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The interaction between the 3rd type III domain from fibronectin and anastellin involves β -strand exchange

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Summary

Anastellin is a small recombinant fragment derived from the extracellular matrix protein fibronectin; it comprises the 1st type III (FN3) domain without the two N-terminal β -strands. It inhibits angiogenesis, tumor growth and metastasis in mouse models and requires endogenous fibronectin for its *in vivo* anti-angiogenic activity. It binds to fibronectin *in vitro* and converts the soluble protein to insoluble fibrils that structurally and functionally resemble fibronectin fibrils deposited in the extracellular matrix by cells. Anastellin binds to several FN3 domains in fibronectin, but how it interacts with these domains and why the interactions lead to aggregation of fibronectin is not well understood. In this work, we investigated the interaction between anastellin and the 3rd FN3 domain (3FN3) from fibronectin. We show that anastellin binds with high affinity to a peptide comprising the two N-terminal β -strands from 3FN3, and we present here the structure of the resulting complex. The peptide and anastellin form a composite FN3 domain, with the two N-terminal β -strands from 3FN3 bound in place of the two β -strands that are missing in anastellin. We also demonstrate using disulfide crosslinking that a similar interaction involving the two N-terminal β -strands of 3FN3 occurs when intact 3FN3 binds to anastellin. 3FN3 adopts a compact globular fold in solution, and to interact with anastellin in a manner consistent with our data, it has to open up and expose a β -strand edge that is not accessible in the context of the folded domain.

Introduction

Fibronectin is a modular extracellular matrix glycoprotein that is composed of multiple type I (FN1), type II (FN2) and type III (FN3) homologous repeats (reviewed in ^{1–4}). It plays a key role in embryonic development ⁵ and is involved in a number of other physiological as

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Supporting Information

Sequences of recombinant proteins used in this study (Table S1), list of NMR experiments acquired for the AB:anastellin complex (Table S2), 2D ¹H-¹⁵N HSQC spectra of the AB:anastellin complex with assignments (Figure S1), and comparison of the AB:anastellin complex with 1FN3 and 3FN3 (Figure S2).

well as pathological processes^{6–10}. It exists in two forms: as a soluble, disulfide-linked dimer, which is abundant in plasma, and as insoluble fibrils, which are an important component of the extracellular matrix. The process by which cells convert soluble fibronectin into insoluble fibrillar aggregates is poorly understood, and no high-resolution information is available about fibronectin conformation and interactions in the fibrils.

Anastellin is a recombinant protein that contains the C-terminal 75 residues from the 1st FN3 domain (1FN3) of human fibronectin; it is based on a proteolytic fragment originally identified by Morla and Ruoslahti¹¹. It inhibits angiogenesis, tumor growth and metastasis in mouse models¹² and requires endogenous fibronectin for its *in vivo* activity^{13, 14}. Addition of anastellin to soluble fibronectin results in formation of insoluble aggregates that are reminiscent of fibronectin fibrils deposited by cells¹⁵. Identification of the structural features that are responsible for the activity of anastellin may therefore enable development of new anti-angiogenic drugs as well as provide novel insight into the properties of fibronectin and the mechanism of fibronectin fibrillogenesis.

The binding sites for anastellin in fibronectin are located in the first three FN3 domains and in the 11th FN3 domain¹⁶. The structures of anastellin and each of these FN3 domains have been determined previously^{17–20} (and D. V. Rusnac, T. C. Mou, S. R. Sprang and K. Briknarová: PDB ID 5DFT) but it is not well understood how anastellin interacts with these domains and why the interactions lead to aggregation of fibronectin. We have now used limited proteolysis, NMR spectroscopy and disulfide crosslinking to investigate the interaction between anastellin and one of its target domains, the 3rd FN3 domain (3FN3) from fibronectin. We identified a peptide from 3FN3 that binds to anastellin with high affinity, and we present here the structure of the peptide:anastellin complex. In addition, we show that interactions similar to those observed in the peptide:anastellin complex also occur when intact 3FN3 binds to anastellin. To engage with anastellin in a manner consistent with our data, 3FN3 has to open up and expose a β -strand edge that is not accessible in the context of the folded domain.

Materials and Methods

Expression and purification of anastellin and 3FN3

M15[pREP4] *Escherichia coli* cells (Qiagen) that contained the pQE12 vector (Qiagen) for expression of anastellin (Table S1) were obtained from Dr. Erkki Ruoslahti (Sanford Burnham Prebys Medical Discovery Institute and University of California, Santa Barbara)^{15, 17}. Anastellin was isolated by affinity chromatography on Ni²⁺-nitrilotriacetic acid (NTA) resin (Qiagen) under denaturing conditions and subsequently refolded by dialysis against phosphate buffered saline (PBS), pH 7.5. It was then further purified by size-exclusion chromatography on Superdex 75 resin (GE Healthcare). 3FN3 (Table S1) was expressed and purified as described previously²⁰.

Limited proteolysis of 3FN3 and matrix-assisted laser desorption ionization time-of-flight (MALDI-ToF) mass spectrometry

3FN3 was digested with α -chymotrypsin (10 μ g α -chymotrypsin per mg 3FN3) for 2 hours at room temperature. After the reaction was stopped with phenylmethylsulfonyl fluoride (PMSF), the 3FN3 digest was combined with anastellin, and the mixture was separated by size-exclusion chromatography on Superdex 75 resin. α -chymotryptic fragments of 3FN3 that co-eluted with anastellin were identified using a Voyager-DE PRO MALDI-ToF Biospectrometry Workstation (Applied Biosystems).

Cloning, expression and purification of AB and CG and preparation of the AB:anastellin complex

The DNA sequences encoding AB and CG (Table S1) were amplified from the 3FN3 expression vector by PCR, and a tobacco etch virus (TEV) protease cleavage site was added to the N-terminus of each sequence in the process. Gateway recombination technology (Life Technologies) was then used to recombine the PCR products into the pDONR 201 vector and to subsequently move the AB and CG encoding sequences into the pDEST 15 and pDEST 17 vectors, respectively. The proteins were expressed in *E. coli* BL21-CodonPlus(DE3)-RIPL cells (Agilent).

The glutathione transferase (GST)-AB fusion protein was purified by affinity chromatography on glutathione-agarose resin. The GST moiety was cleaved off with the TEV protease, which was prepared as described previously²¹, and AB was separated from the majority of the GST by affinity chromatography on glutathione-agarose resin. For titration experiments, AB was further purified by size-exclusion chromatography on Superdex 75 resin. For structural studies of the AB:anastellin complex, AB was combined with anastellin, and unbound AB was removed by affinity chromatography on a HisTrap FF column (GE Healthcare). The complex was then further purified by size-exclusion chromatography on Superdex 75 resin.

The His-tagged CG protein was isolated by affinity chromatography on Ni²⁺-NTA resin under denaturing conditions and subsequently refolded by dialysis against PBS (pH 7.5). The N-terminal His tag was cleaved off with the TEV protease, and CG was separated from the tag and the TEV protease by affinity chromatography on a HisTrap FF column. CG was then further purified by size-exclusion chromatography on Superdex 75 resin.

NMR spectroscopy

All NMR data were acquired at 25°C on a Varian 600 MHz NMR System that was equipped with a triple resonance probe or a ¹³C-enhanced salt-tolerant cold probe. The data were processed with NMRPipe²² and analyzed with CcpNmr Analysis version 2.2.2²³.

Protein samples for NMR spectroscopy were in PBS (pH 7.5) supplemented with 10% ²H₂O. The samples for structural studies of the AB:anastellin complex contained 0.6 mM uniformly ¹⁵N-labeled AB bound to unlabeled anastellin, uniformly ¹³C- and ¹⁵N-labeled AB bound to unlabeled anastellin, uniformly ¹⁵N-labeled anastellin bound to unlabeled AB, or uniformly ¹³C- and ¹⁵N-labeled anastellin bound to unlabeled AB. The

NMR experiments that were acquired for these samples are listed in Table S2. ^1H , ^{13}C and ^{15}N chemical shifts in the AB:anastellin complex were assigned using standard procedures (Fig. S1).

Structure calculations

The structure of the AB:anastellin complex was solved with Aria 2.3²⁴ in conjunction with CNS 1.21²⁵, using partially assigned peaks from the NOESY spectra and Φ and Ψ dihedral angle restraints from TALOS-N²⁶ as input (Table 1). The peaks from filtered NOESY experiments were not included in Aria input but were used instead to guide manual assignment of the peaks in other NOESY spectra as intra- or intermolecular. To prevent generation of artifactual restraints between the highly dynamic N-terminal tails of AB or anastellin and the rest of the complex, chemical shifts for residues preceding A813 in AB and N631 in anastellin were not included in the calculation. Manually identified intraresidual and sequential NOESY peaks stemming from these dynamic residues were also removed from the input²⁰.

The calculation protocol employed spin-diffusion correction²⁷, a quadrupled number of cooling steps during simulated annealing²⁸ and otherwise mostly default Aria parameters and options. In the final run, a total of 80 structures were calculated and 40 of them were refined in explicit water. Nineteen structures that had the lowest energies were selected to represent the ensemble, and their statistics are summarized in Table 1. Figures depicting the structures were prepared with the molecular graphics program Molmol 2K.1²⁹. The chemical shifts and coordinates of the AB:anastellin complex were deposited in the Biomagnetic Resonance Bank (BMRB ID 30061) and the Protein Data Bank (PDB ID 5J6Z), respectively.

Isothermal titration calorimetry (ITC)

Protein samples for ITC were dialyzed extensively against Tris buffer (25 mM Tris, pH 8.0, 150 mM NaCl). ITC experiments were carried out in a VP-ITC instrument (MicroCal, GE Healthcare) at 25°C. 8 μL aliquots of AB were injected into the calorimeter cell containing a 1.43 mL solution of anastellin, and the heats of binding were recorded. The data were analyzed with the ORIGIN software provided with the ITC instrument. Concentration of anastellin lower than 20 μM resulted in a poor signal to noise ratio in the heat measurements, and the concentration was therefore kept in the 50–80 μM range. Under these conditions, however, AB bound tightly to anastellin, and the shapes of the resulting binding curves were not very sensitive to the value of the dissociation constant³⁰.

Disulfide crosslinking experiments

Single cysteine mutations were introduced into 3FN3 and anastellin with the QuikChange II site-directed mutagenesis kit (Stratagene) and were verified by DNA sequencing. Anastellin mutants were prepared essentially as described above for the wild-type protein but were supplemented with 10 mM dithiothreitol (DTT) after elution from the Ni^{2+} -NTA resin, and the PBS buffers for dialysis and size-exclusion chromatography contained 10 mM and 1 mM DTT, respectively. In a similar manner, 3FN3 mutants were isolated by affinity chromatography on a HisTrap FF column in the absence of a reducing agent, then

supplemented with 10 mM DTT, concentrated and further purified by size-exclusion chromatography in a PBS buffer containing 1 mM DTT. The mutant proteins were aliquoted immediately after the size-exclusion chromatography step, flash frozen in liquid nitrogen and stored at -80°C until use. The concentrations of the stock solutions ranged from 120 μM to 200 μM .

For the disulfide crosslinking reactions, each mutant protein was first diluted to a concentration of 16 μM with the crosslinking reaction buffer (20 mM sodium phosphate, pH 7.0, 100 mM NaCl, 10% (v/v) glycerol and 5 mM ethylenediaminetetraacetic acid (EDTA)). 22.5 μL of an anastellin mutant were then combined with 22.5 μL of a 3FN3 mutant, and the crosslinking reaction was initiated by addition of 5 μL of 5 mM Cu(II) (1,10-phenanthroline)₃ solution³¹. After 10 minutes at 25°C , 20 μL of the reaction mixture were removed and the reaction was quenched by addition of 10 μL of nonreducing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer that contained 10 mM EDTA and 40 mM N-ethylmaleimide. The reaction products were resolved by SDS-PAGE and visualized with the Imperial Protein Stain (Pierce).

Results and Discussion

Anastellin binds to β -strands A and B from 3FN3

Purified anastellin and 3FN3 form a complex that can be separated from the unbound components by size exclusion chromatography. We set out to investigate this complex by NMR spectroscopy but the 1D ^1H NMR spectrum of the complex exhibited extensive line broadening, and there were virtually no observable crosspeaks in the 2D ^1H - ^{15}N heteronuclear single-quantum correlation (HSQC) spectra of the complex with either 3FN3 or anastellin ^{15}N -labeled (Fig. 1 and 2A; see also Fig. 4 in³²). Since the complex showed no signs of aggregation in size-exclusion chromatography experiments, the line broadening in NMR spectra is likely a result of conformational exchange.

We tested the effect of temperature, pressure and a number of additives but have so far been unable to identify any conditions that would yield high quality NMR spectra and allow us to determine the structure of the 3FN3:anastellin complex by NMR spectroscopy. Interestingly, though, high quality NMR spectra were observed for our samples on two occasions. In both cases, analysis by SDS-PAGE revealed that the 3FN3 component of the complex was degraded due to uncontrolled proteolysis that occurred during purification or sample storage. This observation suggested that complexes of anastellin with proteolytic or recombinant fragments of 3FN3 may be amenable to high resolution structural studies and provide useful insight into the architecture of the intact complex.

Pursuing this lead, we digested 3FN3 under controlled conditions and prepared complexes of anastellin with the cleaved 3FN3. A high quality NMR spectrum was obtained for ^{15}N -labeled anastellin in the presence of 3FN3 digested with α -chymotrypsin (Fig. 2B). The sample was purified by size-exclusion chromatography, and two α -chymotryptic peptides that co-eluted with anastellin were identified by MALDI-ToF mass spectrometry (Fig. 2D–E). Additional experiments (data not shown) suggested that only one of these fragments,

Fragment 1 in Fig. 2, bound to anastellin. Fragment 1 contains the two N-terminal β -strands, A and B, from 3FN3.

We prepared a recombinant peptide (termed AB) that comprised these two β -strands and investigated its interaction with anastellin using ITC and NMR spectroscopy. AB bound to anastellin with a dissociation constant in the submicromolar range (Fig. 3), and NMR spectra of anastellin with AB were highly similar to those of anastellin with the α -chymotryptic peptide (Fig. 2C). These results unambiguously demonstrate that β -strands A and B from 3FN3 interact with anastellin.

Structure of the AB:anastellin complex

To determine how AB interacts with anastellin, we solved the structure of the AB:anastellin complex. The structure is shown in Fig. 4. Overall, the complex closely resembles an intact FN3 domain, with AB in place of the two N-terminal β -strands that are absent in anastellin (Fig. S2). In the complex, AB adopts a similar conformation as in 3FN3 (Fig. S2) and forms an intermolecular β -sheet with β -strand E of anastellin (Fig. 4). The interactions between the side chains of AB and anastellin are mainly hydrophobic and involve P814, P817, V819, I827, V829 and W831 in AB and Y641, L643, I668, L671, L681, S683, T693, F695 and F697 in anastellin (Fig. 4D).

It was shown previously that complementary fragments of an FN3 domain can assemble into a complex that resembles the native domain³³. The affinity of the interaction between the fragments correlates with the stability of the parent domain³³. Our results now demonstrate that fragments derived from different FN3 domains can also efficiently associate (Fig. 2–4). The high-affinity complementation between AB and anastellin is remarkable given the limited sequence identity (~ 20%) between 1FN3 and 3FN3, the parent domains of anastellin and AB respectively (Fig. 4A). The interaction between AB and anastellin is also quite specific: AB binds only weakly to a construct (termed CG) that contains β -strands C-G from 3FN3 (Fig. 5). The observed low affinity of AB for CG is consistent with the relatively low chemical stability of 3FN3^{20, 34}.

Disulfide mapping of the interface between anastellin and intact 3FN3

The 3-dimensional structure of the AB:anastellin complex now offers, for the first time, an atomic-resolution model for what might be happening when anastellin binds to 3FN3. To investigate whether anastellin indeed interacts with intact 3FN3 in the same manner as observed in the AB:anastellin complex, we employed disulfide crosslinking³¹.

Disulfide crosslinking is a powerful technique that can be used to identify close contacts in proteins or protein complexes and is particularly well suited for challenging systems that are not amenable to high-resolution methods³¹. To probe protein structure by disulfide crosslinking, pairs of cysteines are introduced into a cysteineless protein or a protein complex by site-directed mutagenesis, and the rates of disulfide bond formation are evaluated. Disulfide bond formation can occur only if the two cysteines can approach each other with a maximum distance of 4.6 Å between their C β atoms, and with angular constraints satisfied as well³⁵. Hence, the ability of two cysteines to rapidly form a disulfide bond is usually a signature of proximity within the structure. By measuring the disulfide

formation rates for a number of cysteine pairs, a pattern of rates will often emerge than is consistent with a particular structural model.

In the AB:anastellin complex, β -strand B from AB is hydrogen bonded to β -strand E in anastellin. To investigate the spatial relationship of these two β -strands in the context of the intact complex, we individually mutated several solvent exposed residues in the β -strand B of 3FN3 and in the β -strand E of anastellin to cysteines (Fig. 6A). In addition, we also individually mutated solvent exposed residues in the C–D loop and in the β -strand F of anastellin to cysteines as a negative control (Fig. 6B). The 3FN3 and anastellin cysteine mutants were then subjected to disulfide crosslinking catalyzed by copper phenanthroline, and the observed disulfide bond formation was used to map out spatial proximity between pairs of cysteines in the different positions (Fig. 6C).

The only mutants that efficiently formed intermolecular disulfide crosslinks were anastellin K669C with 3FN3 S826C, anastellin T667C with 3FN3 S826C and V828C, and anastellin S665C with 3FN3 V828C and R830C (Fig. 6C). These pairs of mutants all have cysteine residues in positions that are adjacent in the AB:anastellin complex (Fig. 6A). On the other hand, anastellin K669C with 3FN3 V828C and R830C, anastellin T667C with 3FN3 R830C, and anastellin S665C with 3FN3 S826C formed little or no crosslinks (Fig. 6C). These pairs of mutants have cysteine residues in positions that are more than 9 Å apart in the AB:anastellin complex (Fig. 6A). Finally, the negative control anastellin mutants, V651C and I682C, did not form crosslinks to a significant extent with any of the mutant 3FN3 proteins (Fig. 6C). The observed disulfide crosslinking pattern is consistent with a model in which β -strand B from intact 3FN3 interacts with anastellin in the same manner as in the AB:anastellin complex.

3FN3 has to open up to interact with anastellin

3FN3 adopts a compact globular fold (Fig. 4E and Fig. S2C), with free energy of unfolding of $\sim 4 \text{ kcal mol}^{-1}$ under native conditions^{20, 34}. When the domain is folded, the hydrophobic residues that interact with anastellin are buried, and the edge of β -strand B that interacts with β -strand E in anastellin is hydrogen bonded to 3FN3's own β -strand E (Fig. 4E and Fig. S2C). To engage with anastellin in a manner consistent with our data, 3FN3 has to open up such that β -strand B separates from β -strand E and some of the buried hydrophobic residues become exposed.

The notion that 3FN3 opens up when it interacts with anastellin is supported by results of several other studies^{16, 36}. First, it was shown that 3FN3 bound to anastellin is more susceptible to proteolytic digestion than free 3FN3^{16, 36}. This is in marked contrast to most other protein complexes, which are more resistant to proteolysis than the individual components. Furthermore, 3FN3 protein in which S826 and S868 are mutated to cysteines does not interact with anastellin when the two cysteine residues form a disulfide bridge^{16, 36}. The engineered disulfide bridge locks β -strands B and E together and increases the chemical stability of the domain. However, the mutant protein still binds to anastellin when the cysteine residues are reduced, suggesting that the mutations per se do not affect the interaction. Finally, the rate of binding of anastellin to 3FN3 does not depend on anastellin concentration when the concentration is higher than $\sim 20 \mu\text{M}$. To explain this behavior,

Ohashi et al. suggested that at high anastellin concentrations, the rate of binding is limited by the rate of 3FN3 unfolding^{16, 36}. We subsequently demonstrated that the rate constant for 3FN3 unfolding, $\sim 0.06 \text{ s}^{-1}$ ²⁰, is indeed comparable to the first-order rate constant for anastellin binding to 3FN3 determined by Ohashi et al., $\sim 0.02 \text{ s}^{-1}$ ³⁶. Our structural model for the interaction between 3FN3 and anastellin is consistent with all these earlier observations.

Interactions that involve protein unfolding are unusual but not unprecedented. Some proteins form homo-oligomers by opening up and exchanging (“swapping”) β -strands or other structural elements between the molecules^{37, 38}. Several engineered FN3 domains were observed to form swapped dimers^{39–41}, and β -strand swapping was also proposed to mediate formation of fibrillar aggregates by the thermally destabilized 9th FN3 domain from fibronectin⁴². However, examples of heteromeric interactions that involve protein unfolding are much more limited. We are aware of only one previously reported example, and it, curiously, also involves an FN3 domain. In this case, a high-affinity peptide aptamer interacts with the extra domain B (EDB) from fibronectin by displacing β -strand G in EDB⁴³. Similar to 3FN3 and anastellin, EDB and the aptamer form an intermolecular β -sheet and engage in extensive hydrophobic interactions that involve surfaces otherwise buried in the folded EDB domain. Yu et al. coined the term “coupled unfolding and binding” to describe this unique binding mechanism⁴³. The interactions of anastellin with 3FN3 and of the peptide aptamer with EDB⁴³ both involve β -strand exchange and thus bear some similarity to domain swapping. However, unlike traditional 3D domain swapping, the β -strands that are exchanged between anastellin and 3FN3 and between the peptide aptamer and EDB show limited or no sequence identity.

We suggested previously that the interaction of fibronectin with anastellin involves swapping of β -strands and formation of intermolecular β -sheets, and that such exchange of structural elements leaves some of the previously protected β -strands and hydrophobic surfaces available for new interactions and thus leads to aggregation¹⁷. A similar proposal was also put forward by Ohashi and Erickson¹⁶. The data that we present here show that the interaction of anastellin with one of its target FN3 domains indeed involves an exchange of β -strands, with anastellin binding to β -strands A and B of the target FN3 domain, displacing β -strands C–G in the process (Fig. 7). Our results provide strong support for the current model of anastellin-triggered fibronectin aggregation.

The ability of FN3 domains to open up and engage via β -strand edges that are not accessible in the folded domains might also be important in the process of fibronectin fibril assembly by cells. We proposed earlier that fibronectin fibrillogenesis involves intermolecular β -strand interactions¹⁷. This model was further elaborated by Ohashi and Erickson¹⁶, who pointed out that such interactions might include β -strand exchange between different FN3 domains or addition of β -strands from FN3 domains to the β -sheets of FN1 domains using a mechanism known as the tandem β -zipper⁴⁴. The observations of significant structural rearrangements in EDB and now in 3FN3 suggest that some FN3 domains are metastable and can indeed participate in exchange and/or addition of β -strands during formation and maturation of fibronectin fibrils.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

1FN3	1 st fibronectin type III domain from human fibronectin
3FN3	3 rd fibronectin type III domain from human fibronectin
DTT	dithiothreitol
EDB	extra domain B
EDTA	ethylenediaminetetraacetic acid
FN1	fibronectin type I
FN2	fibronectin type II
FN3	fibronectin type III
GST	glutathione transferase
HSQC	heteronuclear single-quantum correlation
ITC	isothermal titration calorimetry
MALDI-ToF	matrix-assisted laser desorption ionization time-of-flight
NTA	Ni ²⁺ -nitrilotriacetic acid
PBS	phosphate buffered saline
PMSF	phenylmethylsulfonyl fluoride
RMSD	root mean square deviation
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis

TEV tobacco etch virus

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Figure 1.

Effect of anastellin binding on NMR spectra of 3FN3. (A) 2D ^1H - ^{15}N HSQC spectrum of ^{15}N -labeled 3FN3. (B) 2D ^1H - ^{15}N HSQC spectrum of ^{15}N -labeled 3FN3 in the presence of excess anastellin.

Figure 2.

Limited proteolysis of 3FN3 and identification of fragments that bind to anastellin. (A) 2D ^1H - ^{15}N HSQC spectrum of ^{15}N -labeled anastellin bound to unlabeled 3FN3. (B) 2D ^1H - ^{15}N HSQC spectrum of ^{15}N -labeled anastellin bound to unlabeled α -chymotryptic fragments from 3FN3. (C) Same spectrum as in panel B (black), and 2D ^1H - ^{15}N HSQC spectrum of ^{15}N -labeled anastellin bound to unlabeled recombinant AB (red, on top). The spectra are superimposed to highlight their similarity. (D) MALDI-ToF spectrum of the α -chymotryptic fragments from 3FN3 that co-eluted with anastellin in size exclusion chromatography. Internal standards are marked with asterisks (*). (E) Amino acid sequence of 3FN3. Fragments that were identified by MALDI-ToF are marked and their theoretical $(\text{M}+\text{H})^+$ masses are indicated in parentheses.

Figure 3.

Calorimetric titration of anastellin with AB. Representative ITC trace and the binding curve fitted to the data are shown in the upper and lower panels, respectively. In this experiment, 70 μM anastellin was titrated with 720 μM AB. The binding curve is almost rectangular, indicating that the binding is tight (dissociation constant in the 10^{-7} M range or lower), and virtually all added AB binds to anastellin until saturation is reached.

Figure 4.

Structure of the AB:anastellin complex. (A) Aligned amino acid sequences of 1FN3 and 3FN3, the FN3 domains from which anastellin and AB are derived. The residues that are identical in both domains are colored purple. The secondary structure that anastellin and AB adopt in the complex is shown as a diagram next to the sequences. (B) Superimposed backbone traces of the AB:anastellin complex (residues 813–834 of AB and 638–697 of anastellin). (C) A ribbon model of the AB:anastellin complex, with individual β -strands marked. (D) A close-up view of the interface between AB and anastellin. Residues in AB whose side chains interact with anastellin are labeled. The color of AB in all panels changes smoothly from yellow at the N-terminus to orange at the C-terminus, and the color of anastellin changes from blue at the N-terminus to red at the C-terminus. (E) A close-up view of the hydrophobic core in 3FN3. Residues 813–834, which are also present in AB, change color from yellow to orange as in the previous panels; the rest of 3FN3 is colored grey.

Figure 5.

Interaction of AB with CG. (A) 2D ^1H - ^{15}N HSQC spectrum of 0.6 mM ^{15}N -labeled AB. (B) Same spectrum as in panel A (black), and superimposed 2D ^1H - ^{15}N HSQC spectrum of 0.325 mM ^{15}N -labeled AB in the presence of 1.3 mM unlabeled CG (red, on top). The contour levels in the spectrum with CG have been lowered proportionately to compensate for dilution of the labeled protein upon addition of CG. Several new peaks (highlighted with blue circles) become observable when CG is added but the signals from free AB remain strong and the black spectrum is barely visible under the red contours. The data suggest that most of AB remains unbound under these conditions.

Figure 6. Disulfide crosslinking studies of the interaction between anastellin and intact 3FN3. (A and B) A ribbon model of the AB:anastellin complex showing the residues that were selected for mutation to cysteines in the crosslinking experiments. The color scheme is the same as in Fig. 4. The views in panels A and B are related by a 180° rotation around the vertical axis. Short distances between C β atoms of residues in β -strand B of AB and β -strand E of anastellin are shown as dashed lines. (C) The observed disulfide crosslinking pattern between anastellin and intact 3FN3 is consistent with formation of an intermolecular β -sheet between β -strand B of 3FN3 and β -strand E of anastellin. Indicated combinations of anastellin and 3FN3 single-cysteine mutants were subjected to disulfide crosslinking, and the products of the reaction were resolved by SDS-PAGE under non-reducing conditions. The bands that contain disulfide-linked 3FN3:anastellin complexes are highlighted with a red box. The positions of anastellin monomers, 3FN3 monomers, and disulfide-linked anastellin homodimers are also marked. As reported previously, 3FN3 (13 kDa) migrates more slowly in SDS-PAGE than expected for its size³⁶, and it overlaps with the disulfide-linked anastellin homodimers (20 kDa) that are formed by some of the anastellin mutants.

Figure 7.
A schematic representation of the interaction between 3FN3 and anastellin.

Table 1

Experimental restraints and structural statistics for the AB:anastellin complex

Number of peaks in NOESY spectra	
3D aliphatic ¹³ C-edited NOESY (without sensitivity enhancement), ¹³ C- and ¹⁵ N-labeled AB + unlabeled anastellin	342
3D aliphatic ¹³ C-edited NOESY (with sensitivity enhancement), ¹³ C- and ¹⁵ N-labeled AB + unlabeled anastellin	106
3D ¹⁵ N-edited NOESY, ¹⁵ N-labeled AB + unlabeled anastellin	109
3D aliphatic ¹³ C-edited NOESY (without sensitivity enhancement), ¹³ C- and ¹⁵ N-labeled anastellin + unlabeled AB	1223
3D aliphatic ¹³ C-edited NOESY (with sensitivity enhancement), ¹³ C- and ¹⁵ N-labeled anastellin + unlabeled AB	290
3D aromatic ¹³ C-edited NOESY, ¹³ C- and ¹⁵ N-labeled anastellin + unlabeled AB	135
3D ¹⁵ N-edited NOESY, ¹⁵ N-labeled anastellin + unlabeled AB	1050
Number of experimental restraints	
NOE distance restraints in the last ARIA iteration	
Intraresidual	701
Sequential	357
Medium range (2 ≤ i-j ≤ 5)	161
Long range (6 ≤ i-j)	469
Intermolecular	105
Ambiguous	569
Dihedral angle restraints (Φ and Ψ)	134
Number of experimental restraint violations	
NOE violations > 0.5 Å	0 ± 0
Dihedral angle violations > 5°	0 ± 0
Root mean square deviation (RMSD) from experimental restraints	
NOE distance restraints (Å)	0.013 ± 0.001
Dihedral angle restraints (°)	0.4 ± 0.1
RMSD from idealized geometry	
Bonds (Å)	0.0033 ± 0.0001
Angles (°)	0.45 ± 0.01
Impropers (°)	1.2 ± 0.1
RMSD of residues 638–697 and 813–834 from mean coordinates	
Backbone atoms (N, Ca, C') (Å)	0.64 ± 0.09
Heavy atoms (Å)	1.09 ± 0.12
Distribution of Φ and Ψ dihedral angles of residues 638–697 and 813–834 in Ramachandran plot ⁴⁵	
Most favored regions (%)	89.0
Additional allowed regions (%)	10.8
Generously allowed regions (%)	0.1
Disallowed regions (%)	0.1