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Characterization and crystallization of a novel haemoglobinase from pathogenic *Escherichia coli*

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A haemoglobin-degrading enzyme from pathogenic *Escherichia coli* has been cloned, expressed and purified to homogeneity. The pure protein proteolyses haemoglobin and binds haem. *In vivo*, its role is to remove haem from haemoglobin and pass it to the bacteria, allowing them to overcome the limiting concentration of iron available in the body. The protein has been crystallized using polyethylene glycol to give crystals in a hexagonal space group with unit-cell parameters $a = b = 114.6$, $c = 434.3$ Å. X-ray data have been collected to 2.5 Å resolution. This is the first member of the SPATE (serine protease autotransporters of Enterobacteriaceae) family of autotransporter proteins to be crystallized.

1. Introduction

Recently, we discovered a novel haem-acquisition system in a human pathogenic *Escherichia coli* strain (Otto *et al.*, 1998). A haemoglobin protease, called haem-binding protein (Hbp), is secreted in the culture medium by this pathogen. This protein degrades haemoglobin and subsequently binds the released haem, which it somehow transfers to the bacteria. Specialized haem-acquisition systems are a prerequisite for successful multiplication of pathogenic bacteria in their host (Crosa, 1989; Mietzner & Morse, 1994; Lee, 1995; Schrijvers & Stojiljkovic, 1999; Otto *et al.*, 1992). Hbp appears to be an important virulence factor that plays a significant role in the pathogenesis of *E. coli* infections (Otto *et al.*, 2002).

Hbp is an autotransporter protein; the C-terminal domain of the precursor protein mediates transport of the N-terminal passenger domain across the outer membrane (Henderson *et al.*, 1998). Hbp (the passenger domain) is released from the cell by an unknown protease (van Dooren *et al.*, 2001). Autotransporter proteins have been implicated as virulence factors in many Gram-negative pathogens and there is a growing interest in their functional role (Pohlner *et al.*, 1987; Henderson *et al.*, 1998). Many autotransporters like Hbp have been discovered but their structures and mechanisms remain unknown. Autotransporters can be classified into several subfamilies. Hbp belongs to the SPATE (serine protease autotransporters of Enterobacteriaceae) subfamily (Guyer *et al.*, 2000). All members of this protein family show a high degree of sequence similarity and all show toxicity or proteolytic activity. It is hypothesized that these proteins contain homologous domains, which could be excellent targets for the development of novel antibacterial drugs. The shape of apo-Hbp in solution has been determined recently by a combined approach using solution X-ray scattering, bead-modelling and analytical centrifugation (Scott *et al.*, 2002).

In this study, we show that purified Hbp is capable of binding haem and possesses haemoglobin protease activity. The protein has been crystallized and X-ray data collected to 2.5 Å resolution.

2. Material and methods

Apo-Hbp was purified from the culture supernatant by gel filtration (van Dooren *et al.*, 2001). Holo-Hbp was obtained by first buffer exchanging the purified apo-Hbp into phosphate-buffered saline (PBS) to reduce the high salt concentration. The protein was then incubated anaerobically with haematin at a molar ratio of 1:1.3 in PBS at 310 K for 2 h. Finally, unbound haem was removed using a desalting column.

2.1. Haem-detection assay

100 µl protein samples were spotted onto a nitrocellulose membrane with a Bio-Dot microfiltration apparatus (BioRad Laboratories, Veenendaal, The Netherlands). Haem–protein complexes were detected by chemiluminescence as described in Dorward (1993). The chemiluminescence substrate Lumi-Light Plus (Roche Diagnostics, Almere, The Netherlands) was used to visualize haem. The membranes were analyzed by photoimaging using the BioRad Fluor-S MultiImager.

2.2. Haemoglobin protease activity

A slightly modified endopeptidase assay developed by Sroka *et al.* (2001) was used to determine the haemoglobin protease activity of the purified Hbp protein. Human haemoglobin (3 μ*M*) (Sigma-Aldrich Corporation) or fivefold diluted human serum (ICN Biomedicals Inc., Costa Mesa, CA, USA) were incubated with 2.4 μ*M* of Hbp in PBS pH 6.5 and 10 m*M* $CaCl_2$ at 310 K for 4 h. After incubation, the samples were analyzed by Western blotting and photoimaging.

2.3. Crystallization

The protein (4 mg ml^{-1} in 10 m*M* Tris–HCl pH 8.0, 300 m*M* NaCl) was crystallized using 15–20% polyethylene glycol 6000, 0.1 *M* sodium acetate pH 4.6, 200 m*M* ammonium sulfate and 10%(*w*/*v*) glycerol. Crystals were grown using the hanging-drop method at 289 K, with 4 μl drops and 1 ml of mother liquor in the well. X-ray data were collected to 2.5 Å resolution using synchrotron radiation (BL40-B2, SPring-8, Hyogo-ken, Japan) and a Rigaku R-AXIS image-plate detector. The data were processed with *MOSFLM* and scaled with *SCALA* (Collaborative Computational Project, Number 4, 1994).

3. Results and discussion

3.1. Molecular weight of purified Hbp

The cleavage sites of the translocator unit (TU; Stathopoulos *et al.*, 1999) and the leader peptide of Hbp have been determined (Otto *et al.*, 1998), giving an expected molecular weight of 111 875.23 Da for mature Hbp. Mass-spectrometry analysis revealed the molecular weight to be 111 880.23 Da.

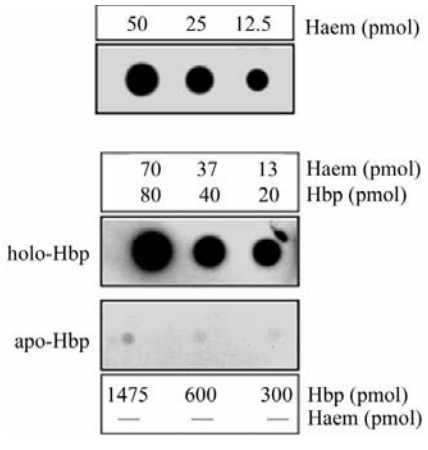

Figure 1
Haem-binding properties of purified Hbp. Holo-Hbp was obtained by incubating apo-Hbp anaerobically with haematin in PBS buffer. The upper panel represents a serial dilution of a stock solution of haemin. The lower panels show the amounts of haem bound by Hbp. The amounts of Hbp and haem present in a spot are expressed in picomoles.

3.2. Haem-binding properties and proteolytic activity of Hbp

The Hbp protein was purified exclusively in its apo form. The holo form of Hbp was obtained by incubating apo-Hbp with haematin under anaerobic conditions in PBS buffer. It appears that under these conditions Hbp binds haem in a 1:1 molar ratio (Fig. 1). Denaturing Hbp or the presence of high-salt buffers completely eliminated the haem-binding properties of the protein, indicating that a specific conformation of Hbp is needed for the binding of haem (data not shown). A negative effect on the binding of haem by Hbp was also found when the samples were incubated under aerobic conditions; only 30–50% of the Hbp was then found in its holo form (data not shown). No known haem-binding motif is apparent in the primary sequence of Hbp. Recently, another haemoglobin protease named THAP has been isolated from the hard tick *Boophilus microplus* and analyzed (Sorgine *et al.*, 2000). This aspartic proteinase also binds haem with a 1:1 stoichiometry and lacks a haem-binding motif. The THAP haem-binding site functions as a docking site that recognizes haem bound to haem proteins. Thus neither Hbp nor THAP has a classical haem pocket and it is possible that neither interacts with the Fe atom. Sorgine *et al.* (2000) proposed that the interaction of THAP with haem in haemoglobin occurs through the propionate radicals of protoporphyrin IX.

The purified Hbp was tested for endopeptidase activity and showed a haemoglobin protease activity of 1400 pmol h^{-1} per milligram of Hbp. Omission of $CaCl_2$ from the reaction mixture gave a lower activity of 1150 pmol h^{-1} per milligram of Hbp, suggesting that Hbp is a calcium-dependent protease. Previously, the activity was found to be 300 pmol h^{-1} per milligram of Hbp (Otto *et al.*, 1998). In those experiments, Hbp was purified by means of ion-exchange chromatography. The purification method seems to have reduced the endopeptidase activity of Hbp in some way, possibly by removing bound calcium. The observed haemoglobin protease activity in the present study is comparable to the activity of a haemoglobin protease of *Plasmodium falciparum*. In this parasite, an endopeptidase with an activity of 2400 pmol h^{-1} per milligram of enzyme has been characterized (Goldberg *et al.*, 1991). The haemoglobin protease activity of Hbp was also tested in human serum. This substrate contains 60 n*M* haemoglobin and 2.5 m*M* calcium. Hbp very efficiently degraded the haemoglobin with or without the addition of $CaCl_2$ (data not shown).

Table 1
Data-collection statistics.

	All data	Outer shell
Resolution range (Å)	15–2.5	2.64–2.5
No. reflections measured	189529	26782
No. unique reflections	58236	7860
Multiplicity	3.6	3.6
Completeness (%)	98.3	98.3
$R_{\rm merge}$ (%)	6.8	40.7
Mean $I/\sigma(I)$	12.0	2.7

3.3. Crystallographic characterization

Apo-Hbp crystals grew as hexagonal prisms over a period of about two weeks and reached a maximum size of about 0.3 × 0.3 × 0.8 mm, although they were generally rather smaller. The unit-cell parameters are $a = b = 114.6$, $c = 434.3$ Å and the space group is $P6_n22$, n currently being undetermined. The crystals were readily cryocooled to 100 K by washing them briefly in a mother liquor identical to that from which they grew but having a higher concentration of glycerol (20%). Given the long c axis, data collection to the diffraction limit was difficult and required mounting a crystal with this axis along the spindle. Data were collected from a single crystal in two sweeps, the first collecting the higher resolution spots to 2.5 Å using 0.6° oscillations and the

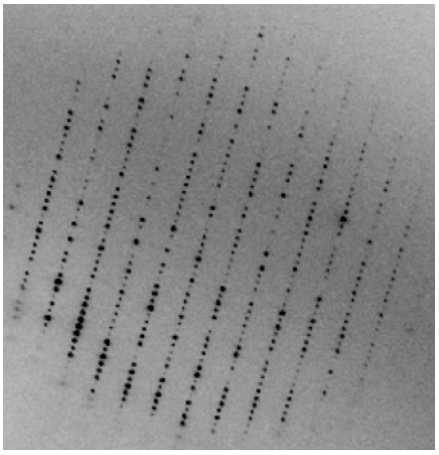

Figure 2
A detail of a diffraction image of Hbp. The upper right corner of this image corresponds to a resolution of 2.8 Å. The closeness of the spots caused by the long c axis is apparent. Incident radiation of 1.0 Å wavelength was used.

second a low-resolution pass using 1.2° oscillations (Fig. 2). Data were collected at beamline BL40B2 at the SPring-8 synchrotron source in Japan. The final data set (processed in space group *P*622) has an $R_{\rm merge}$ of 9.2% with a multiplicity of 3.6 (Table 1). Heavy-atom compounds are currently being screened to find useful derivatives.

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