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# A comparison between the unfolding of fibronectin and contactin

**A Dąbrowska[1], K Lebed, M Lekka, J Lekki and W M Kwiatek**

The Henryk Niewodniczański Institute of Nuclear Physics, Polish Academy of Sciences, Radzikowskiego 152, 31-342 Kraków, Poland

E-mail: Aleksandra.Dabrowska@ifj.edu.pl

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**Abstract**

The mechanical unfolding of two proteins belonging to the immunoglobulin superfamily, fibronectin (an extracellular matrix protein) and contactin (a neuronal adhesion protein), was studied by means of atomic force microscopy (AFM). The mean unfolding forces and characteristic lengths describing unfolding events of two types of the immunoglobulin module observed for contactin were compared with results obtained for fibronectin. The results showed that the FnIII-type domain present in both proteins, i.e. in contactin and fibronectin, requires a similar force of about 100 pN to be unfolded. However, the IgC2-type domains of contactin, normally remaining intact in view of the intra-domain disulfide bonds, reveal rather lower stability in the presence of the reducing agent. The force needed to unfold a single IgC2 domain was calculated and established to be about 70 pN.

Initially, natural human fibronectin was chosen only as a reference protein for studies of contactin unfolding force values. However, interesting results were obtained and used as a reference in further analysis of the contactin unfolding pathway. Two characteristic length values were obtained for the FnIII domain type of both studied proteins; thus for both domains the ability to unravel in two different pathways was concluded.

## 1. Introduction

Fibronectin is a long glycoprotein existing as a soluble form in plasma and also as an insoluble form constituting an important component of extracellular matrix where it participates in cell adhesion and cell mobility [1, 2]. Fibronectin possesses several important binding sites enabling the interaction with other proteins (e.g. collagen), fibrins or cells. Contactin is an axonal cell adhesion molecule occurring in neuronal cell surface and areas where neurons contact with each other [3]. It is mainly involved in neurite outgrowth [4, 5]. As a cell surface

[1] Author to whom any correspondence should be addressed.

  

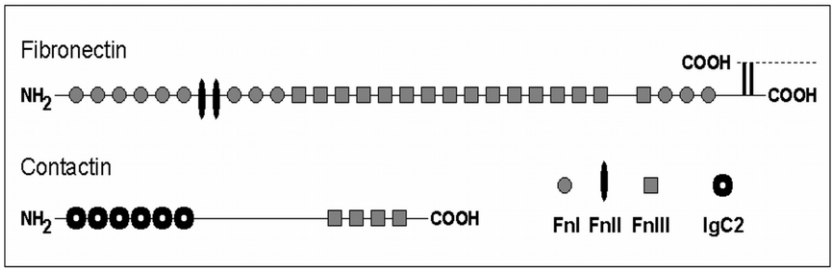


**Figure 1.** Diagram of the protein structure for fibronectin and contactin. For fibronectin, only one monomer is drawn. The double black line close to its carboxyl terminus symbolizes two disulfide bonds responsible for dimer formation. Different types of modules are marked with different symbols described in the figure (adapted from [13, 3]).

signal-transducing molecule it participates in cellular communication [6–10]. Apart from that, it is also involved in intercellular adhesion of neurons by the formation and maintenance of stable contacts between neuronal cells [3, 11].

From a mechanical point of view the axonal cell adhesion proteins play an important role in the formation and maintenance of functional neuronal networks. Since such proteins are localized at the synaptic sites they are supposed to be involved in the plasticity of synaptic connections [10]. Information about the mechanical features may be useful in the study of some of the contactin functions related with axonal plasticity [12]. Therefore, the mechanical behaviour of contactin, a neuronal cell adhesion protein, was studied in detail in the unfolding experiment and is compared with the results of a similar experiment performed for fibronectin, a well known adhesion protein.

Both proteins belong to the immunoglobulin-like superfamily; thus, both are composed of Ig-like domains. Fibronectin consists of two nearly identical monomers, each of about 2300 amino acids long, linked together by disulfide bridges located near their carboxyl termini (see figure 1). Contactin is a monomer composed of about 1000 amino acids.

Fibronectin contains in its tertiary structure three distinct domain types: 12 domains of type I (FnI), 2 domains of type II (FnII) and 15–16 domains of type III (FnIII) [13]; whereas contactin posses four FnIII-type modules near its carboxyl terminus and six immunoglobulin-like domains of C2 type (IgC2) near the amino terminus. On average, the FnIII domain is composed of 90 and 95 amino acids, for fibronectin and contactin, respectively. The calculated contour length, estimated as a product of the number of amino acids composing the protein fragment multiplied by the length of the monomer unit (0.38 nm), is approximately 34 and 36 nm, correspondingly. The IgC2 domain consists of about 87 amino acids, which corresponds to approximately 33 nm in contour length.

The secondary structure of all three types of domain, i.e. fibronectin FnIII, contactin FnIII and IgC2, are dominated by $\beta$-sheets. Each domain remains in the folded state mainly due to hydrogen bonds occurring between particular strands of the domain. The difference between IgC2 and FnIII domain types lies in different orientation of hydrogen bonds with respect to the direction of protein stretching. In the first case, the hydrogen bonds between a pair of strands are parallel to the unfolding direction and, therefore, the hydrogen bonds are ruptured in turn. Such a spatial configuration is called a zipper H-bonded configuration [14]. Another type of configuration, called a shear H-bonded configuration, occurs in FnIII domains. In this case, the orientation of the hydrogen bonds is perpendicular to the unfolding direction. The unravelling of such domain begins from simultaneous ruptures of all hydrogen bonds between a pair of strands. In consequence, in the second case a larger force is required to unfold such a domain.

Contactin has not been unfolded so far by means of atomic force microscopy (AFM). A similar domain type is present in synaptotagmin extracted from synaptic vesicle membrane protein. A polyprotein composed of similar domain type $(C2A)_9$ was studied by means of AFM, previously [14]. The important difference between synaptotagmin C2A and contactin IgC2 domains is the presence of internal disulfide bonds stabilizing the structure of the latter. It is known from previous AFM studies that domains, which are stabilized by internal disulfide bonds, do not unfold under usual experimental conditions. Oberhauser *et al* [15] reported that only domains of type III could be unfolded during stretching of natural fibronectin, while both FnI and FnII domain types stay intact because of the presence of stabilizing disulfide bonds. Carl *et al* observed the unfolding of five Ig-type domains (from melanoma cell adhesion molecules) after using an agent to reduce the disulfide bonds [16]. Therefore, to enable unfolding of contactin IgC2 domains the chosen reducing agent was added into the solution.

Despite the fact that the mechanical unfolding of fibronectin domains was widely studied by means of atomic force microscopy [15, 17–20], each experiment, slightly different in terms of measurement condition or way of sample preparation, sheds light on the problem of protein unfolding.

## 2. Materials and methods

### *2.1. Reagents*

Natural fibronectin from bovine plasma (Fn, Sigma) and recombinant contactin-4 (Cont4, R&D System, Biokom) were chosen. Tris (2-carboxyethyl) phosphine hydrochloride (TCEP, Sigma) was used in a 5 mM concentration. This denaturing agent reduces the disulfide bonds selectively and very efficiently [21]. Besides, it does not interact covalently with the protein being reduced [22]. Phosphate buffered saline (PBS, ICN Biomedicals, pH 7.4, containing 10 mM of $PO_4^{2-}$, 137 mM of NaCl and 27 mM of KCl) was used to prepare all protein solutions.

### *2.2. Sample preparation*

The protein concentration was 0.1 mg $ml^{-1}$ and 0.03 mg $ml^{-1}$ for fibronectin and contactin, respectively. As a substrate, freshly cleaved mica was used. For protein immobilization it was incubated in the protein solution for 30–60 min. Then, after protein deposition, the sample was rinsed with the PBS buffer or 5 mM TCEP solution and immediately measured.

### *2.3. AFM measurements*

The measurements were carried out using a home-built atomic force microscope [23]. Standard silicon nitride cantilevers (MLCT-AUHW, Veeco) with a nominal spring constant of 0.01 N $m^{-1}$ or 0.03 N $m^{-1}$ were used. No special treatment was applied to the cantilevers. During unfolding measurements the cantilever deflection as a function of the relative sample position was recorded. Then, the measured values were converted into force and the protein extension. Force curves were acquired within an area of about 1 $\mu m^2$ at different locations on the sample surface. The scanner step size was always less than 1 nm (it varied in the range from 0.30 nm to 0.97 nm). The typical pulling speed was about 1 $\mu$m $s^{-1}$; however the loading rate value, as the more appropriate measurement condition parameter, is always indicated.

From force curves the increase of length, also called the unfolding length or characteristic length, and the unfolding force at which unfolding of a given domain occurs were obtained. The force-peaks were analysed excluding each first and last ones because of the possibility of non-specific interactions occurring near the contact point and the protein detachment from the

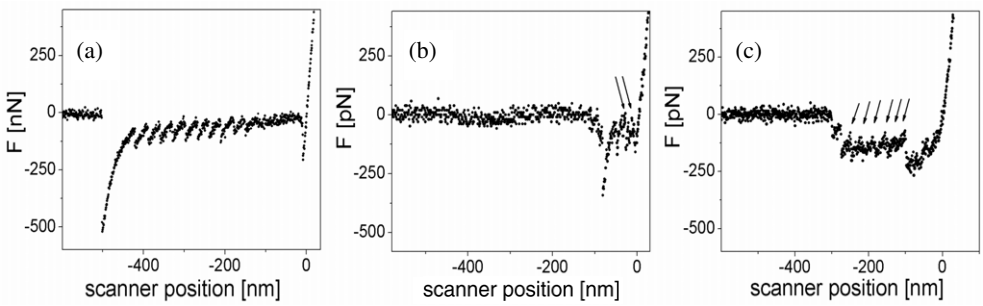


**Figure 2.** An example of force curve presenting: (a) the regular saw-tooth pattern resulting from the unfolding of subsequent FnIII domains in PBS, (b) two unfolding event (marked by arrows) recorded for contactin unfolding in PBS, (c) six regular unfolding events obtained from contactin unfolding in the presence of 5 mM TCEP.

tip at the end of stretching [24]. All unfolding length and force mean values obtained from fitting corresponding distributions were given with a standard deviation (SD) values.

## 3. Force curves

Figure 2 presents the examples of force curves measured during the unfolding of fibronectin and contactin, respectively. Since fibronectin monomer is composed of 15 FnIII domains, a long saw-tooth pattern corresponding to multiple unfolding events was easily observed during force curve acquisition (see figure 2(a)). In contrast to fibronectin, contactin contains only four domains that could be unfolded in normal measurement conditions (i.e. in PBS buffer). Indeed, the measured force curves showed a very short saw-tooth pattern composed of single unfolding events (see figure 2(b)). Those events were ascribed to the unravelling of FnIII modules. The remaining six IgC2 domains in their native state are stabilized with internal disulfide bonds; they are thus assumed to remain intact during protein stretching and do not contribute to the saw-tooth pattern.

The results of contactin unfolding were expected to change after addition of TCEP. The experiment mentioned in section 1 performed on the polyprotein $(C2A)_9$ showed that such C2A Ig-like domains have rather low mechanical stability [14]. Thus, two levels of unfolding forces on force curves obtained from contactin unfolding in the presence of TCEP were expected.

But in our case, the experiment performed in the presence of TCEP resulted in force curves in which any difference in height of force-peaks cannot be noticed at first sight (see figure 2(c)). On the other hand, force curves with the number of unfolding events larger than four were recorded in this experiment; therefore, since a single contactin molecule contains only four FnIII domains, at least two force-peaks on such curves have to be ascribed to the unfolding of IgC2 domains.

To study the process more precisely the number of unfolding events $n$ in each stretching cycle was analysed and presented as a frequency of $n$-domain unfolding in a normalized histogram (see figure 3). Typically, three domains were unfolded, independently in both experiments, performed in PBS and in PBS supplemented with TCEP. However, there is a difference between these two cases in the maximum of the $n$ value. During the experiment in the presence of the reducing agent, up to eight unfolding events on a single curve were observed as well. This shows that TCEP reduces the disulfide bonds and thus enables the unfolding of contactin IgC2 domains.

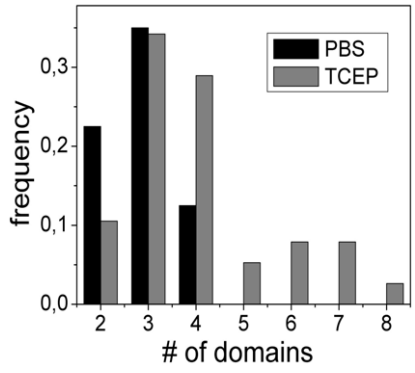


**Figure 3.** The frequency of $n$-domain unfolding observed in the measurements of contactin unfolding carried out in PBS (black) and in 5 mM TCEP (grey). The numbers of events corresponding to the unfolding of a given number of modules were normalized to the total number of curves acquired in a given measurement.

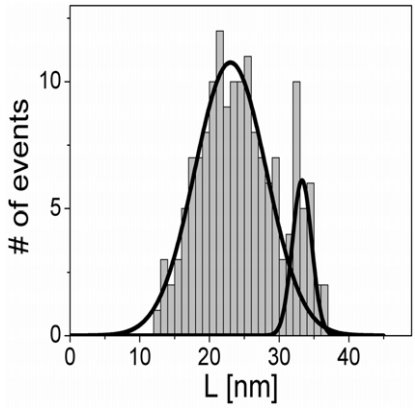


**Figure 4.** Length histograms obtained for fibronectin unfolding at the loading rate of 2.93 nN $s^{-1}$. Two Gauss functions were fitted, centred at $L = (23.0 \pm 5.3)$ nm and $(33.3 \pm 1.2)$ nm. The total number of unfolding events $n = 160$.

## 4. Unfolding lengths

The sets of force curves were used to create histograms of the unfolding length for both proteins, fibronectin and contactin (see figures 4 and 5). In figure 4 two clear maxima characterizing the unfolding of fibronectin FnIII domains were fitted with Gauss functions. At the loading rate of 2.93 nN $s^{-1}$ the most probable characteristic lengths values were $(23.0 \pm 1.0)$ nm and $(33.3 \pm 1.0)$ nm. An identical measurement performed previously for the recombinant Fn fragment $^{4-12}$FnIII resulted in similar values of $(23.8 \pm 1.0)$ nm and $(32.6 \pm 1.0)$ nm, correspondingly [25]. On the basis of detailed analysis of those two measurements both characteristic lengths were ascribed to unfolding of Fn type III domains. The occurrence of two lengths reveals the ability of FnIII domains to unfold in two different ways. The larger length from each pair of length values corresponds to the unfolding of entire domains (the mean theoretical length estimated for $^{1-15}$FnIII domains is 34 nm). The smaller length was previously related with unfolding of a part of the domain [25]. The numerical simulations of unfolding performed for $^{9}$FnIII [26] and $^{10}$FnIII [27] confirmed the possibility of the presence of such an intermediate state (at the reported value of about 24 nm).

The length histograms obtained for the unfolding of contactin performed in two different experimental conditions, in PBS buffer and in the presence of TCEP, are shown in figures 5(a) and (b), correspondingly. The measurement in PBS resulted in length values of $(18.6 \pm 5.1)$ nm and $(29.2 \pm 6.7)$ nm. The measurement in 5 mM TCEP provided a pair of similar values, namely $(18.9 \pm 11.9)$ nm and $(28.9 \pm 5.9)$ nm.

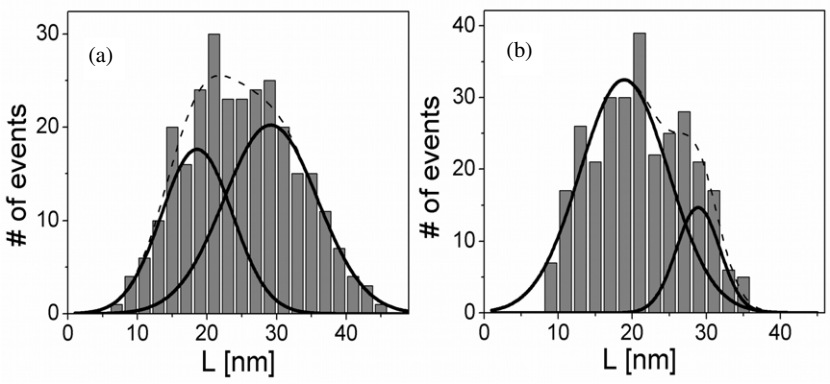


**Figure 5.** (a) Length histogram obtained for contactin unfolding in PBS at the loading rate of 3.41 nN $s^{-1}$. The Gauss functions are centred at $L = (18.6 \pm 5.1)$ nm and $(29.2 \pm 6.7)$ nm. The dashed line shows the Gaussian sum. The total number of unfolding events $n = 282$. (b) Length histogram obtained for contactin unfolding in the presence of 5 mM TCEP at the loading rate of 3.57 nN $s^{-1}$. The Gauss functions are centred at $L = (18.9 \pm 11.9)$ nm and $(28.9 \pm 5.9)$ nm. The dashed line shows the Gaussian sum. The total number of unfolding events $n = 294$.

Independently of the measurement condition, all obtained histograms repeatedly showed two maxima (see figures 5(a) and (b)). However, the reason for their presence is not clear. Previously, two unfolding pathways for fibronectin FnIII domains were reported [25]. Thus, in the case of contactin stretched in PBS, where only FnIII-type domains were assumed to unfold, one of the explanations for the occurrence of two characteristic lengths could be the structural similarity between contactin FnIII domains and fibronectin ones.

The histogram obtained for contactin stretched in the presence of TCEP (see figure 5(b)) is a mixture of events corresponding to several increases of length, i.e. two resulting from FnIII domain unfolding and one (or more) resulting from the unfolding of the IgC2 domain.

Since the mean contour lengths of contactin FnIII and IgC2 are comparable (the difference of 3 nm is below the measurement precision), the lack of an additional separate length peak in histogram 5(b) is not surprising.

In figure 5(a) the areas under the Gauss peaks fitted to the length histogram are nearly the same. Thus, unfolding of FnIII domains in PBS results in shorter and longer increases of length with a similar probability. The addition of the reducing agent to the solution does not affect the way of unfolding of FnIII domains. The clear difference in areas under the two Gauss functions fitted to the length histogram in figure 5(b) could be explained with the appearance of new events which are the unfolding of IgC2 domains.

In figure 6(b) (see the inset) the unfolding events for FnIII and IgC2 type were separated by fitting two Gauss functions to the force histogram. As can be concluded from the analysis of the areas under those two curves, in the presence of TCEP the contribution of unfolding events for contactin IgC2 and FnIII domains is comparable.

## 5. Unfolding forces

As mentioned in section 3 section, Carrion *et al* observed a relatively low stability of the C2A Ig-like synaptotagmin domain which is structurally close to the studied one [14]. The reported unfolding force was of about 60 pN at pulling speed 0.6 $\mu$m $s^{-1}$. Figure 6(a) shows the force histograms obtained for fibronectin unfolding in PBS at the loading rate of 2.93 nN $s^{-1}$. Figure 6(b) presents the results of contactin unfolding in two different experimental conditions:

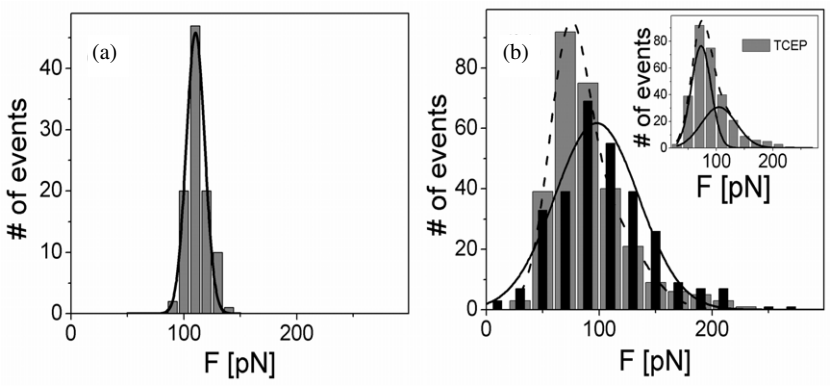


**Figure 6.** (a) Force histogram obtained for fibronectin unfolding in PBS at the loading rate of 2.93 nN $s^{-1}$. It shows a narrow singular maximum centred at $F = (110 \pm 8)$ pN. The total number of events $n = 100$. (b) Force histograms obtained for contactin unfolding in PBS and 5 mM TCEP at the loading rate of 3.41 and 3.57 nN $s^{-1}$, respectively. They were fitted with Gauss functions centred at $(98 \pm 35)$ pN for the PBS measurement (solid line) and at $(80 \pm 24)$ pN for the TCEP measurement (dashed line). The total number of events $n = 296$ and 294, correspondingly. Inset: the same force histogram corresponding to the contactin unfolding in TCEP with two Gauss functions fitted (solid line) and centred at $(73 \pm 17)$ pN and $(105 \pm 31)$ pN.

in PBS and in PBS containing 5 mM TCEP obtained at the loading rate of 3.41 and 3.57 nN $s^{-1}$, respectively.

Since one contactin molecule is composed of four FnIII and six IgC2 domains, two distinct unfolding values were expected. At first sight both force histograms presented in figure 6(b) show a single maximum corresponding to the most probable unfolding force. However, there is a slight difference between those two values. The histogram resulting from measurement in TCEP is shifted by about 20 pN towards lower forces.

The force histograms resulting from both measurements, in PBS and in the presence of reducing agent, are very wide (see figure 6(b)). However, since the lower stability of IgC2 domains was shown previously [14], the shifting of the second histogram (the grey one in figure 6(b)) can result from the contribution of lower forces sufficient to unfold the IgC2 domain.

Therefore, two Gauss functions were fitted to the histogram (see inset in figure 6(b)). One of them, centered at $(105 \pm 31)$ pN, can be ascribed to the unfolding of FnIII-type domains, and the other one, centered at $(73 \pm 17)$ pN, to IgC2 domain unfolding. The force value obtained is larger than the reported force of approximately 60 pN; however, the difference results probably from the fact that our experiment was performed at higher pulling speed, and thus at larger loading rate.

## 6. Conclusion

Fibronectin FnIII domains unfold at relatively high forces. The occurrence of two increases of lengths reveals the ability of FnIII domains to unfold in two different ways at the same force value.

A similar ability was concluded for contactin FnIII-type domains on the basis of the results of protein unfolding under the same experimental condition, in PBS buffer. For both fibronectin and contactin FnIII domains the same force value was obtained. This allows the conclusion that both these modules have the same stability and the similar mechanical behaviour.

Contactin IgC2 domains, which are normally stabilized by disulfide bonds preventing their unfolding, were unfolded in the presence of the reducing agent.

On the basis of the qualitative analysis of the areas under the Gauss functions fitted to each length histogram it could be postulated that contactin domains of type IgC2 unfold more frequently, giving shorter increases of length (about 20 nm) than longer ones, allowed by the contour length of this domain (about 33 nm). However, an unambiguous description of IgC2 domain unfolding could be provided by the results of the unfolding experiment performed using the $(IgC2)_6$ fragment of contactin.

It was observed that the mechanical unfolding of contactin Ig-like domains of type C2 requires considerably lower forces [14]. The force value obtained corresponding to contactin IgC2 domain unfolding is comparable with the force reported for the same type of domains from synaptotagmin, another neuronal protein.

To sum up, it turned out that contactin reveals a similar mechanic behaviour as fibronectin, a protein whose mechanical function is already well known. Thus, the results obtained for contactin could be used for better understanding of the same aspect of contactin functioning in neuronal networks.

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