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Crystal structure of the transport unit of the autotransporter *adhesin involved in diffuse adherence* from *Escherichia coli*

Iris Gawarzewski [a,c], Frank DiMaio [b], Elisa Winterer [c], Britta Tschapek [a], Sander H.J. Smits [a], Joachim Jose [c], Lutz Schmitt [a,*]

[a] *Institute of Biochemistry, Heinrich-Heine University Düsseldorf, 40225 Düsseldorf, Germany*
[b] *Department of Biochemistry and HHMI, University of Washington, Seattle, WA 98195, USA*
[c] *Institute of Pharmaceutical and Medicinal Chemistry, PharmaCampus, Westphalian Wilhelms-University Münster, 48149 Münster, Germany*

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ABSTRACT

Several serious gastrointestinal diseases, which are widespread all over the world, are caused by enteropathogenic *Escherichia coli*. The monomeric autotransporter AIDA-I (*adhesin involved in diffuse adherence*) represents an important virulence factor of these strains and is involved in adhesion, biofilm formation, aggregation and invasion into host cells. Here, we present the crystal structure of the transport unit of AIDA-I at 3.0 Å resolution, which forms a 12-stranded β-barrel harboring the linker domain in its pore. Mutagenesis studies of the C-terminal amino acid demonstrated the great impact of this terminal residue on membrane integration of AIDA-I and passenger translocation.

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1. Introduction

Many diarrheagenic diseases, especially among livestock and infants in third world countries, are caused by Gram-negative enteropathogenic *Escherichia coli* (EPEC) strains (Nataro and Kaper, 1998; Ngeleka et al., 2003). The *adhesin involved in diffuse adherence* (AIDA-I) glycoprotein, which was initially discovered in a patient suffering from infantile diarrhea (Benz and Schmidt, 1989), is an important virulence factor of EPEC strains that mediates auto-aggregation, biofilm formation, adhesion and invasion into host cells (Charbonneau et al., 2006). AIDA-I was assigned to the group of monomeric autotransporters (AT), which belong to the type V secretion systems (T5SS) (Gawarzewski et al., 2013a; Leo et al., 2012). Like all monomeric AT, preAIDA-I consists of different functional domains (Supplementary Fig. 1A): (I) an N-terminal signal peptide for Sec-dependent transport across the inner membrane (IM), (II) a passenger (α-) domain harboring biological activity in the extracellular space, (III) a linker domain and (IV) a β_2-domain, which is predicted to form a β-barrel in the outer membrane (OM) (Henderson et al., 2004; Jose, 2006; Jose et al., 1995; Leyton et al., 2012; Maurer et al., 1997). Additionally, AIDA-I contains an autochaperone domain termed β_1-domain, which is located at the C-terminus of the α-domain (Supplementary Fig. 1A) (Benz and Schmidt, 2011; Konieczny et al., 2001; Maurer et al., 1997). PreAIDA-I is translated as a 132 kDa protein comprising 1286 amino acids (Benz and Schmidt, 1992). After Sec-dependent transport across the IM, the signal peptide (amino acids 1–49) is cleaved off and proAIDA-I is released into the periplasm. The β_2-domain of proAIDA-I is integrated into the OM, while the α-domain is translocated. Finally, proAIDA-I is auto-proteolytic cleaved at the cell surface to form (I) the 100 kDa mature AIDA-I and (II) the 47.5 kDa $AIDA_C$ (Supplementary Fig. 1A) (Benz and Schmidt, 1992; Suhr et al., 1996). Mature AIDA-I, which remains strongly associated with $AIDA_C$ by an unknown mechanism (Suhr et al., 1996), mediates adhesion, biofilm formation, aggregation and invasion into host cells (Charbonneau and Mourez, 2007; Charbonneau et al., 2006). Despite the huge variety of α-domains

* Corresponding author. Address: Institute of Biochemistry, Heinrich-Heine University Düsseldorf, Universitätsstrasse 1, 40225 Düsseldorf, Germany. Fax: +49 211 81 15310.
E-mail address: lutz.schmitt@hhu.de (L. Schmitt).

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among monomeric AT, the transport unit displays a high structural similarity as observed by the structures of for example NalP, EspP, EstA, BrkA (Barnard et al., 2007; Oomen et al., 2004; van den Berg, 2010; Zhai et al., 2011) and Hbp (Tajima et al., 2010). Based on crosslinking experiments, mutational analysis and protease accessibility assays with the monomeric AT EspP, a secretion model was proposed (Ieva et al., 2008, 2011; Pavlova et al., 2013), which is a combination of the hairpin and the BamA model (Henderson et al., 2004; Voulhoux et al., 2003). This model suggests that the AT adopts a stable conformation in the periplasm by interaction with chaperones forming a partly folded β-barrel with a hairpin-like linker domain in the pore. BamA, also termed Omp85, recognizes a C-terminal signature sequence within the β-barrel and subsequently integrates this incompletely folded β-barrel into the OM. This supports the translocation of the α-domain to the cell surface. Most members of monomeric AT, which are assigned to the subgroup 'a' of T5SS, are involved in virulence processes. Thus, the underlying mechanisms of processing as well as the translocation across the outer membrane are important aspects of pathogenesis. Here, we present the crystal structure of the transport unit AIDA-I-linker-β_2 at 3.0 Å. Structural analysis revealed a 12-stranded β-barrel with the linker domain accommodated in its hydrophilic pore. Mutagenesis studies demonstrated the impact of the C-terminal amino acid as part of the signature sequence on membrane integration.

2. Experimental procedures

2.1. Cloning, expression and purification of AIDA-I-linker-β_2

The sequence of the linker and β_2-domain of AIDA-I encoded by pET-SH3 (Jose and Handel, 2003) was cloned into the plasmid pJM007 for constitutive expression (Maurer et al., 1997). A his_6-tag was introduced N-terminal to the α-domain, which carried an epitope (PEYFK) for immunodetection (Gawarzewski et al., 2013b). The expression of the encoded fusion protein FP-HisN163 carrying the transport unit termed AIDA-I-linker-β_2 (MW: 50.9 kDa, Supplementary Fig. 1B) from the resulting plasmid pIG501 was performed in *E. coli* UT5600 (DE3) at 30 °C and 170 rpm for 18 h in shaking flasks. The sequence of the cloned β_2-domain carried four amino acid exchanges (K1008R, I1013R, R1210E, R1212S, Supplementary Fig. 2) when compared to the reference sequence (Uniprot accession No. Q03155). In order to verify that they have no influence on transport efficacy, we used site-directed mutagenesis and replaced the mutated amino acids by the "original" ones as given by the Uniprot accession No. Q03155. As can be seen in Supplementary Fig. 2 this had not influence on the transport efficacy of the PEYFK epitope, which had however no effect on outer membrane integration or α-domain translocation, as judged by our mutational analysis (Supplementary Fig. 3). Solubilization with n-dodecyl-N,N-dimethylamine-N-oxide (LDAO) and purification were conducted as described (Gawarzewski et al., 2013b).

2.2. Crystallization and X-ray analysis

Purified FP-HisN163 with a final concentration between 7 and 28 mg/ml was mixed with an equal volume of reservoir solution (0.1 M sodium cacodylate, pH 6.5, 27.5% PEG 2000 MME) and incubated at 18 °C in hanging-drop vapor diffusion plates (Qiagen). Crystal harvesting, data collection and analysis was performed as described (Gawarzewski et al., 2013b). Data collection and refinement statistics are summarized in Table 1.

2.3. Structure determination and refinement

The initial molecular replacement solution was obtained using a homologous autotransporter structure (EspP(N1023S); PDB code: 3SLT) with 15% sequence identity over 252 residues in the aligned region as template. The initial hit was fairly strong, with a phaser TFZ score of 7.5, but autobuilding was unable to improve this initial solution (McCoy et al., 2007). The model was subsequently refined in MR-Rosetta (DiMaio et al., 2011). Starting from the initial alignment to 3SLT, 1000 homology models were built, constrained by density from the initial molecular replacement hit. Each of these models was then evaluated against the reciprocal space data (using Phaser's MR_RNP mode). The best model according to this measure was used to rephase the data, and chain tracing was performed with phenix.autobuild (Terwilliger et al., 2008). Starting from the MR-Rosetta model, phenix.autobuild built and placed 294 residues, with an R_{work}/R_{free} of 0.32/0.39. The subsequent map was readily interpretable. Manual refinement with COOT (Emsley et al., 2010) and REFMAC5 (Murshudov et al., 1997, 2011) resulted in a final resolution of 3.0 Å with a crystallographic R_{work}/R_{free} of 24.8%/30.6% (Table 1). The structure of AIDA-I-linker-β_2 has been deposited in the Protein Data Bank (Berman et al., 2000) under accession code: 4MEE (www.rcsb.org/pdb).

Table 1
Data collection statistics. Values in parentheses correspond to the highest resolution shell. $R_{merge} = \Sigma_{hkl} \Sigma_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl} \sum_i I(hkl)$, where $I_i(hkl)$ is the ith observation of reflection hkl and $\langle I(hkl)\rangle$ is the weighted average intensity for all observations i of reflection hkl.

Parameter	Value
Cell dimensions	
Space group	$P2_12_12_1$
a, b, c (Å)	40.33, 85.83, 134.05
$\alpha = \beta = \gamma$ (°)	90
Data collection	
Wavelength (Å)	0.8726
Resolution range (Å)	50.0–3.0
R_{merge} (%)	9.7 (61.9)
$I/\sigma(I)$	13.61 (2.39)
Completeness (%)	92.6 (94.4)
Redundancy	4.65 (4.73)
Matthews coefficient (Å^3/Da)	2.32
Overall B factor (Å^2)	48.29
Refinement	
Resolution (Å)	39.67–3.0 (3.1–3.0)
No. of reflections	36648
R_{work}/R_{free} (%)	24.8/30.6
No. of atoms	
Protein	2234
B-factor (Å^2)	61.81
RMSD	
Bond lengths (Å)	0.015
Bond angles (°)	1.65
Residues in Ramachandran plot (%)	
Most favored	96.61
Additional allowed	3.39
Disallowed	0

2.4. Mutagenesis of the C-terminal phenylalanine and labeling for fluorescence-activated cell sorting (FACS)

Based on the autotransporter sequence encoded on plasmid pET-SH3 (Jose and Handel, 2003), five different autotransporter mutants were generated using the Quick Change Site-Directed Mutagenesis Kit as described in the manufactures' manual (Stratagene). Customized primer pairs (Sigma Aldrich) for every point mutation in the DNA-sequence were applied resulting in an exchange or the deletion of the C-terminal phenylalanine. The resulting plasmids pEW006 (F1286W), pEW003 (F1286Y), pEW004 (F1286H), pEW008 (F1286V) and pEW005 (F1286Δ) were transferred in *E. coli* UT5600 (DE3) for protein expression. For FACS measurements, cells carrying a plasmid for the wild type or a

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