from a single crystal. Molecular-replacement results using these data indicate that *H. pylori* LAP is a hexamer with 32 symmetry.

1. Introduction

Helicobacter pylori colonizes the stomachs of roughly 50% of the population and is associated with numerous severe gastroduodenal diseases, including gastric and duodenal ulcers and gastric adenocarcinoma (Marshall & Warren, 1984; Uemura *et al.*, 2001; Peek & Blaser, 2002). The eradication of *H. pylori* reduces the recurrence of gastric cancer in patients who have received endoscopic resection of early cancer and the recurrence of ulcers in patients with peptic ulcer disease (Ford *et al.*, 2011; Uemura *et al.*, 1997). At present, there is no specific single drug that can effectively cure *H. pylori* infection; current treatments involve the use of a combination of a proton-pump inhibitor with two broad-spectrum antibiotics (mainly clarithromycin and either amoxicillin or metronidazole; Treiber *et al.*, 1998). An increasing prevalence of resistance to the antibiotic components of such regimens (Broutet *et al.*, 2003) requires better understanding of the molecular mechanisms of *H. pylori* pathogenesis and the identification of new drug targets for the development of effective anti-*Helicobacter* agents.

Cytosolic leucyl aminopeptidase (LAP, leucine aminopeptidase, cathepsin III; EC 3.4.11.1), found in many microorganisms, plants and animals, catalyses the removal of N-terminal leucine residues and, at a lower frequency, other amino-acid residues from peptides and proteins. LAPs are classified into two families, M1 and M17, which have distinctly different structures, catalytic mechanisms and biological roles (for a review, see Matsui *et al.*, 2006). LAPs are important housekeeping proteins that are involved in many essential physiological processes such as peptide and protein turnover, catabolism of endogenous and exogenous proteins, modulation of gene expression and antigen processing (Matsui *et al.*, 2006). Analysis of the crystal structures of LAPs from *Escherichia coli* (Sträter *et al.*, 1999), bovine eye lens (Burley *et al.*, 1991), *Pseudomonas putida* (Kale *et al.*, 2010) and *Plasmodium falciparum* (McGowan *et al.*, 2010) revealed that LAPs in peptidase family M17 have two globular α/β domains: a smaller variable N-terminal domain and a larger conserved C-terminal domain that contains the active site. Their activities require two metal ions and are inhibited by bestatin.

LAP from *H. pylori* is a 54.3 kDa protein that is upregulated in metronidazole-resistant *H. pylori* (Kaakoush *et al.*, 2009), which

suggests that, in addition to having an important housekeeping role, LAP contributes to the mechanism of drug resistance. *H. pylori* LAP shows low similarity to other members of the M17 family of metallo-peptidases with, for example, only 26 and 24% amino-acid sequence identity to the LAPs from *E. coli* and human, respectively (Dong *et al.*, 2005). Despite the low level of sequence homology, the residues essential for the metal binding and catalytic activity of M17 LAPs are conserved in *H. pylori* LAP (Dong *et al.*, 2005), suggesting a common catalytic mechanism for this enzyme. Previous kinetic studies showed that *H. pylori* LAP is an allosteric enzyme with sigmoid rather than hyperbolic velocity *versus* substrate-concentration plots (Dong *et al.*, 2005). This clearly distinguishes the *H. pylori* enzyme from most of the other known M17 LAPs, which generally show Michaelis–Menten saturation kinetics (Thierry & Janine, 1996). In this paper, we report the cloning, purification, crystallization and preliminary X-ray analysis of recombinant *H. pylori* LAP in complex with bestatin. Analysis of the crystal structure of this enzyme would be an important step towards understanding the structural basis of its unique allosteric behaviour.

2. Materials and methods

2.1. Cloning and overexpression

The coding sequence for LAP (UniProtKB ID O25294) was PCR-amplified from genomic DNA of strain 26695 of *H. pylori* using *Pfu* DNA polymerase (Stratagene) and the primers CACCTTAAAAATCAAATTAGAAAAAACAC (forward) and TCAAGCCTTTTCAAAGCTC (reverse). The amplified fragment was cloned into the pET151/D-TOPO vector using the TOPO cloning kit (Invitrogen) to produce an expression vector that contains an N-terminal His₆ tag followed by a TEV protease cleavage site. The expression clone was confirmed by DNA sequencing. The recombinant protein used for crystallization comprised residues 1–495 of LAP plus six additional residues from the TEV protease cleavage site and vector (GIDPFT). The vector was transformed into *E. coli* strain BL21(DE3) (Novagen). The cells were grown in LB medium containing 50 mg l⁻¹ ampicillin at 310 K until an OD₆₀₀ of 0.8 was reached, at which point overexpression of LAP was induced by adding 0.5 mM IPTG and growth was continued for a further 3 h. The cells were then harvested by centrifugation at 6000g for 15 min at 277 K.

2.2. Purification

The cells were resuspended in buffer A (20 mM sodium phosphate pH 7.4, 200 mM NaCl, 1 mM PMSF) and lysed by sonication. Cell debris was removed by centrifugation at 12 000g for 30 min at 277 K. The supernatant was collected and clarified by ultracentrifugation at 105 000g for 20 min at 277 K. NaCl and imidazole were then added to the supernatant to final concentrations of 500 and 10 mM, respectively, after which the supernatant was loaded onto a 5 ml HiTrap Chelating HP column (GE Healthcare) pre-washed with buffer A containing 500 mM NaCl. The column was washed with 20 column volumes of buffer B (20 mM sodium phosphate pH 7.4, 500 mM NaCl, 60 mM imidazole) and the protein was eluted with buffer B containing 500 mM imidazole. The N-terminal tag was cleaved off with His₆-TEV protease (Invitrogen) overnight at 277 K whilst dialysing the sample against buffer C [50 mM Tris–HCl pH 8.0, 2 mM DTT, 200 mM NaCl, 1% (v/v) glycerol]. NaCl and imidazole were then added to the sample to final concentrations of 500 and 15 mM, respectively, and the TEV protease and uncleaved protein were removed on a HiTrap Chelating HP column. The flowthrough was concentrated to 2 ml in a Vivaspinn 30 000 Da cutoff concentrator and

Table 1

Data-collection statistics.

Values in parentheses are for the outermost resolution shell.

Space group	P1
Unit-cell parameters (Å, °)	$a = 97.5$, $b = 100.2$, $c = 100.4$, $\alpha = 75.4$, $\beta = 60.9$, $\gamma = 81.8$
V_M (Å ³ Da ⁻¹)	2.54
Mosaicity (°)	1.7
No. of crystals	1
Temperature (K)	100
No. of images	220
Rotation per image (°)	0.5
Wavelength (Å)	1.54
Resolution range (Å)	30–2.8 (2.9–2.8)
Completeness (%)	72 (76)
Observed reflections	86018
Unique reflections	57157
Mean $I/\sigma(I)$	4.8 (1.8)
$R_{\text{merge}}^{\dagger}$	0.096 (0.291)

$\dagger R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $I_i(hkl)$ is the intensity of the i th observation of reflection hkl .

passed through a Superdex 200 HiLoad 26/60 gel-filtration column (GE Healthcare) equilibrated with buffer D (50 mM HEPES pH 8.0, 200 mM NaCl). The protein concentration was determined using the Bradford assay (Bradford, 1976). The protein purity was estimated to be greater than 98% (Fig. 1).

2.3. Crystallization and preliminary X-ray analysis

Prior to crystallization, the protein was concentrated to 14.3 mg ml⁻¹, mixed with bestatin (final concentration 1 mM) and centrifuged for 20 min at 13 000g to clarify the solution. Initial screening of crystallization conditions was carried out by the hanging-drop vapour-diffusion method using an automated Phoenix crystallization robot (Art Robbins Instruments) and Crystal Screen HT and PEG/Ion HT (Hampton Research). The initial crystallization droplets consisted of 100 nl protein solution mixed with 100 nl reservoir solution and were equilibrated against 50 µl reservoir solution in a 96-well Art Robbins CrystalMation Intelli-Plate (Hampton Research). Crystals appeared after 2 d from condition No. 78 of Crystal Screen HT, which consisted of 10% (w/v) polyethylene glycol (PEG) 6000, 5% (v/v) 2-methyl-2,4-pentanediol (MPD), 100 mM HEPES pH 7.5. This condition was subsequently refined to improve the quality of the crystals, yielding

Figure 1

Reduced Coomassie Blue-stained 12.5% SDS-PAGE gel of purified recombinant *H. pylori* LAP.

optimal PEG 6000, protein and bestatin concentrations of 9%(v/v), 9.2 mg ml⁻¹ and 1 mM, respectively. For data collection, the crystals were flash-cooled to 100 K after soaking in a cryoprotectant solution consisting of 12%(w/v) PEG 6000, 6%(v/v) MPD, 100 mM HEPES pH 7.5, 2 mM bestatin, 25%(v/v) glycerol. X-ray data were collected from the crystal to 2.8 Å resolution using a Rigaku MicroMax-007 microfocus rotating-anode generator and a Rigaku R-Axis IV imaging-plate detector. A total of 220 images were collected using 0.5° oscillations. The data were processed and scaled using *d*TREK* (Pflugrath, 1999). The statistics of data collection are summarized in Table 1.

2.4. Molecular replacement

In a search for a suitable model for molecular replacement, a sequence-similarity search against the structures deposited in the RCSB Protein Data Bank was performed. The two most similar sequences were those of LAPs from *P. putida* and *E. coli* (Sträter *et al.*, 1999; Kale *et al.*, 2010). The 495-residue LAP from *H. pylori* shares 35% identity with both the 497-residue LAP from *P. putida*

and the 503-residue LAP from *E. coli* in a 375-residue overlap. Molecular replacement was performed with the *Phaser* program (McCoy *et al.*, 2005) using all data to 2.8 Å resolution. The search model was prepared using *CHAINS*AW (Stein, 2008) based on the coordinates of *P. putida* LAP (PDB entry 3h8g; Kale *et al.*, 2010).

3. Results and discussion

3.1. Overexpression, purification and biochemical characterization

Recombinant *H. pylori* LAP was expressed from the pET151/D-TOPO plasmid in *E. coli* BL21(DE3) upon induction of T7 polymerase. The enzyme was purified to >98% electrophoretic homogeneity based on Coomassie Blue staining of SDS-PAGE gels (Fig. 1). The protein migrated on SDS-PAGE with an apparent molecular weight of 53 kDa, which is close to the value calculated from the amino-acid sequence (54.3 kDa).

When subjected to gel filtration, the protein eluted as a single peak with an apparent molecular weight of approximately 260 kDa (Fig. 2), indicating that *H. pylori* LAP forms an oligomer in solution. The particle weight value estimated from the mobility of the gel-filtration column was approximately midway between the calculated masses of a tetramer (217.2 kDa) and a hexamer (325.8 kDa). Previous studies of LAPs from different plants, animals and microorganisms consistently reported a hexameric state for this family of enzymes (Matsui *et al.*, 2006; Stack *et al.*, 2007). The elution profile of *H. pylori* LAP is therefore likely to represent a hexamer rather than a tetramer.

3.2. Crystallization and preliminary crystallographic analysis

Crystals of the *H. pylori* LAP–bestatin complex (Fig. 3) were obtained using a sparse-matrix crystallization approach. Test data collected from a cryocooled crystal using an in-house Rigaku rotating-anode generator showed diffraction to 2.5 Å resolution

Figure 2
Gel-filtration trace of purified recombinant *H. pylori* LAP. The molecular weight was determined from a calibration plot of log(molecular weight) (log MW) versus the retention volume [$V_{\text{retention}} \text{ (ml)} = 549.3 - 73.9 \times \log \text{ MW}$] available at the EMBL Protein Expression and Purification Core Facility website (http://www.embl.de/pepcore/pepcore_services/protein_purification/chromatography/hiload26-60_superdex200/index.html).

Figure 3
Crystals of the *H. pylori* LAP–bestatin complex.

Figure 4
A representative 0.5° oscillation image of the data collected using an in-house Rigaku rotating-anode generator. The arrow indicates reflections at 2.5 Å resolution.

(Fig. 4). Analysis of the diffraction data using the autoindexing routine in *d*TREK* showed that the crystals belonged to the primitive triclinic space group *P*1, with unit-cell parameters $a = 97.5$, $b = 100.2$, $c = 100.4$ Å, $\alpha = 75.4$, $\beta = 60.9$, $\gamma = 81.8^\circ$. The V_M value calculated under the assumption of a hexamer in the asymmetric unit was $2.54 \text{ Å}^3 \text{ Da}^{-1}$, which falls within the range observed for protein crystals (Matthews, 1968) and corresponds to a solvent content of approximately 52%. A molecular-replacement search was performed using *Phaser* with a monomer of *P. putida* LAP as a model. Six copies of the search model were found in the asymmetric unit, which made up a hexamer with 32 symmetry that was very similar to the hexamers observed in the crystals of the LAPs from *P. putida* and *E. coli* (Kale *et al.*, 2010; Sträter *et al.*, 1999), indicating that the molecular-replacement solution is correct. Attempts to optimize the crystal quality and collect higher resolution diffraction data and structure refinement are currently in progress.

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