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The solution structure of the invasive tip complex from Afa/Dr fibrils

Ernesto Cota¹, Celine Jones², Peter Simpson¹, Harri Altroff², Kirstine L. Anderson¹, Laurence du Merle³, Julie Guignot⁴, Alain Servin⁴, Chantal Le Bouguénec³, Helen Mardon², and Stephen Matthews¹

¹Division of Molecular Biosciences, Biochemistry Building, Imperial College London, South Kensington, London SW7 2AZ, UK

²Nuffield Dept. Obstetrics and Gynaecology, Division of Medical Sciences, University of Oxford Women's Centre, Level 3, John Radcliffe Hospital, Headington, Oxford, OX3 9DU

³Unite de Pathogénie Bactérienne des Muqueuses, Institut Pasteur, 28 rue du Docteur Roux, 75724 Paris CEDEX 15, France

⁴Institut National de la Sante et de la Recherche Medicale, Unite 510, Faculte de Pharmacie Paris XI, Chatenay-Malabry, France

Summary

Afa/Dr family of adhesins are produced by pathogenic *E. coli* strains that are especially prevalent in chronic diarrheal and recurrent urinary tract infections. Most notably, they are found in up to 50% of cystitis cases in children and 30% of pyelonephritis in pregnant women. Afa/Dr adhesins are capped surface fibrils that mediate recognition of the host and subsequent bacterial internalisation. Using the newly solved three-dimensional structure of the minimal invasive complex (AfaDE) combined with biochemical and cellular assays, we reveal the architecture of the fibrillar cap and identify a novel mode of synergistic integrin recognition.

Introduction

Diffusely-adherent *E. coli* (DAEC) strains are present in a large proportion of patients with recurrent urinary tract infections and diarrhea. These infections often manifest as cystitis and pyelonephritis, and are especially prevalent in pregnant women and young children. Among all *E. coli* pathogens, DAEC serotypes can be distinguished by their diffuse pattern of adherence to epithelial Hep-2 and HeLa cells *in vitro* (Servin, 2005)

An important feature of DAEC strains is the presence of the Afa/Dr group of adhesins. Afa/Dr adhesins display an afimbrial/fimbrial morphology and are exported to the bacterial surface by the chaperone-usher pathway, a widespread system among gram-negative pathogens for the secretion of fimbrial proteins (Sauer *et al.*, 2000). These adhesins are the main virulence determinants in DAEC and account for the recognition of different host cell receptors. Prominent examples are the AfaE adhesin and the AfaD invasin of the plasmid-borne *afa* operons (Le Bouguenec *et al.*, 2001). AfaE mediates the primary adhesion event via binding to GPI-anchored molecules, the decay accelerating factor (DAF or CD55) (Nowicki *et al.*, 1990) and some carcinoembryonic antigen-related molecules (CEACAMs) (Berger *et al.*, 2004; Guignot *et al.*, 2000). β_1 integrins are also recruited by the AfaD invasin around Afa/Dr adherent bacteria, an essential interaction for the subsequent internalisation of bacteria and the maintenance of chronic infections (Guignot *et al.*, 2001; Plancon *et al.*, 2003).

Understanding how innate immunity responses are triggered by the interaction of DAEC with epithelial cells requires a detailed description of the fimbrial architecture and the interplay between fimbrial adhesins and host cell receptors. Our recent structural work revealed that AfaE acts as a pilin domain that assembles into a flexible fibre via the chaperone-usher pathway (Anderson *et al.*, 2004a). An absent β -strand is initially provided by the chaperone in a process termed donor strand complementation (DSC) (Sauer *et al.*, 2000). Subunits destined to join the lengthening fibre possess a free N-terminal strand that takes over the role previously performed by the chaperone (donor strand exchange - DSE). It has been proposed that the AfaD protein caps the AfaE fibrils, where it could efficiently perform its role as an invasin, however, the architecture of the polymer tip and its interaction with integrins is still poorly understood (Anderson *et al.*, 2004a). To address this, we have determined the high resolution structure of an active tip complex between AfaD and AfaE, and characterised a novel synergistic interaction with $\alpha 5\beta 1$ and $\alpha v\beta 3$ integrins.

Results

Production and functional characterization of AfaDE-dsc

We have previously described how the construction of a monomeric, self-complemented form of the AfaE-III adhesin from the *afa-3* operon (AfaE-*dsc*) facilitated the characterisation of its structural and functional properties (Anderson *et al.*, 2004a; Anderson *et al.*, 2004b). In a similar manner, to investigate the optimal invasive region of the Afa/Dr adhesins, we constructed donor strand self-complemented versions of AfaD-III from the *afa-3* gene cluster (Garcia *et al.*, 1994; Le Bouguenec *et al.*, 1993) (AfaD-*dsc*) and a complex between AfaD-III and AfaE-III (AfaDE-*dsc*). The AfaD invasin does not possess an N-terminal donor strand, which defines its role as a fimbrial capping domain (Anderson *et al.*, 2004a; Cota *et al.*, 2004). Construction of AfaD-*dsc* involved the extension of its C-terminal sequence with the N-terminal donor strand from AfaE (first 16 residues of the mature protein) via a 4-residue linker, DNKQ (Anderson *et al.*, 2004a; Anderson *et al.*, 2004b; Cota *et al.*, 2004). Similarly, a construct of the AfaDE-*dsc* complex was created by fusion of the intact AfaD-*dsc* and AfaE-*dsc* coding sequences (Fig. 3A and 3B).

We then tested the ability of these constructs to promote host-cell entry. Carboxylated polystyrene beads were covalently coupled to AfaDE-*dsc*, AfaD-*dsc* or AfaE-*dsc*. AfaE-*dsc* beads, like the bacteria producing only AfaE-III, adhered to the HeLa cells but were never observed internalized (Anderson *et al.*, 2004a). The AfaD-*dsc* beads exhibited a limited interaction with cells and no significant internalization was observed (Data not shown). In contrast, beads coated with AfaDE-*dsc* were observed both interacting with the cell surface and internalized into the HeLa cells (Fig. 1A). The $\beta 1$ subunit of integrins has been shown to be recruited around adherent bacterial harboring *afa* operons (Guignot *et al.*, 2001; Plancon *et al.*, 2003). Immunofluorescence labeling and confocal microscopy analysis was therefore used to determine whether $\beta 1$ integrin is recruited around cell-associated AfaDE-coated beads. HeLa cells incubated with AfaDE-coated beads exhibit a clear clustering of $\beta 1$ integrin molecules beneath cell-associated beads, which can be seen as forming rings delineating their circumference (Fig. 1B). Clustering of an unrelated control receptor was not observed confirming the specificity of the integrin interaction. Taken together these data (Fig. 1) confirm that only AfaDE-*dsc* coated beads displayed a host cell invasion pattern similar to Afa/Dr-associated bacteria, indicating that it provides all the necessary molecular interactions for attachment and invasion.

The solution structure of AfaDE-dsc

We also determined the high resolution three dimensional structures of AfaD-*dsc*, and AfaDE-*dsc*, by NMR (Figs. 2-4). Using a final combination of manual and automated

assignment methods, NOESY data were comprehensively analyzed for AfaD-*dsc* and final family of solution structures was calculated. NMR spectra of AfaDE-*dsc* are very similar to of its free component subunits indicating that the domains structures are unchanged; specifically, the majority of the chemical shift differences between free AfaD/AfaE and AfaDE-*dsc* are limited to the domain-domain interface (Fig. 2). Based on refined structures of AfaD-*dsc* and AfaE-*dsc* together with 150 residual dipolar coupling measurements recording on AfaDE-*dsc*, a high resolution structure for an AfaDE-*dsc* complex was finally derived, revealing a well-defined, extended domain arrangement (Figs. 3, 4 and Table 1). The final structure of the AfaD domain exhibits an Immunoglobulin-like topology (Figs 3 and 4), in which the two β -sheets pack against each other in a similar fashion to the AfaE subunit. Furthermore, AfaD superimposes with an RMSD of 2.6 Å over 115 equivalent backbone C β atoms of the related invasin DraD. Subtle differences occur in loops D-D' and C-C', and in the donor strand position which is replaced by a non-native histidine tag in the DraD crystal structure (Jedrzejczak *et al.*, 2006). An effective isotropic overall correlation time of ~16 ns for AfaDE-*dsc*, (~8 ns AfaD-*dsc* and AfaE-*dsc* alone) was estimated from T $_1$ and T $_2$ ^{15}N relaxation data (data not shown). Further analysis, using the program TENSOR, revealed that an axially symmetric diffusion tensor with $D_{\parallel}/D_{\perp}=1.95$ yielded the best description of the data, confirming that in solution the average AfaDE-*dsc* structure approximates a cigar shape. The buried surface area at the AfaDE domain interface is $325 \pm 80 \text{ Å}^2$, which is comparable to the 330 Å^2 buried by the FnIII9-10 interface of fibronectin (Leahy *et al.*, 1996). Some interdomain flexibility at the 9/10 interface is important in maximizing the integrin interactions from the two domains and our structures suggests that integrin recognition by AfaDE may be similar in this respect.

AfaD exhibits an Immunoglobulin-like topology, in which the two β -sheets pack against each other in an analogous fashion to AfaE and archetypal pilin domains; superimposition over equivalent residues gives RMSDs ~2.5 Å. The structure also illustrates that the free N-terminus in AfaE contributes a complementing strand to the fold of AfaD and provides high resolution detail for the incorporation of AfaD into the AfaE polymer. The conformation of the incoming strand reveals an identical strand-strand 'register' in both domains, highlighted by a conserved donor strand cleft that accommodates the side-chains of C-terminal Thr and Leu residues (TTKLLTVT) (Fig. 5). Interestingly, this pocket is open to solvent in AfaD but closed in AfaE, which correlates with the weaker interaction between the donor strand and AfaD, and its ability to detach itself from the bacterial fibrils in culture (Gounon *et al.*, 2000; Jouve *et al.*, 1997). Furthermore, AfaD does not possess a free N-terminal extension which defines its role as the initiator of fimbrial assembly and therefore the cap of the fibrillar structure.

AfaDE-*dsc* displays synergistic binding to $\alpha_5\beta_1$ and $\alpha_v\beta_3$ integrins

The integrin binding capacities of AfaD-*dsc*, AfaE-*dsc* and AfaDE-*dsc* were subsequently compared using a series of ELISA-based binding assays. Figure 6 illustrates data revealing direct interactions between AfaDE-*dsc* and two integrin families present on both epithelial cells and enterocytes, $\alpha_5\beta_1$ and $\alpha_v\beta_3$, which have also been identified as targets for other pathogens (Boyle and Finlay, 2003; Zarate *et al.*, 2004). The interaction between AfaDE-*dsc* and integrin has a $K_d \sim 700 \text{ nM}$ (Table 2). This affinity is significantly lower than that measured for surface proteins from truly invasive bacteria: the invasin from *Yersinia pseudotuberculosis* binds the $\alpha_5\beta_1$ integrin with a $K_d = 5 \text{ nM}$ (Van Nhieu and Isberg, 1991). The comparatively low affinity of AfaDE-*dsc* for integrins correlates with the low levels of invasion observed for Afa/Dr bacteria and their subsequent ability to persist within a host for long periods of time. The eventual release of bacteria into the lumen or urine provides a mechanism for establishing chronic/recurrent intestinal or urinary tract infections.

Surprisingly, a markedly weaker affinity for integrins (>10 fold higher K_d) is observed for AfaD in isolation, where as AfaE alone provides no measurable interaction (Fig. 6). To test whether the increased integrin-binding affinity for AfaDE-*dsc* was a consequence of a second site interaction or enhanced AfaD domain stabilization, we performed equilibrium denaturation experiments (Fig. 7). While the AfaD monomer is significantly less stable than AfaE, neither domain is substantially stabilized upon formation of the complex, implying that the increased binding affinity is most likely due to a weak second site interaction from AfaE and/or the AfaDE interface.

Discussion

Cell entry by Afa-associated *E. coli* strains is primarily mediated by the recruitment of integrins (Guignot *et al.*, 2001; Plançon *et al.*, 2003). In this process, the AfaD invasin is directly involved in the recognition of the integrin $\beta 1$ chain (Plançon *et al.*, 2003). Integrins are a family of cell surface receptors responsible for interactions between the cell surfaces and the extracellular matrix as well as several important cell-cell adhesion events (Hynes, 1992). The binding properties and the arrangement of domains in the AfaDE complex are reminiscent of the synergistic, 'two-site' mode of integrin $\alpha 5 \beta 1$ binding by fibronectin and invasin from *Y. pseudotuberculosis* (Leong *et al.*, 1995). Cell attachment by fibronectin occurs primarily via an RGD-loop motif from FIII10, while maximal binding and subsequent integrin activation is provided by the synergy region from the adjacent FnIII9 (Copie *et al.*, 1998; Takagi *et al.*, 2003). However, the exact nature of the contribution of FIII9 to maximal $\alpha 5 \beta 1$ activity and signaling is not clear (Altroff *et al.*, 2004). In the invasin from *Y. pseudotuberculosis* two separate motifs from adjacent domains are critical for efficient integrin binding and invasion (Hamburger *et al.*, 1999; Leong *et al.*, 1995).

In addition to the RGD motif, integrin ligands can bind via RGD-like sequences (reviewed in (Ruoslahti, 1996)). A phage-display peptide library used to identify RGD mimetics has found variants with comparable affinity to the canonical RGD, notably those containing the DGR and NGR tripeptides (Koivunen *et al.*, 1993). Initial inspection of the sequence alignment for the AfaD family of invasins suggested a similar binding mechanism might exist, as they contain such tripeptides in five different loop regions. In particular, the invasin used in this study, AfaD-III contains prominent DGR tripeptides in loops between strands C1-C2 and C2-D (residues 46-48 and 58-60, respectively), and an RDG sequence between residues 17-19. We have shown that non-conservative substitutions in these motifs have no significant effect on integrin binding (Manuscript in preparation). These results suggest that AfaDE binding to integrins is mediated by a non-sequential motif, such as the one previously described for the *Y. pseudotuberculosis* invasin (Hamburger *et al.*, 1999; Leong *et al.*, 1995), in which two aspartic acids separated by 100 amino acids are critical for binding. Additional mutagenesis and NMR-based experiments are in progress to define the exact nature of this interaction.

Alternative mechanisms of cell internalisation by Afa/Dr strains have also been described. Garcia *et al.* (2000) have shown that strains bearing the *afa-3* operon can trigger cell invasion at reduced levels in the absence of the AfaD invasin. Similarly, it has been shown that binding of the Dr adhesin (97% identity with the AfaE-III adhesin) is able to promote cell invasion via the short consensus repeat 3 domain of the GPI-anchored decay accelerating factor, DAF (Selvarangan *et al.*, 2000). The affinity of this interaction has been characterised and falls within the micromolar range (16 μ M) (Anderson *et al.*, 2004a). Details regarding the specificity of each mechanism to different cell types and their relative contribution to the invasion process remain to be elucidated.

The ability to form a stable fimbriae and the large ΔG_{D-N} of monomeric AfaE (AfaE-dsc) show that residues involved in the donor strand 'cleft' provide the optimal interactions for efficient polymerisation. This tight interaction is in contrast with the weaker binding of the donor strand by AfaD, as shown by the limited number of contacts and a more exposed cleft. The plasticity of this region is further highlighted by the structure of a DraD in which the cleft accommodates the terminal histidine tag of an adjacent monomer in the crystal (Jedrzejczak *et al.*, 2006). In summary, the low levels of invasion, the relatively low binding K_d of AfaE and AfaD to receptors, the reduced stability of the AfaD monomer and its release from the bacterial fibril provide a molecular basis for the chronic and recurrent nature of infections caused by this pathogen.

Experimental procedures

Cloning, Expression and Purification of AfaE-dsc, AfaD-dsc and AfaDE-dsc

The ability to self complement AfaD and AfaE without altering their structure provides us with the opportunity of synthesising the multisubunit complexes as single polypeptide chains (Anderson *et al.*, 2004b; Cota *et al.*, 2004). This can be accomplished by extending the mature sequence of AfaD-III (122aa residues) (Garcia *et al.*, 1994; Le Bouguenec *et al.*, 1993) with a full-length, self-complemented AfaE creating the AfaDE-dsc (285aa residues, Fig. 3A). The constructs, AfaE-dsc, AfaD-dsc and AfaDE-dsc were expressed cytoplasmically using the pRSETA plasmid (Promega) in the BL21(DE3) *E. coli* strain (Novagen). ^{15}N , ^{13}C double-labelled samples of AfaE-dsc and AfaD-dsc were produced in minimal media, containing 0.07% $^{15}\text{NH}_4\text{Cl}$ and 0.2% ^{13}C -glucose, supplemented with 50 $\mu\text{g/ml}$ ampicillin. A ^2H , ^{15}N , ^{13}C triple-labelled sample of AfaDE-dsc was produced from Silantes media *E. coli* CDN OD1. All samples were purified in denaturing conditions (50 mM sodium phosphate buffer, pH 8.0, 0.3 M NaCl and 8 M urea) using the binding of the N-terminal hexahistidine tag to Nickel-bound agarose beads. Purified proteins were refolded by dialysis into 50 mM sodium phosphate buffer, pH 7.0 and concentrated to approximately 0.5 mM for NMR.

NMR Spectroscopy and Structure Calculation

Backbone and side-chain assignments on individual domains were completed using standard double and triple-resonance assignment methodology (Sattler *et al.*, 1999). H_α and H_β assignments were obtained using HBHA(CBCACO)NH (Sattler *et al.*, 1999). The side-chain assignments were completed using HCCH-total correlation (TOCSY) spectroscopy and (H)CC(CO)NH TOCSY (Sattler *et al.*, 1999). 3D ^1H - ^{15}N / ^{13}C NOESY-HSQC (mixing time 100 ms at 500 MHz and 800 MHz) experiments provided the distance restraints used in the final structure calculation. Heteronuclear ^1H - ^{15}N NOE data with minimal water saturation were acquired using the sequence described by Farrow *et al.* (Farrow *et al.*, 1994).

The ARIA protocol (Linge *et al.*, 2003) was used for completion of the NOE assignment and structure calculation for AfaD-dsc and AfaE-dsc. The frequency window tolerance for assigning NOEs was ± 0.04 ppm and ± 0.06 ppm for direct and indirect proton dimensions and ± 0.7 ppm for nitrogen and carbon dimensions. The ARIA parameters, p, Tv, and Nv, were set to default values. The 15 lowest energy structures had no NOE violations greater than 0.5 Å and dihedral angle violations greater than 5°. These domain structures were subsequently refined against a comprehensive set of amide residual dipolar couplings (RDCs) recorded on monomeric samples. The full structural statistics are presented in Table 1. Additionally, an adapted HADDOCK approach (Dominguez, et al., 2003) was used for structure calculation of the AfaDE complex. The RDC-refined structures of AfaD and AfaE monomer were semi-rigidly docked under the influence of distance restraints describing the intermolecular NOEs between domains and 150 RDCs recorded on the AfaDE complex

from two alignment media (70 and 80 from AfaD and AfaE, respectively). The best structures adjudged by the RDC energy term were selected for torsion angle dynamics and subsequent Cartesian dynamics in an explicit water solvent (see Table 1 and Fig. 4D). ^{15}N T_1 and ^{15}N T_2 were measured using methods described elsewhere (Farrow *et al.*, 1994). Relaxation delays of 50, 250, 400, 650, 950 and 1400 ms were employed for T_1 measurements, and delays of 16.6, 33.2, 49.8, 66.4, 83.0, 99.6, 149.4 and 199.2 ms for T_2 . Relaxation analysis and derivation of diffusion tensors was performed according to standard techniques using the program TENSOR (Dosset *et al.*, 2000).

Cell-invasion assay

The coating of beads with AfaE-*dsc* and immunofluorescence adherence assay were performed as described previously (Plancon *et al.*, 2003). Fluorescent crimson carboxylated microspheres (Molecular Probes, Interchim, Montluçon, France) were coated with proteins. HeLa cells were used after 18 h of culture. To analyse the entry of beads into cells after 3 h incubation period, cells were washed several times and fixed. Extracellular beads were then labelled by incubating unpermeabilized cells with rabbit anti-rAfaD-III antibodies (Garcia *et al.*, 1996). The particles were washed three times and then incubated with FITC-labelled anti-rabbit IgG antibodies.

Integrin-clustering assay

Purified proteins (2 mg/ml) were coated onto 1 μm polystyrene beads (Interfacial dynamics corporation, Interchim, France) according to the manufacturer's instruction. For clustering assay, HeLa cells were grown at 37°C in 5% CO_2 in Dulbecco's modified Eagle medium (DMEM)/HamF12 containing glutamax and supplemented with 5% foetal calf serum (FCS), 1% sodium pyruvate and 1% non essential amino-acids. HeLa cells were used for clustering assay after 18 hrs of culture. Beads were added to the cells (10^7 beads/mL) for 1 hour. The cells were washed with phosphate buffer saline (PBS) 6 times then fixed in 3% paraformaldehyde in PBS pH 7.4 for 15 min at room temperature then treated with 50 mM NH_4Cl for 10 min.

For immunostaining, coverslips were washed twice in PBS containing 0.1% saponin, incubated overnight at 4 °C with primary antibodies (rabbit polyclonal anti-Dr adhesin (gift from B. Nowicki) and mouse anti- $\beta 1$ integrin (clone 18, BD transduction laboratories) diluted in 10% horse serum, 0.1% saponin in PBS). Coverslips were then washed twice with 0.1% saponin in PBS and incubated for 1 hour with secondary antibodies (anti-rabbit TRITC and anti-mouse FITC, Jackson). Coverslips were washed twice in 0.1% saponin in PBS, once in PBS and once in H_2O , and mounted in Dakocytomation fluorescent mounting medium (Dako). Samples were analysed using a confocal laser scanning microscope (LSM510, Zeiss). Images were processed using Adobe photoshop 5.0.

Integrin-binding assays

Solid phase binding assays were performed as described previously with purified placental integrins $\alpha\text{v}\beta 3$ and $\alpha 5\beta 1$ (Altroff *et al.*, 2001; Altroff *et al.*, 2004). Human placentas, obtained with informed consent and in accordance with the requirements of the Oxford Research Ethics Committee. Assays were performed in triplicate, and background antibody binding in the absence of ligand was subtracted from the readings. Nonspecific binding of His-tag fusion proteins to uncoated wells containing BSA only was measured separately for each ligand concentration point and subsequently subtracted from the corresponding values for total binding. Dose-response data from the assays were analyzed by non-linear regression using a sigmoidal curve fit (Prism, GraphPad Software).

Equilibrium denaturation experiments

Fluorescence measurements (excitation at 278 nm and emission at 360 nm) were performed in 50 mM sodium phosphate buffer, pH 7.0, 50 mM NaCl and a range of guanidine thiocyanate (GuSCN) concentrations at 25°C. For all proteins, final protein concentration was 3 μ M. ΔG_{D-N} values were determined with the method described by Clarke and Fersht (Clarke and Fersht, 1993). For the analysis of fluorescence data, *denaturant activity* units, rather than molar concentration of GuSCN, have been used, as this method yields a constant *m*-value at all GuSCN concentrations and therefore an accurate determination of ΔG_{D-N} values in the absence of denaturant (Cota and Clarke, 2000; Pandya *et al.*, 1999).

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A **B** **Figure 1.**

A) Microscopic examination of AfaDE-coated beads with HeLa cells. Extracellular beads were labelled with rabbit anti-His6-AfaD-III and were visualized by the green fluorescence of FITC-labelled anti-rabbit IgG antibodies. Intracellular beads were visualized by the intrinsic red fluorescence of the beads. Some non-internalised beads appear yellow from the combination of FITC and intrinsic red fluorescence.

B) Detection of integrin recruitment beneath adherent AfaDE-coated beads (left and expansion right). β 1 integrin was labelled with mouse anti- β 1 integrin and visualized by the green fluorescence of FITC-labelled anti-mouse IgG antibodies. AfaDE-coated beads were visualized by red fluorescence using anti-Dr adhesin antibody. Beads were visualized by

intrinsic red fluorescence. Arrows indicate rings of clustered integrin molecules beneath adherent beads. A control experiment using antibodies against Transferrin which does not cluster is also shown.

Figure 2.

An assigned ^1H - ^{15}N HSQC NMR spectrum of the 32kDa AfaDE-*dsc* at pH 7 and 303 K (black). Spectra of AfaD-*dsc* (green) and AfaE-*dsc* (blue) are also shown for comparison. Key spectral differences are annotated and congregate at the AfaDE interface indicating that the domain structures are unchanged. Remaining assignments are available from the Biological Magnetic Resonance Bank under accession codes 5946 and 5947 (Anderson *et al.*, 2004b; Cota *et al.*, 2004).

A

B

Figure 3.

A) Amino acid sequence and localisation of β -strands (black bars) in AfaDE-dsc. The mature AfaD sequence is shown in light green and mature AfaE in light blue. The N-terminal donor strand from AfaE is shown in bold, dark blue and linker regions (DNKQ) in bold, dark red.

B) Topology diagram for the structure of AfaDE-dsc. β -strands are shown in light blue for AfaE and light green for AfaD with the exception of the self-complementing strand, which is light blue/white.

Figure 4.

A) C α traces representing the ensemble of NMR-derived structures for AfaDE-*dsc* (AfaD, green; AfaE, blue).

B) Ribbon representation of a representative structure for the AfaDE-*dsc* (AfaD, green; AfaE, blue).

C) Surface representation of a representative structure for the AfaDE-*dsc* (AfaD, green; AfaE, blue). Key interfacial residues are shown in red.

D) Plot of experimental residual dipolar couplings (RDCs) against calculated RDCs for the NMR-derived structure of AfaDE-*dsc*

A **B** **Figure 5.**

A) Donor strand binding cleft in AfaD. The body of the protein is represented using a surface map (AfaD green and complementary F and A strands in white). The AfaE donor strand is shown in wire representation with side chains of residues TTKLTVT as red sticks.

B) Donor strand binding cleft in AfaE. The body of the protein is represented using a surface map (AfaE blue and complementary F and A strands in white). The AfaE donor strand is shown in wire representation with side chains of residues TTKLTVT as red sticks.

A **B** **Figure 6.**

A) Binding experiments illustrating the interaction between $\alpha_5\beta_1$ integrin with FnIII9-10, AfaDE-*dsc*, AfaD-*dsc*, AfaE-*dsc*. A fit of these data is shown yielding estimates for the dissociation constants (Table 2). No binding could be observed for AfaE-*dsc*.

B) Binding experiments illustrating the interaction between $\alpha_v\beta_3$ integrin with FnIII9-10, AfaDE-*dsc*, AfaD-*dsc*. A fit of these data is shown yielding estimates for the dissociation constants (Table 2). No binding could be observed for AfaE-*dsc*.

Figure 7.

A) Plot of relative fluorescence intensity at different wavelengths for native and denatured forms of AfaD-*dsc*, AfaE-*dsc* AfaDE-*dsc*. Data were taken at the start of the transition region (at 0.286 denaturant activity units, DA, for AfaD-*dsc* and AfaDE-*dsc* and at 1.151 DA for AfaE-*dsc* and AfaDE-*dsc*) and at the end of the transition region (at 0.569 DA for AfaD and AfaDE and at 1.469 DA for AfaE-*dsc* and AfaDE-*dsc*). Open circles: native AfaD-*dsc*, closed circles: denatured AfaD-*dsc*. Open squares: native AfaDE-*dsc*, closed squares: denatured AfaDE-*dsc*. Open triangles native AfaE-*dsc*, closed triangles: denatured AfaE-*dsc*. Open diamonds: native AfaDE-*dsc*, closed triangles denatured AfaDE-*dsc*.

B) Fraction of unfolded AfaD-*dsc*, AfaE-*dsc* and AfaDE-*dsc* as a function of GuSCN concentration. Denaturant concentrations are expressed in *denaturant activity* units, DA (see *Equilibrium denaturation experiments* in *Materials and Methods*). For all proteins, ΔG_{D-N} (in kcal/mol) and $D_{50\%}$ (in DA units), respectively: Afa**DE**-*dsc*, -5.33 ± 1.53 and 0.45 ± 0.01 ; AfaDE**E**-*dsc*, -10.35 ± 1.12 and 1.52 ± 0.02 ; Afa**D**-*dsc*, -4.29 ± 1.63 and 0.39 ± 0.01 ; Afa**E**-*dsc*: -10.78 ± 0.36 and 1.46 ± 0.01 . The bold, underlined D or E letters indicate the respective data for AfaD and AfaE in the monomeric forms or in the AfaDE complex.

Table 1Structural statistics for AfaE-*dsc*, AfaD-*dsc* and AfaDE-*dsc*

<i>Construct and PDB code for deposited coordinates</i>	<i>AfaE-dsc (1RXL)</i>	<i>AfaD-dsc (2FVN)</i>	<i>AfaDE</i>	
<u>Number of experimental restraints</u>	4,258	4,010		
Total NOE-derived	4,099	3,839	After refinement of AfaE and AfaD monomer structures with 100 and 99 RDCs (recorded on monomers), respectively	
Ambiguous	1,542	1,423		
Unambiguous	2,557	2,416		
Intraresidue	864	1037		
Sequential	587	693	HADDOCK semi-rigid body docking under the influence interdomain NOEs and 150 RDCs (recorded on dimer)	
Medium-range ($ i - j = 4$)	194	167		
Long-range ($ i - j > 4$)	912	1054		
Talos (ϕ/ψ)	58	72		
<u>RMSD from experimental restraints</u>				
Distance (Å)	0.02 ± 0.003	0.03 ± 0.003	Distance (Å)	0.02 ± 0.001
Dihedral angle (deg.)	0.52 ± 0.1	1.1 ± 0.3	RDCs (Hz)	0.8 ± 0.1
<u>RMSD from idealized covalent geometry</u>				
Bonds (Å)	0.0045 ± 0.0001	0.0054 ± 0.0003	0.0045 ± 0.0001	
Angles (deg.)	0.59 ± 0.02	0.62 ± 0.04	0.59 ± 0.06	
<u>Energies (kcal mol⁻¹)</u>				
ENOE	84.8 ± 22.5	60.9 ± 20	2.5 ± 1	
Ebond	43.8 ± 2.5	63.2 ± 8	43.8 ± 2.5	
Eangle	205 ± 12.4	231 ± 34	205 ± 12.4	
Evdw	-1259 ± 20.3	-1130 ± 18	-1259 ± 20.3	
<u>Coordinate RMSD (Å)</u>				
Backbone atoms in secondary structure	0.49 ± 0.05	0.59 ± 0.1	1.07 ± 0.5	
Heavy atoms in secondary structure	0.91 ± 0.08	1.10 ± 0.1	1.91 ± 0.5	
<u>Ramachandran plot^a</u>				
Residues in most favoured regions (%)	75	60	72	
Residues in additionally allowed regions (%)	19	30	20	
Residues in generously allowed regions (%)	4	7	7	
Residues in disallowed regions (%)	2	3	3	

^aStructural quality was evaluated using PROCHECK_NMR (Laskowski *et al.*, 1996)

Table 2

Calculated dissociation constants for integrin-Afa/Dr adhesins interaction

Integrin ligand	Dissociation constants (μM)	
	$\alpha 5\beta 1$	$\alpha v\beta 3$
AfaE	No binding	No binding
AfaD	8.3 ± 2	7.6 ± 2
AfaDE	0.78 ± 0.3	0.72 ± 0.2
FnIII9-10	0.015 ± 0.003	0.13 ± 0.04